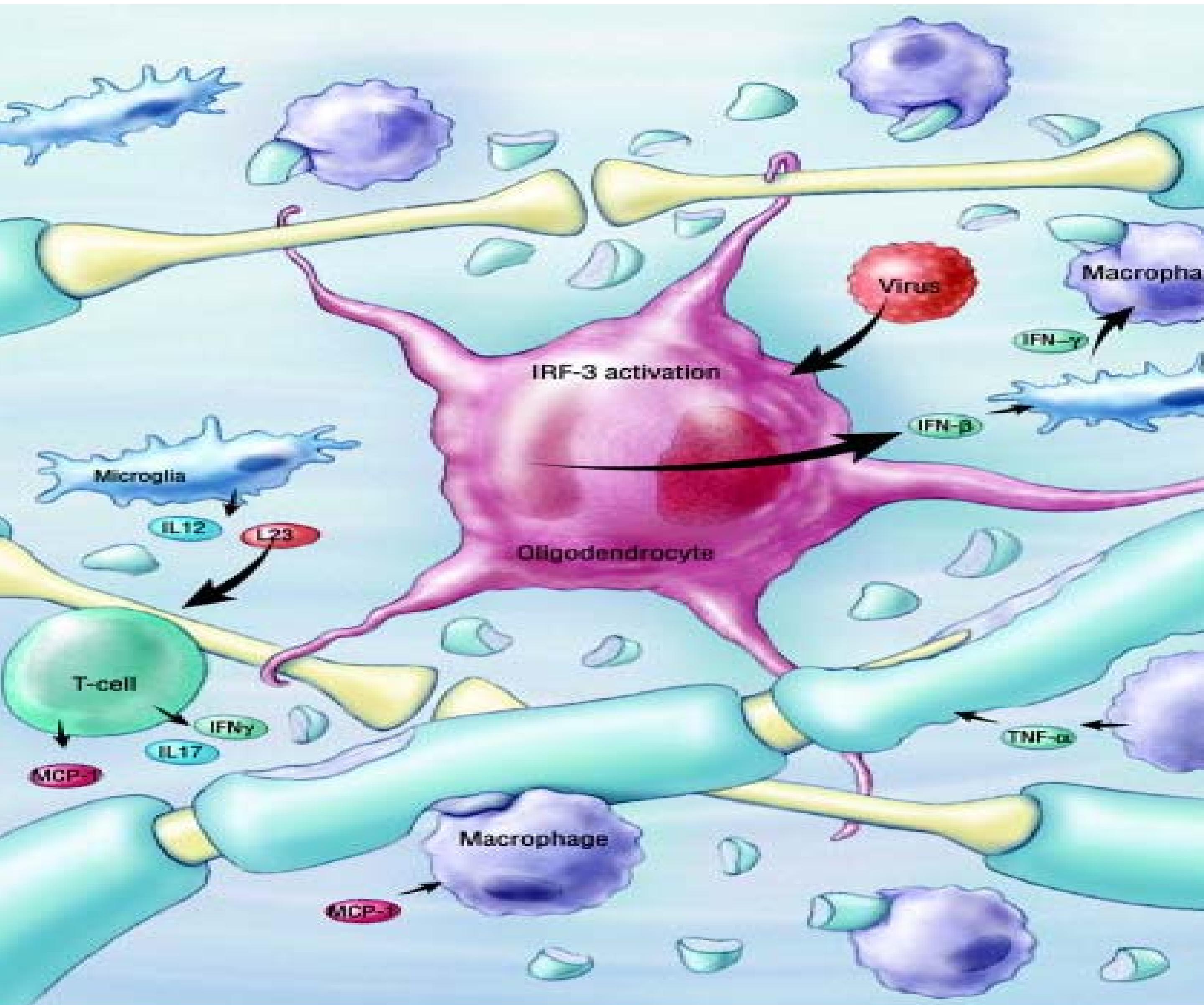
CANDIDATE GENE ASSOCIATIONS WITH MULTIPLE SCLEROSIS: IL2 GENE FUNCTIONAL STUDY

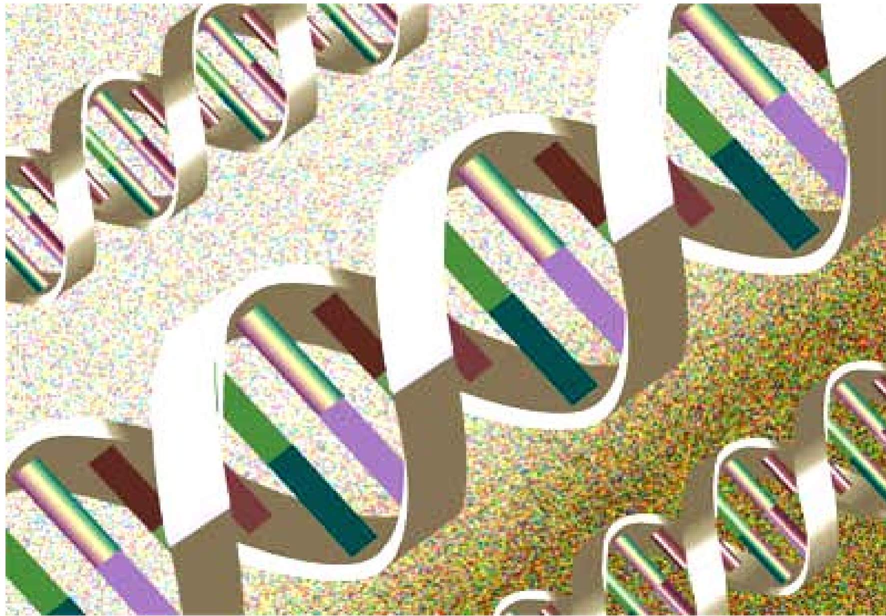
MARIA FEDETZ



Granada, 2009

TESIS DOCTORAL

ASOCIACIÓN DE GENES CANDIDATOS A ESCLEROSIS MÚLTIPLE: ESTUDIO FUNCIONAL DEL GEN DE LA IL-2



Memoria presentada por la licenciada

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para optar al grado de Doctor por la Universidad de Granada

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Granada, 2009

Editor: Editorial de la Universidad de Granada
Autor: María Fedetz
D.L.: GR 2283-2009
ISBN: 978-84-692-3079-4

Front cover illustration. Cartoon of new lesion formation in multiple sclerosis hypothetically initiated by viral infection of an oligodendrocyte. From: Lipton HL, Liang Z, Hertzler S, Son KN. A specific viral cause of multiple sclerosis: one virus, one disease. Ann Neurol. 2007; 61: 514-23.

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Don ANTONIO ALCINA MADUEÑO y Doña FUENCISLA MATESANZ DEL BARRIO, Doctores en Ciencias Biológicas,

CERTIFICAN:

Que el trabajo que presenta Dña. MARÍA FEDETZ, con el título "Asociación de genes candidatos a esclerosis múltiple: estudio funcional del gen de la IL-2" ha sido realizado en Instituto de Parasitología y Biomedicina "López Neyra" bajo nuestra dirección y consideramos que tiene el contenido y rigor científico necesario para ser sometido al juicio del tribunal que ha nombrado la Universidad de Granada para optar al grado de Doctor.

Y para que así conste firmamos el presente certificado en Granada a 23 de Abril de 2008.

Fdo. Antonio Alcina Madueño   Fuencisla Matesanz del Barrio

A mi familia

A la memoria de mí querida mamá

Моей семье

AGRADECIMIENTOS

El trabajo presentado en esta tesis está realizado en el Instituto de Parasitología y Biomedicina "López Neyra". Yo quiero expresar mi gratitud a todas las personas que han contribuido a mi investigación, me han ayudado y apoyado e hicieron estos años inolvidables. Hay muchas personas a las cuales yo quería agradecer especialmente:

En primer lugar, al Dr. Antonio Alcina y a la Dra. Fuencisla Matesanz, los directores de la tesis, quienes me han dado la oportunidad de trabajar en el laboratorio, han creído en el proyecto "Dra. M.Fedetz" y no han perdido la esperanza a pesar de los pesares... Yo les agradezco por toda su ayuda durante este tiempo, tanto en el trabajo como en los consejos de la vida cotidiana en este país, nuevo para mi.

Igualmente, al Dr. Oscar Fernández por darme la excelente oportunidad para continuar el trabajo en el campo de la esclerosis múltiple. También a los miembros de su gran equipo, especialmente a las Dras. Laura Leyva y Gloria Fernández por la atención.

A mi tutor por la Universidad de Granada, Dr. Ignacio Molina, por la atención a todos los problemas y ayuda.

A mis antiguos y contemporáneos compañeros del laboratorio 101(205) Ana, Melania, Leyre, Alfredo, Dorothy, Toni, por compartir el espacio, el tiempo, los problemas y las soluciones. Al camarada Alfredo, también, por entender mi mentalidad post-soviética☺…

A los becarios del "López" durante todos estos años, por la posibilidad única de vivir con ellos la vida estudiantil por

segunda vez en mi vida, por su ayuda y alegría, por las fiestas y apoyo de cada día. Son muchos, antiguos y de hoy, os agradezco a todos.

A compañeros no-becarios del Instituto, quien me han ayudado en el trabajo y en la "inserción" a la vida española. En primer lugar a Gema, quien me dio su apoyo incondicional el primer y mas difícil año lejos de mi familia, también a sus padres, Herminia y José, quienes considero como familiares. A mi otra casi hermana española, Maria José y su gran familia, a mi camarada cubana Nieves y su familia. A Vicente, Enrique, Ángeles, Mari Carmen S, José Antonio P, Alfonso, Aurora, Marta, Almudena, Antonio L, Antonio M, Victoria, José Manuel, Manolo y otros- gracias a todos por todo el bueno.

A mi ex jefe Dr. Alexey Pavlovich Belozorov por enseñar en su propio ejemplo que a la creatividad no le afectan los malos tiempos y que "no el puesto hace la persona importante, sino al contrario", por su amistad, sus sabios consejos y ayuda en todo lo posible e imposible. A mis compañeros de institutos en Ucrania, quienes se han convertido en amigos, con quienes "se comió mucha sal" y además en los tiempos difíciles.

Al final, a mí familia ruso - ucraniano – española, mi marido Juan Manuel, sus padres Manuela y José, tíos, primos y sobrinas españoles, mi padre Iván, hermana Olga, cuñado Víctor, sobrina Tania y mis tíos y primos rusos y ucranianos, todos siempre dispuestos a prestarme su ayuda.

A mí querida mamá Elena - la Persona, Madre y Científica inconfundible, que no llego a ver estos días, por toda mi vida…

ABBREVIATIONS

AI	autoimmunity
AID	autoimmune disease
APC	antigen presenting cell
BBB	blood-brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
DCs	dendritic cells
EAE	experimental autoimmune encephalomyelitis
EBNA	EBV nuclear protein
EBV	Epstein-Barr virus
GWAS	genome-wide association study
HATs	histone acetyltransferases
HDACs	histone deacetylases
HERVs	human endogenous retroviruses
HHV-6	human herpes virus 6
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
IFNs	interferons
IFN-γ	interferon-γ
LD	linkage disequilibrium
MBP	myelin basic protein
MRI	magnetic resonance imaging

MS	multiple sclerosis
MZ	monozygotic twins
NK	natural killer
OCBs	oligoclonal bands
PPMS	primary progressive MS
RA	rheumatoid arthritis
RRMS	relapsing-remitting MS
SLE	systemic lupus erythematosus
SNPs	single nucleotide polymorphisms
SPMS	secondary progressive MS
Stat	signal transducer and activator of transcription
T1D	type 1 diabetes mellitus
Th	T helper cells
Tr1	T regulatory cells 1
Tregs -	regulatory CD4+CD25+T cells

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RESUMEN

RESUMEN

La esclerosis múltiple (EM), un desorden inflamatorio del sistema nervioso central, se caracteriza por la perdida de mielina, gliosis y varios grados de patología en axones y oligodendrocitos. Como en todas las enfermedades complejas, en EM se cree que existe una interacción entre factores ambientales, aun no identificados, y genes de susceptibilidad (Dyment, 1997). Conjuntamente, estos factores desencadenan una cascada de efectos, que comprenden la implicación del sistema inmune, inflamación aguda de axones y glia, recuperación de funciones y reparación de estructuras, gliosis post-inflamatoria y neurodegeneración. La implicación secuencial de estos procesos se refleja en el curso clínico caracterizado por episodios de recuperación, de deficiencias en el estado físico del paciente y una progresión secundaria (Lublin & Reingold, 1996).

La EM presenta un amplio rango de fenotipos clínicos y patológicos. Un único mecanismo predominante de desarrollo de la enfermedad no ha surgido todavía aunque estudios inmunopatológicos en EM mas datos en modelos animales presentan evidencias convincentes de una patología inmuno-mediada (Lassmann. 2008).

EM es una de las enfermedades descapacitantes crónicas más comunes del sistema nervioso central que afectan más frecuentemente a población caucasiana (hasta un 15%), con alrededor de 2,5 millones de individuos afectados en el mundo. En España la prevalencia de la EM es de 50 a 70 individuos por cada 100.000 habitantes (Fernandez et al., 1994; Ares et al., 2007). La enfermedad comienza normalmente en adultos jóvenes y afecta a mujeres con más frecuencia que a hombres.

Estudios epidemiológicos y de familias apoyan fuertemente el papel de los factores genéticos en la patogénesis de la EM. Los miembros de familias con algún miembro afectado con EM tienen un mayor riesgo de desarrollar la enfermedad comparado con los esposos o hijos adoptados de los pacientes de EM (Sadovnick et al., 1993). El riesgo es mayor en gemelos y hermanos y

menor en hijos y medio-hermanos. La distribución del riesgo en familias es indicativa de un modelo de heredabilidad poligénico.

Numerosos estudios genéticos de ligamiento realizados a finales de los 90 a partir de familias o estudios de asociación con un número discreto de marcadores y bajo poder estadístico determinaron más de 50 loci con una muy baja coincidencia entre diferentes estudios a excepción de la región del complejo mayor de histocompatibilidad (HLA), aunque proporcionaron información sobre el bajo efecto de los loci individuales en la enfermedad sugiriendo que un número considerable de genes con efectos relativamente pequeños estarían implicados en la susceptibilidad a EM.

Al principio de esta tesis el único locus de susceptibilidad a EM claramente confirmado en múltiples estudios tanto de ligamiento como de asociación era *HLA* de clase II. Posteriormente se ha definido el haplotipo *HLA- DQB1*0602-DQA1*0102 -DRB1*1501-DRB5*0101* como el que incrementa el riesgo a padecer EM (Oksenberg et al., 2008). Sin embargo, se desconoce si los propios alelos de susceptibilidad en los genes de HLA o otros en desequilibrio de ligamiento con ellos son los responsables del incremento de susceptibilidad en los pacientes.

Sistema de la Inrterleuqina-2 (IL2) / receptor de la IL2 (IL2R) y su implicación en la EM.

El reciente descubrimiento de la importancia de las células T reguladoras (Treg) (CD4+CD25high) en la tolerancia inmunológica y regulación inmune (Fontenot et al., 2005) hacen a los genes del sistema de la IL2/IL2R interesantes candidatos para la susceptibilidad a EM.

La autoinmunidad asociada con deficiencias en IL2/IL2R puede deber principalmente al fallo en la producción de células Treg. Esta dependencia crucial de IL-2 en el desarrollo tímico, homeostasis periférica y transmisión de señales en células Treg es mediada por el IL2R (Malek, 2008). Se ha sugerido que defectos persistentes en la función efectora de las Treg en pacientes de EM son responsables de la inadecuada respuesta específica de antígeno en células T (Viglietta et al., 2004; Haas et al., 2007). También se ha visto

afectada la función de las células periféricas Treg en pacientes de diabetes tipo 1 (DT1), soriasis, miastenia gravis autoinmune y artritis reumatoide (AR) (Sugiyama et al., 2005; Lindley et al., 2005; Balandina et al., 2003).

Estudios en el modelo experimental de EM, encefalitis autoinmune experimental (EAE), proporcionaron evidencias de la implicación del locus del *IL2* en la susceptibilidad y severidad a EM, sugiriendo que esta citoquina pudiera jugar un papel crucial en procesos autoinmunes del sistema nervioso central (Petitto et al., 2000; Encinas et al., 1999).

Polimorfismos en el promotor mínimo del *il2* de ratón que afectan la actividad transcripcional se han propuesto como candidatos a susceptibilidad en varios modelos de enfermedades autoinmunes como EAE, diabetes dependiente de insulina (en ratones NOD) y disgénesis ovárica autoinmune. Se ha visto que una menor producción intrínseca de la IL2 en el modelo de ratón NOD aumenta la susceptibilidad a diabetes (Yamanouchi et al., 2007).

La secreción de IL-2 se encontró significativamente inhibida en PBMCs estimuladas de pacientes de EM tanto en remisiones como recaídas (Selmaj et al., 1988; Wandinger et al., 1997), de forma similar, a lo que se ha descrito para otras enfermedades autoinmunes como DT1 (Roncarolo et al., 1988) y síndrome de Sjörgen (Miyasaka, 1984).

Estudios genéticos de ligamiento del todo genoma en familias con EM mostraron una asociación potencial de la región 10p15 con la enfermedad (Akesson et al., 2003). Esta región contiene el gen que codifica para la cadena alpha del receptor de la IL2 (*IL2Rα/CD25*). Esta asociación fue descrita también para DT1 mediante estudios de ligamiento de genoma completo y como gen candidato mediante la estrategia de Tag-SNP (Vella et al., 2005).

Genes de susceptibilidad comunes entre enfermedades de tipo autoinmune.

Se ha mostrado cierta coincidencia de loci de susceptibilidad entre diferentes enfermedades autoinmunes y también comparando las regiones génicas conservadas implicadas en modelos animales de diferentes enfermedades (Becker et al., 1998). Estas coincidencias apoyan la hipótesis de

que en ciertos casos enfermedades autoinmunes clínicamente diferentes puedan estar controladas por un conjunto común de genes.

EM muestra varias características comunes con otras enfermedades incluyendo heredabilidad poligénica, susceptibilidad parcial conferida por genes de HLA y evidencias de contribución ambiental. La predisposición genética en algunos pacientes con EM favorece el desarrollo de otras enfermedades autoinmunes como DT1 (Nielsen et al., 2006), enfermedad inflamatoria intestinal (Gupta et al., 2005) o lupus eritomatoso sistémico (LES) (Corporaal et al., 2002). Estos datos sobre factores de riesgo comunes a enfermedades autoinmunes inducen a plantear como genes candidatos a EM aquellos determinados para otras enfermedades.

Las citoquinas, por tener un papel esencial en la regulación de la respuesta inmune, son candidatos a participar en la etiologia de enfermedades autoinmunes. De hecho variantes de algunas de ellas y sus receptores se han visto implicadas en varias enfermedades de tipo autoinmune. Las citoquinas proinflamatorias se conocen por jugar un importante papel en la progresión y daño tisular en enfermedades de tipo autoinmune como EM, AR o LES. IL6 se considera como una de más importantes citoquinas proinflamatorias. Polimorfismos en el promotor de la *IL6* que afectan la expresión génica fueron asociados con susceptibilidad a AR (Fishman et al., 1998) y DT1 (Jahromi et al., 2000). Además, la IL6 contribuye a diferentes neuropatologías humanas y se ha implicado en la etiología del la EAE en ratones transgénicos para *IL6*. Ratones "knockout" para la *IL6* mostraron ser altamente resistentes a desarrollar EAE (Okuda et al., 1998). IL6 ejerce una actividad inhibitoria en la diferenciación de Th1 y T reg y en el cambio del balance de Treg a células Th17, sugerido como determinante en la patogénesis de la EAE.

Otro de los genes que se ha visto implicado en varias patologías de tipo autoinmune es el *FCRL3* que codifica una glicoproteína que es un miembro de la superfamilia de receptores de inmunoglobulinas. Aunque su función se desconoce, FcRL3 contiene motivos ITAM en su dominio citoplásmico sugiriendo un papel del receptor en regulación inmune (Ravetch & Bolland, 2001).

Tratamiento y respuesta a interferón.

En el tratamiento con base inmunológica de la EM el uso de los IFNs tipo I ha obtenido los resultados más prometedores. Su empleo en pacientes con EM se debió a la hipótesis de que la enfermedad podía estar causada por una infección viral persistente o latente del sistema nervioso central en personas con un sistema inmunitario alterado. Los IFN poseen actividad antiviral, antiproliferativa, inmunomoduladora y hormonal (Weinstock-Guttman, 1995). Sin embargo, a pesar de los buenos resultados de la terapia con IFNβ, existe un 30% de pacientes que no experimentan una mejoría con dicho tratamiento. Esta falta de respuesta puede ser causada por factores genéticos entre otros.

Entre las proteínas cruciales con actividad antiviral inducidas por IFNs de tipo 1 está la enzima OAS1 de la familia de las 2´,5´ adenilato sintetasas involucrada en la respuesta antiviral innata. Dado que las proteínas OAS se expresan de forma constitutiva a bajos niveles pueden actuar no solo como proteínas efectoras sino como receptores en el citoplasma de la célula reconociendo patrones de RNA (PRRs) de doble cadena para la detección de virus dsRNA. El RNA degradado por la RNasa L puede activar otros PRRs citoplásmicos, resultando en la inducción de la expresión IFN de tipo I (Malathi et al., 2007). Esto es un mecanismo de amplificación de la producción de IFN mediante la activación de la ruta de OAS-RNasa L.

La importancia directa de la proteína OAS 1 en la respuesta antiviral ha sido puesta de manifiesto por estudios genéticos que muestran que polimorfismos en el sitio aceptor de "splicing" del gen *OAS1* (que da lugar a dos isoformas de la enzima con diferente actividad) correlaciona significativamente con la respuesta antiviral a la fiebre amarilla. Por otra parte, estos polimorfismos se han visto asociados a susceptibilidad a DT1 (Bonnevie-Nielsen et al., 2005).

Estudios de Cribado de genoma completo en EM

Hasta la fecha se han realizado tres cribados de genoma completo (GWAS, Genome Wide Association Studies) en EM. En el estudio Wellcome TCCC (2007), aunque no es propiamente un estudio de genoma completo, se

analizaron 14.500 SNPs no sinónimos. Este estudio fue decepcionante ya que no logró determinar nuevos factores de riesgo para EM y hasta la fecha no se han replicado ninguna de las asociaciones publicadas. Otro de estos estudios lo realizó el consorcio formado por grupos americanos y británicos denominado IMSGC (Internacional Multiple Sclerosis Genetic Consortium) con el apoyo de la WTCCC con chips de 500,000 SNPs (IMSGC, 2007). Se ha estudiado en familias con EM el desequilibrio de transmisión de las variantes y replicación de los mejores asociaciones en un estudio posterior caso-control. El tercer estudio GWAS ha sido realizado por un grupo Norteamericano (Baranzini et al., 2008) analizando más de 550.000 SNPs en un estudio caso-control de caucasianos de origen europeo. Es interesante destacar que no hay una gran coincidencia entre los loci de riesgo detectados en los estudios con la excepción *del HLA, IL2Ra e IL7Ra*. Las razones de esta baja réplica de los loci detectados es que los estudios de GWAS por su carácter multiensayo y los bajos "odds ratio" de estas asociaciones producen un gran número de falsos positivos y requieren poblaciones muy grandes para confirmar o descartar las asociaciones detectadas.

Objetivo

El objetivo de esta tesis es identificar genes involucrados en la susceptibilidad a padecer EM y en la evolución de la enfermedad con la estrategia de genes candidatos y estudios funcionales con el fin de de revelar los mecanismos fisiopatológicos de la enfermedad. La identificación de los genes implicados en la susceptibilidad a padecer EM permitiría el descubrimiento de marcadores moleculares que tengan aplicación en su diagnóstico así como el planteamiento de posibles nuevas terapias fruto de un mejor conocimiento de los factores genéticos y rutas moleculares involucradas en la enfermedad.

Objetivos propuestos

1. Estudio del sistema de *IL2/IL2Ra* en relación con la susceptibilidad a EM:
    - Análisis de asociación del locus 4q27 donde se localiza el gen de la *IL2*

- Análisis funcional del polimorfismo *-330* del promotor de la *IL2* asociados al curso de la EM.
- Análisis de asociación con EM de polimorfismos del gen *IL2Rα* localizados en zonas reguladoras del gen.
- Análisis comparativo de los polimorfismos asociados a EM y DT1 y localización de los SNPs causales de estas asociaciones.

2. Estudio en EM de genes asociadas a otras enfermedades de tipo autoinmune:

- Análisis de asociación de variantes del gen de la *IL6* en EM previamente asociado a AR.
- Análisis de asociación de variante del gen *FCRL3* en EM previamente asociado a AR, DT1 y LES.

3. Validación de la asociación determinada por estudios GWAS en EM con variantes del gen *IL7Rα*.

4. Análisis en EM del gen *OAS1* de respuesta a interferón respecto a susceptibilidad y respuesta al tratamiento con IFNβ.

Pacientes y métodos

La selección de los genes candidatos en relación a la susceptibilidad a EM se ha realizado combinando diferentes criterios para identificar y dar la prioridad a los genes de susceptibilidad:

- Selección basada en localización en regiones previamente determinadas por su asociación a EM en estudios de ligamiento y GWAS.
- Selección de acuerdo a criterios funcionales, tanto por haberse demostrado su implicación en EM en estudios de modelos animales como por haberse demostrado su importancia en autoinmunidad.
- Selección por haberse visto asociado a susceptibilidad con otras enfermedades autoinmunes.
- Selección por tener una función importante en la ruta de señalización de INF tipo I.

Las variantes analizadas en esta tesis son variaciones de una sola base (SNPs), polimorfismos que suponen la mayor fuente de variabilidad entre personas. El criterio de selección de estos SNPs dentro de un locus es tener potencial efecto funcional. En este sentido se seleccionaron SNPs que supusieran cambios de aminoácidos, que se localizaran en regiones aceptoras o donadoras de "splicing", en regiones UTR (regiones que no se traducen) y zonas promotoras o reguladoras de la expresión.

Se emplearon mayoritariamente dos sistemas de genotipado para la determinación de las variantes – PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism) técnica que radica en el corte diferencial con endonucleasas de restricción de los productos amplificados por PCR y Taqman, este ensayo utiliza la actividad 5' nucleasa de la polimerasa junto con dos sondas TaqMan para discriminar entre los dos alelos de un SNP.

Varios ensayos funcionales se realizaron para determinar el efecto de los polimorfismos localizados en el promotor. Estos se realizan mediante un gen reportador, luciferasa, en transfecciones transitorias y medidas de la actividad luciferasa en los extractos celulares.

Los ensayos cuantitativos de la expresión de diferentes genes fueron realizados por transcripción reversa del RNA celular y posterior PCR a tiempo real utilizando SYBR Green como fluoroforo.

Las muestras de ADN y ARN utilizados en este trabajo proceden de una cohorte de pacientes de EM y controles sanos que a día de hoy cuenta con 800 pacientes de EM no relacionados procedentes de cuatro hospitales de Andalucía (Hospital Clínico San Cecilio de Granada y Hospital Virgen de las Nieves de Granada, Hospital Carlos Haya de Málaga y Hospital Virgen Macarena de Sevilla). Los controles sanos procedentes de bancos de sangre de cuatro provincias de Andalucía (Granada-Almeria, Malaga, Sevilla).

RESULTADOS

Genes del sistema de *IL2/IL2Ra*

Se ha dedicado una parte significativa de esta tesis al estudio de papel de los genes de la *IL2* y del *IL2Ra* en la susceptibilidad a EM.

Tras el análisis de varios polimorfismos en la región promotora y codificante del gen de la *IL2* respecto a la susceptibilidad a padecer EM en estudios caso-control, determinamos que la variante *-330 G* (rs2069762) se encontraba sobre-representada en pacientes de EM aunque no alcanzaba significación estadística, sin embargo este alelo se vio asociado a susceptibilidad con la forma secundaria progresiva (SP) de la enfermedad. Esta asociación pudimos confirmarla posteriormente en una cohorte más amplia manteniéndose lo observado en el estudio original. La estratificación de los pacientes por los cursos de la enfermedad, remitente recidivante (RR) y SP, podría aumentar la potencia estadística del estudio debido a una mayor homogeneidad clínica de los pacientes y permitir la determinación de la asociación del polimorfismo *-330 G/T* respecto a la enfermedad. La transición del curso RR a SP ocurre en aproximadamente 50% de los pacientes RR con un periodo de transición en tiempo variable e impredecible (Tremlett et al., 2008)

Se analizaron los efectos del polimorfismo *330G/T* sobre la expresión del gen de la *IL2* viéndose un efecto alelo especifico sobre la expresión. Los estudios de transfección en células Jurkat con el gen reportador de la luciferasa controlado por las dos variantes del promotor *G* o *T*, mostraron que la construcción que portaba el alelo *G* era dos veces más activa que la portadora de *T*. Por otra parte, ensayos de cuantificación relativa de la expresión del gen de la *IL2* en PBMCs activadas provenientes de diferentes personas mostró una menor expresión de los transcritos provenientes del promotor con la variante *G*. Estos datos estuvieron en consonancia con los obtenidos tras la cuantificación total de la expresión del gen de la *IL2* por PCR a tiempo real, observándose mayores niveles de expresión de *IL2* en muestras de individuos con genotipo *TT* y *GT* que *GG*.

Esta divergencia encontrada entre los resultados "in vitro" con ensayos de transfección en células Jurkat e "in vivo" en PBMC de diferentes individuos sugiere que el polimorfismo *-330* del promotor "in vivo" podría estar modulado por otras variantes de las que se habría aislado en el estudio "in vitro" al analizar un pequeño fragmento de la región 5' del gen. Estos

resultados reflejan el complejo mecanismo de regulación de la expresión del gen de la *IL2* e indican que la influencia del polimorfismo *-330* está modulada por otros elementos. Por otra parte, estas alteraciones de expresión estarían implicadas en la EM dada la asociación de este SNP funcional con la enfermedad.

Posteriormente al trabajo mencionado, el locus *TENR-IL2-IL21* se vio asociado en GWAS a DT1, enfermedad de Graves, enfermedad celiaca, AR y soriasis (Todd et al., 2007; van Heel et al., 2007; Liu et al., 2008). Nosotros analizamos los polimorfismos que se habían descrito asociados a estas enfermedades en un estudio caso control con nuestra colección de pacientes y controles de EM. No pudimos replicar la asociación para el SNP rs6822844 teniendo una potencia estadística de 94%. Tampoco observamos asociación con EM del SNP rs3136534. Por tanto, nuestros resultados no mostraron mayor influencia del locus *TENR-IL2-IL21* en la susceptibilidad a padecer EM, este estudio no podía descartar asociaciones con OR inferiores a 1,15. Se requerirían estudios con una cohorte mayor así como una mayor densidad de marcadores en la región para descartar o confirmar el papel del locus de la *IL2* en la EM.

La cadena alpha del receptor de la IL2 tiene un papel regulador esencial en la función de la IL2. Nosotros planteamos un estudio con el objeto de analizar la posible implicación del gen *IL2Ra* en EM. Para ello analizamos cuatro polimorfismos localizados en regiones reguladoras de la expresión del gen. Encontramos una asociación significativa del alelo *T* del polimorfismo rs1570538 con susceptibilidad a padecer EM. Este polimorfismo se localiza en la región 3' UTR del gen de la *IL2Ra*. Este estudio implica por primera vez el *IL2Ra* en susceptibilidad a EM. En estudios posteriores GWAS realizados por el IMSGC se confirmo este locus como factor de riesgo a EM (IMSGC, 2007).

A pesar de que la asociación del locus con EM es un hecho, se requería la determinación de la variante causal de la asociación para determinar las implicaciones funcionales en la enfermedad. Por esta razón profundizamos en el estudio del locus realizando un mapeo fino analizando 8 SNPs localizados a lo largo del gen y en la parte 5' de este. Estas variantes fueron seleccionadas

tomando como base un estudio de mapeo fino del locus de la *IL2Rα* en relación a DT1 (Lowe et al., 2007), así como el estudio GWAS realizado en EM por IMSGC y nuestro propio estudio. Por otra parte incrementamos la potencia del análisis aumentando el número de muestras a 800 pacientes y un número equivalente de controles sanos. Con este estudio confirmamos la asociación del locus de la *IL2Rα* con EM aunque se encontraron diferencias entre las variantes asociadas a diabetes tipo 1 y EM. El polimorfismo más asociado con diabetes (rs41295061) no mostró asociación con EM. Por otra parte, el SNP rs35285258, perteneciente a otro grupo de asociación a DT1 independiente del SNP rs41295061 mostró máxima asociación en EM pero con alelo de riesgo contrario a DT1. Desconocemos si estos polimorfismos tienen algún efecto funcional sobre la expresión del *IL2Rα* ya que ninguno de ellos se localiza en regiones reguladoras conocidas del gen. Sin embargo, otros grupos han relacionado el polimorfismo rs35285258 con variaciones en la concentración del IL2-Rα soluble lo que sugiere que esta variante u otra de la región que se encuentre en desequilibrio de ligamiento con ella podrían modular la expresión del gen (Lowe et al., 2007). Por otra parte, nuestros resultados y obtenidos simultáneamente por otro grupo para DT1 (Maier et al., 2009) sugieren que los efectos de estas variantes en DT1 y EM son contrarios y por tanto con una implicación diferente en los mecanismos inmunopatológicos de la enfermedad.

Genes comunes entre diferentes enfermedades de tipo autoinmune.

La implicación del gen *IL6* en susceptibilidad a varias enfermedades de tipo autoinmune y su potencial papel modulador del curso de la enfermedad demostrado en el modelo EAE nos impulsó a investigar variantes del gen de la *IL6* en relación a la susceptibilidad a la enfermedad.

Previamente a este estudio se había determinado asociación de un SNP funcional en posición *-174* del gen de la *IL6* con AR juvenil y DT1. Nosotros analizamos este polimorfismo en relación con susceptibilidad a EM no encontrando diferencias en la distribución de los genotipos de la variante *-174 IL6* con respecto al grupo enfermos y controles, también estratificando por

género o curso de la enfermedad. No obtuvimos ninguna evidencia que sugiera que la variante funcional del promotor de la *IL6* tenga un papel en la patología de la EM a diferencia de otras enfermedades autoinmunes.

Otro gen que se ha visto implicado en susceptibilidad a varias enfermedades autoinmunes es el *FCRL3*. Nosotros analizamos en nuestra cohorte de enfermos de EM y controles sanos la implicación en la susceptibilidad a EM de tres polimorfismos que mostraron máxima asociación in distintas poblaciones y enfermedades autoinmunes. Los polimorfismos seleccionados fueron: una variante funcional en el promotor del gen *FCRL3* en posición *-169* (*FCRL3_3* (rs7528684) que altera la unión del factor de transcripción kappa B al promotor y que regula la expresión del gen, una variante en posición *-110* (rs11264799) y un polimorfismo en el tercer exon que produce un cambio de aminoácido Asp/Asn.

Observamos una asociación del alelo C del polimorfismo *-169 C/T FCRL3* con protección a EM. El alelo *C* esta relacionado con una mayor expresión del gen y es un factor de riesgo para AR, LES y enfermedad tiroidea autoinmune (Kochi et al., 2005; Thabet et al., 2006), mientras que es un factor protectivo en EM. El análisis de haplotipos mostró que el haplotipo portador del alelo *C* del polimorfismo *-169 C/T FCRL3* estaba fuertemente asociado con protección. Sin embargo, no podemos descartar que otra variante dentro del mismo bloque de desequilibro de ligamiento en la región 5' del en *FCRL3* o el gen *FCRL2* que se encuentra adyacente sea el causante de la asociación.

Validación de loci asociados a EM por estudios de GWAS

Simultáneamente a la realización de esta tesis el consorcio internacional para la esclerosis múltiple (IMSGC) realizo un estudio de cribado de genoma completo con más de 500000 marcadores en cerca de 931 tríos (hijo enfermo, padre y madre sanos). De este estudio se determinaron una serie de locis asociados a EM, algunos como el *HLA* o el *IL2Ra* ya se conocían como factores de riesgo a la EM. Otros loci fueron descritos por primera vez en asociación a la enfermedad. Sin embargo, estas asociaciones determinadas por GWAS deben ser confirmadas en otras poblaciones de enfermos de EM

dado que este tipo de estudios produce un gran número de asociaciones espurias por su carácter multiensayo.

Uno de estos loci contenía el gen que codifica para el receptor de la IL7 (*IL7Rα*). En otro estudio independiente se determinó que la variante *T244I* del gen de *IL7Rα* (rs6897932), se asociaba a EM (Gregory et al., 2007). Esta variante parecía alterar el "splicing" de los transcritos de la *IL7Rα* modificando el balance entre dos formas del tránscrito con diferente composición de exones lo que podría dar origen al aumento de una forma soluble del receptor en detrimento de la forma unida a la membrana. Nosotros decidimos analizar esta variante en nuestra cohorte de pacientes y controles de EM con el objeto de confirmar o descartar la asociación con la enfermedad. Nuestros datos mostraron una mayor representación del alelo C de la variante rs6897932 en casos respecto a controles en consonancia con los datos publicados para otras poblaciones sugiriendo una implicación de la IL7 en la EM. De hecho la IL7 juega un papel fundamental en la supervivencia, diferenciación y proliferación de las células T lo que la sitúa como una molécula clave en el control de la autoinmunidad.

Genes de la ruta del interferón respecto a la susceptibilidad a EM y respuesta al tratamiento

Uno de los tratamientos con más éxito en la actualidad para la EM es el IFNβ. Sin embargo, hay una gran diversidad por parte de los pacientes con respecto a la respuesta a este tratamiento. En la actualidad se desconoce la causa de esta falta de respuesta aunque los factores genéticos podrían tener un papel fundamental. Por otra parte, nuestro grupo, en varios trabajos, ha demostrado que algunos genes de respuesta a IFN tipo I están implicados en la susceptibilidad a EM.

En esta tesis nos propusimos estudiar la influencia de variantes del gen *OAS1* (2',5'- oligoadenylate synthetase 1) respecto a la susceptibilidad a EM así como respuesta al tratamiento. Se analizaron SNP situados en el aceptor de "splicing" del exon 7 (rs10774671) y un SNP no sinónimo (Ser162Gly) (rs3741981), situado en el exon 3.

Nosotros encontramos que el haplotipo GA creado por el alelo G en la variante rs10774671 y el alelo A de rs3741981 es 4.6 veces mas frecuente en enfermos de EM respecto controles sanos. No se encontró ninguna asociación ni de alelos, genotipos o haplotipos de estos marcadores respecto a la respuesta al tratamiento. Estos resultados conjuntamente con otros que muestran asociación de variantes de genes de respuesta a IFN tipo I como Interferon regulatory factor 5 (*IRF5*) (Kristjansdottir et al., 2008) o de las cadenas del receptor evidencian la implicación de esta ruta en susceptibilidad a EM (Leyva et al., 2005). Dada la función antivírica de la ruta del interferón es tentativo sugerir que su implicación en la EM podría estar relacionada con la susceptibilidad a ciertas infecciones víricas relacionadas con la patogenía.

CONCLUSIONES

1. El polimorfismo *-330* del promotor del gen de la IL2 se asocia con susceptibilidad a la forma secundaria progresiva de la EM. Este polimorfismo modifica los niveles de expresión alélica y su efecto es modulado por otros elementos reguladores "in vivo". La expresión de la IL2 se ve alterada en pacientes de EM lo que sustenta la importancia de esta citoquina en la enfermedad.
2. Los polimorfismos -475 y -631 del promotor del gen de la IL2 no contribuyen a la susceptibilidad a EM.
3. Nuestros resultados descartan una importante influencia de las variantes del locus *TENR-IL2-IL21* asociadas con DT1, AR y enfermedad celiaca aunque este locus parece tener un papel en progresión de la enfermedad.
4. Se ha determinado por primera vez la asociación de locus del *IL2Ra* con susceptibilidad a padecer EM. El análisis de polimorfismos en este locus reveló una heterogeneidad entre EM y DT1 con respecto a la asociación genética sugiriendo una implicación diferente de esta ruta en la inmunopatología de estas enfermedades.
5. No se encontraron evidencias de que la variante del promotor del gen de la IL6 asociada a otras enfermedades autoinmunes sea un factor de riesgo para la EM.
6. La variante funcional *-169C* del promotor del gen *FCRL3* determinada como factor de riesgo para varias enfermedades autoinmunes es un factor de protección para EM.
7. La variante funcional rs6897932 *C* del *IL7Ra* se confirma como factor de riesgo a EM en la población española.
8. Haplotipos pero no variantes independientes revelaron una asociación respecto EM del gen *OAS1* involucrado en respuesta innata antivírica. Estos datos apoyan la implicación de la ruta del IFN tipo 1 en EM y sugieren que la asociación de *OAS1* con la enfermedad podría deberse a su papel antivírico.

INTRODUCTION

INTRODUCTION

1. CLINICAL ASPECTS OF MS

Multiple sclerosis (MS) is a chronic, inflammatory and demyelinating neurological disease affecting the central nervous system (Ebers et al., 1995). In most Caucasian populations, MS is second only to trauma as a cause of acquired neurologic disability arising in early to middle adulthood.

A general consensus is that MS begins with the formation of acute inflammatory lesions characterized by breakdown of the blood-brain barrier (BBB). Such lesions are often clinically "silent" and have been estimated to be about ten times more frequent than episodes of clinical worsening (Stone et al., 1995). Disruption of the BBB typically lasts for about a month and then resolves leaving an area of damage that can be visualized by conventional magnetic resonance imaging (MRI). Both immunological and genetically determined vulnerability factors of CNS tissue may influence the outcome of these acute lesions.

As the name of the disease implies, affected individuals exhibit hardened (or "sclerotic") tissue in many (or "multiple") parts of the brain and spinal cord. The lesions observed in MS are typically characterised by a demyelinated core separated by a very sharp border from normally myelinated surrounding tissue. These lesions are characterized by a perivascular infiltration by immune cells, notably CD4+ and CD8+ T cells during the acute inflammatory phase of lesion formation (Junker et al., 2007). Inflammation in the progressive disease phase is not restricted to focal lesions and appears to be responsible for a more diffuse pattern of tissue damage affecting the nervous system as a whole. This diffuse white matter damage is difficult to visualise with classical histological and imaging techniques, and its importance has only recently been appreciated. Axonal damage can be present from early in the disease course, with numerous transected axons containing amyloid precursor protein. Finally, end-state lesions contain glial

scar tissue generated by activated astrocytes, it is visible macroscopically (Stadelman & Brück, 2008). This scar tissue in the CNS impair the saltatory conduction along axons that is necessary for normal functioning of nerve impulses thus leading to progressive physical disability.

MS is classically regarded as a white matter disease. However, recent histopathological studies have convincingly shown that grey matter regions are also heavily affected. Grey matter damage starts early in the disease and substantially affects clinico-cognitive functioning (Geurts & Barkhof, 2008) with a particularly high prevalence of cortical demyelination being observed in progressive forms of the disease.

MS is a clinically heterogeneous disease that varies according to the location of plaques or lesions in the CNS. Common symptoms include visual disturbances, loss of balance and coordination, spasticity, sensory disturbances, bladder and bowel incontinence, pain, weakness, fatigue, and paralysis. This debilitating disease also causes cognitive impairment in an estimated 45–65% of patients—with symptoms ranging from bradyphrenia to language deficits. Despite the impairment and deterioration seen in the disease, life span of affected individuals is only slightly shortened—creating a significant impact on the quality of life for patients and on a nation's healthcare system.

The course of MS varies considerably among affected individuals. Cases may be episodic or progressive, severe or mild, and disseminated or primarily affecting the spinal cord and optic nerve. Disease progress can range from an occasional nuisance over a lifetime to mortality within a year. In addition to clinical criteria, magnetic resonance imaging, evoked potential recordings, and cerebrospinal fluid examination can be used to confirm clinical diagnosis. A common abnormality detected in MS patients is also increased intrathecal immunoglobulin synthesis reflected by raised IgG indices and an oligoclonal pattern in CSF.

The course of MS is divided into major subtypes: relapsing–remitting and primary progressive. At the onset of the disease, 85–90% of the patients

present a clinical course characterized by discrete attacks followed by periods of partial or total recovery - relapsing-remitting MS (RRMS). Many relapsing–remitting cases cease to remit and exhibit progression of at least one symptom in a slow or step-wise manner over at least 6 months. This form of the disease is referred to as secondary progressive MS (SPMS). Transition from RR to SP MS course occurs in more than 50 % of RR patients, it can take different periods of time and is still unpredictable (Lublin & Reingold, 1996; Tremlett et al., 2008). A continuous and irreversible loss of brain tissue occurs during the chronic stages of MS despite a dramatic reduction or paucity in new demyelinating lesions. The progressive increase in ventricular volume highlights the brain atrophy that occurs as most MS patients age (Trapp, 2008) (Figure 1). The primary progressive MS is a less common form (10% at onset) characterized by slow and steadily worsening symptoms involving sites of the CNS that do not remit from initial onset.

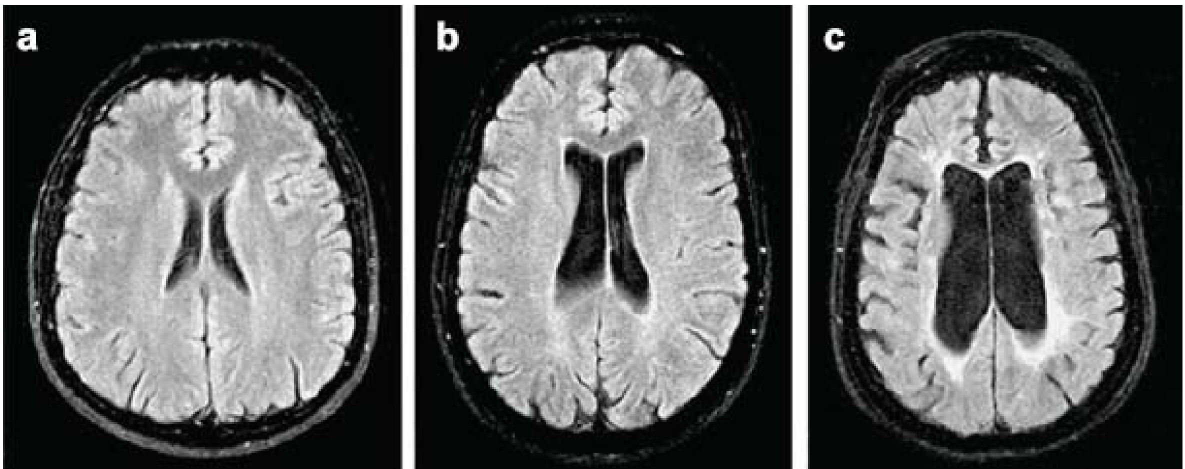

Figure 1. A continuous and irreversible loss of brain tissue occurs during chronic stages of MS. a. Normal brain. b. Brain of a relapsing-remitting MS patient. c. Brain of a secondary progressive MS patient at end-stage disease (Trapp, 2008).

Considerable heterogeneity has been observed in the demyelinating plaques of MS. Pathological analysis of *actively* demyelinating lesions has led

to identification of four distinct patterns of demyelination, based on parameters, such as the presence/ absence of Ig and complement deposition, myelin protein loss, patterns of oligodendrocyte degeneration and T cell-/macrophage-dominated immune responses. In addition to T-cell–mediated or T-cell plus antibody–mediated autoimmune encephalomyelitis and demyelination in the most common types I and II plaques, there were demonstrated other patterns (III and IV) highly suggestive of a primary oligodendrocyte dystrophy, reminiscent of virus- or toxin-induced demyelination rather than AI (Lucchinetti et al., 2000). Nevertheless initial heterogeneity of demyelinating lesions in the *earliest* phase of MS lesion formation may disappear over time in *established* MS as consistent presence of complement, antibodies, and Fcγ receptors in phagocytic macrophages has permited to suggest that antibody- and complement-mediated myelin phagocytosis is the dominant mechanism of demyelination in *established* MS (Breij et al., 2008). However the detection of plaques that are distant from blood vessels and are characterized by prevalence of microglial cells and myelin loss associated with significant oligodendrocytic apoptosis suggested the existence of alternative mechanisms of etiopathogenesis (Barnett & Prineas, 2004; Kutzelnigg et al., 2007; Lucchinetti et al., 2000; Pittock & Lucchinetti, 2007). Several pathological studies of lesions even suggest that MS is an overlapping spectrum of related disorders (Genain, 1999,; Raine et al., 1999; Lucchinetti et al., 2000; Kornek & Lassmann, 2003). Evolving views of the pathogenesis of MS encompass both inflammatory and degenerative components (Frohman et al., 2006).

2. ETIOLOGY OF MS

The basic pathology of MS was already recognized in the 18th century. Immunology, neuroscience, epidemiology and genetics today provide new insight; nevertheless important aspects of its cause and pathogenesis remain unknown. MS is thought to result from a complex interaction between genetic and environmental factors (Dyment et al., 1997). Whether infectious agents,

environmental toxins, mutagenesis, or nutrient deficiencies cause MS is uncertain; the nature of the triggering event leading to exposure of immunogenic myelin-derived peptides remains unanswered.

There are several lines of evidence to support the long-held belief that MS is a disease with an autoimmune etiology. One hypothesis suggested that autoimmune demyelination is triggered by a mechanism of "molecular mimicry" to viral or bacterial proteins after a non-specific initial infection (Libbey et al., 2007). Alternative hypotheses have implicated mechanisms that are intrinsic to the myelinating cells and depend on aberrant regulation of gene expression in myelinating oligodendrocytes (Casaccia-Bonnefil et al., 2008) or posttranslational modifications of myelin proteins as the deimination of myelin basic protein (MBP) associated with the exposure of an immunodominant epitope of the molecule (Moscarello et al., 2007; Musse & Harauz, 2007). An alternative possibility has been suggested by the recent identification of autocatalytic anti- MBP antibodies in the sera of MS patients, characterized by proteolytic activity against the auto-antigen (Belogurov et al., 2008).

Several reports now exist which question the evidence that MS is an autoimmune disease suggesting a much more complex pathophysiology; underscoring that demyelination, neuro-dysfunction and possibly neurodegeneration can occur in MS brain independent of the inflammatory lesion (Trapp, 2004). Evidence has also been advanced to support an infectious etiology for MS (see part 4.2).

3. MS PATHOGENESIS

Autoimmune attack drives MS? Although the primary role of the immune system in MS has been questioned, the pathological hallmark of active MS lesions is the presence of mononuclear immune cell infiltrates and demyelination (Figure 2). Substantial recent progress in MS therapy through early and aggressive immunotherapy with monoclonal antibodies targeting

lymphocytes underscores the fact that perturbation of the immune system drives the disease through its early stages (Lassmann, 2008).

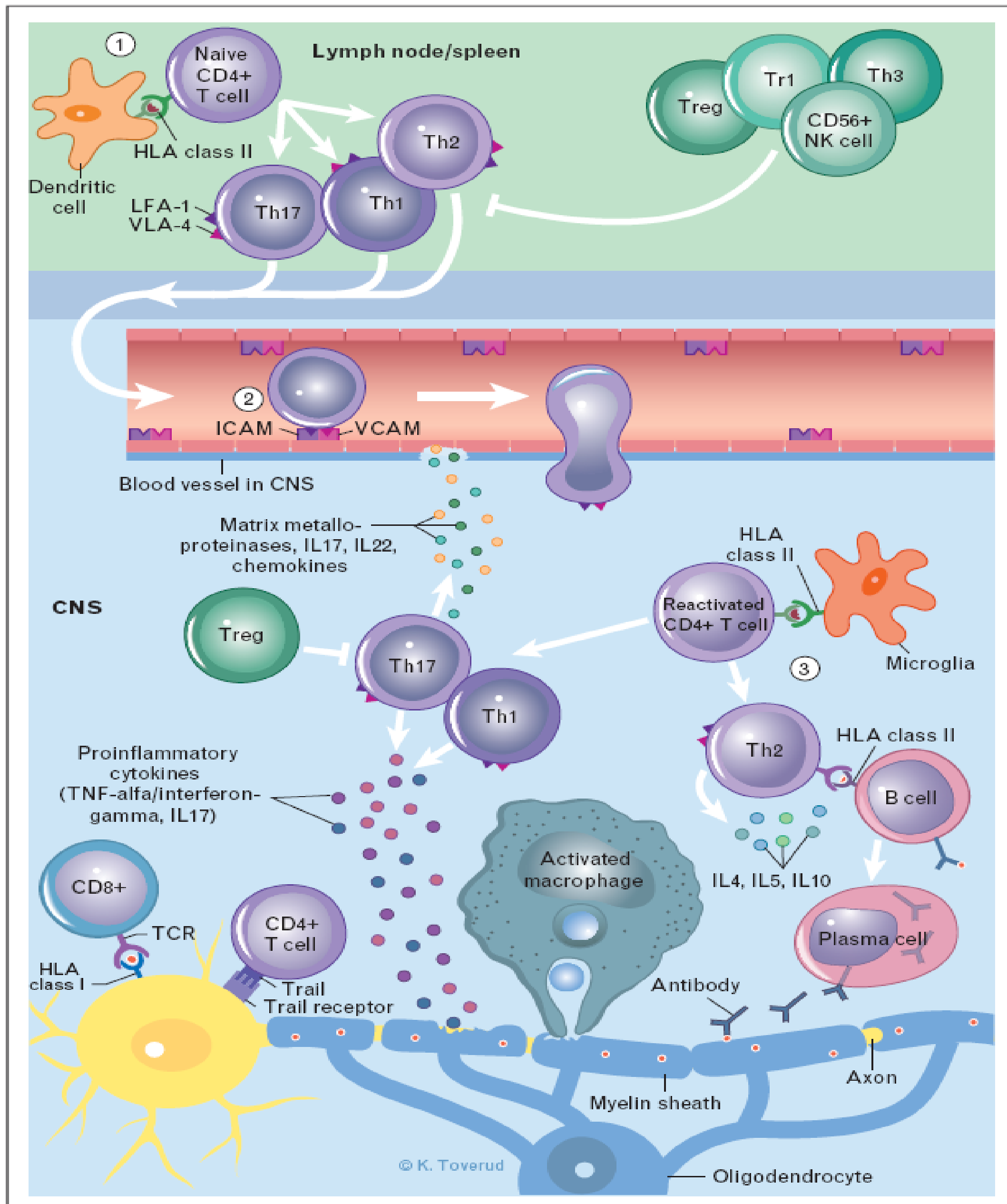

Figure 2. Pathogenesis of MS (Holmøy & Hestvik, 2008)

3.1. The role of T cells in the pathogenesis of MS

3.1.1. CD4+ T cells

The data that acute lesions are initiated by CD4+ T helper cells in a way similar to what has been demonstrated in EAE are wide accepted. It is believed that T lymphocytes react against myelin components and activate microglia and macrophages, leading to damaged myelin sheaths with impaired nerve conduction (Steinman et al., 2002).

By the prevailing theory of molecular mimicry it is suggested that CD4+ T cells are activated by a foreign antigen cross-reacting with myelin (Libbey et al., 2007). Only nonactivated myelin reactive T cells are present in the blood of controls, in contrast, activated myelin-reactive CD4+ T cells are found in the blood and CSF of MS patients (Zhang et al., 1994).

Considerable interest has developed about the role of T-cell responses to MBP as a potencial autoantigen in MS. Oligoclonally expanded Th cells receptors specific for MBP are found in MS lesions (Oksenberg et al. , 1993) and some MS patients show an immunodominant MBP peptide complexed with HLA-DR2 on APCs at sites of demyelination (Krogsgaard et al., 2000). The involvement of a CD4+ T cell–mediated process is consistent with the association of genetic risk with HLA class II molecules (Olerup & Hillert, 1991)

MBP-reactive T cells from MS patients are found to be less responsive to CTLA-4 blockade compared to those from healthy controls (Oliveira et al., 2003), signifying that in MS patients these T cells are not subject to the normal regulatory mechanisms. Although the contribution of MBP-reactive T cells to the pathogenesis of MS is currently unknown, their differential phenotype and costimulatory requirements indicate a memory and potentially dysregulated cell population.

Nevertheless, primary target antigens are still largely unknown. Moreover, it is not known what factors trigger the autoreactive T cells to migrate into the CNS and mediate disease, though environmental factors, such as infections have long been suspected (Sibley et al., 1985).

3.1.2. CD4 Th1 or Th17 cells?

The interleukin-12 (IL-12)/ T-helper 1 (Th1) pathway was initially thought to be responsible for the pathogenesis of multiple chronic inflammatory diseases, including psoriasis, inflammatory bowel disease (IBD), arthritis (RA), or MS, mainly through their production of interferon-gamma (IFNγ) and its effects on macrophage activation and chemokine production (Zamvil & Steinman, 1990). Later it was shown that loss of IFNγ did not prevent the development of AI that raised the possibility of involvement of other Th subsets, different from Th1 cells, in the induction of autoimmunity (AI) (Becher et al., 2002; Gran et al., 2002). Evidence now indicates that CD4+ Th 17 cells may be the major cell type involved in orchestrating tissue inflammation and AI in EAE.

Naïve T cell differentiation into Th17 and their further expansion require an adequate mix of cytokines acting on specific nuclear factors (Figure 3) (Benghiat et al., 2009).

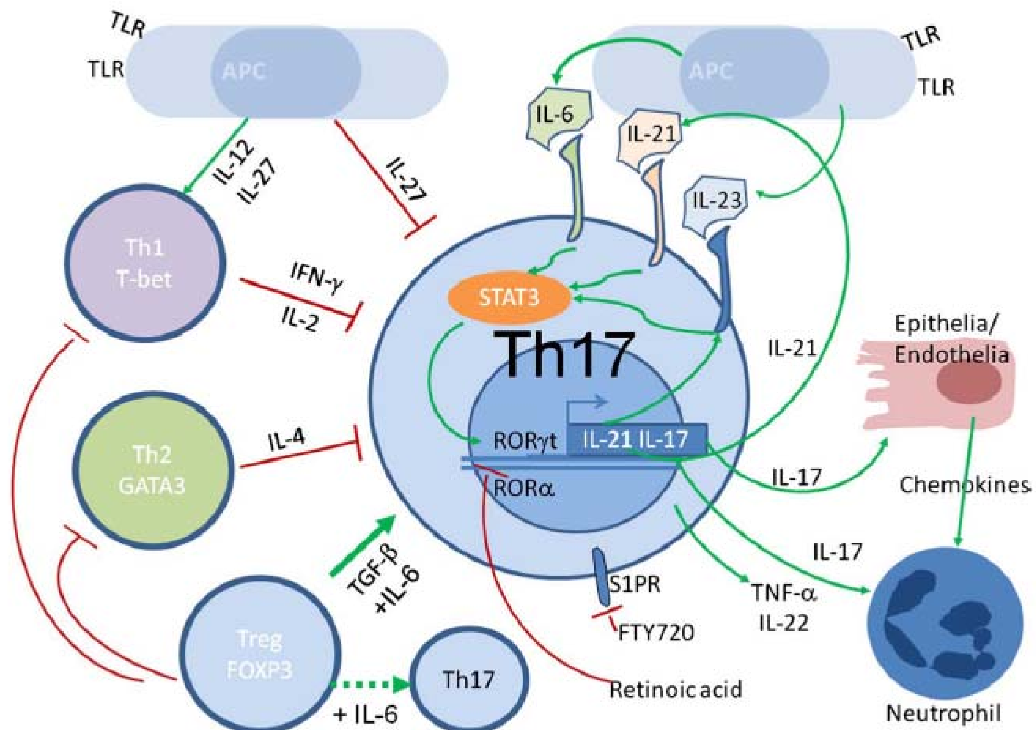

Figure3. Th17 cell differentiation and regulation (Benghiat et al. ,2009)

It is observed that in the natural course of EAE both Th1 and Th17 cells infiltrate the CNS together with $Foxp3^+$ Treg cells, but the Th17 cells in the CNS peaked earlier than the Th1 cells (Korn et al., 2007). Although it is not clear how Th1 and Th17 cells interact with each other and promote autoimmune tissue injury. Thus, the debate is open on the role of Th1 and Th17 and their interaction in the patogenesis of MS. Interestingly, human Th1 cells and Th17 cells express different sets of chemokine receptors (Sato et al. 2007), indicating that they might be recruited to different types of inflammatory lesions or to different anatomical sites.

In various EAE models of MS, it is shown that Th1 and Th17 cells are independently capable of inducing paralysis and inflammation nevertheless the pathology in EAE lesions induced by Th1 cells more closely resembles MS than the pathology induced by Th17 cells (Kroenke et al., 2008). It remains a matter of debate whether classic Th1 cells in humans play a protective role against the pathogenic activity of Th17 cells or contribute alongside them to the pathogenesis of autoimmune as well as chronic inflammatory disorders (Annunziato et al., 2008).

New data point that Th17 cells can act not only as disease inducers but also as innocent bystanders or protectors (Steinman, 2008). Mice deficient in T-bet and STAT-4, and thus lacking Th1 cells have overwhelmingly large numbers of Th17 cells and are resistant to EAE (Chitnis et al., 2001; Bettelli et al., 2004; Lovett-Racke et al., 2004).

A further point in the Th1 vs Th17 debate is the observation that a considerable population of cells secreting both IL-17 and IFNγ can consistently be detected *in vivo* in the inflamed CNS. This may indicate the existence of a transitional cell that expresses both IFNγ and IL-17 *in vivo*. Some reports propose that lineage commitment of T helper cells may be occurring in the CNS and that CNS-derived antigen presenting cells might have the capacity to differentially activate and possibly further differentiate Th17 cells, Th1 cells and Treg cells depending on the phase of EAE (Deshpande et al., 2007).

In the recent study Kryczek and coworkers (2008) on the role of IFNγ in the development and balance between Th17 and Th1 in humans, the authors

propose a novel dynamic between Th1 and Th17 in the course of inflammation as follows: Th1-mediated inflammation is attenuated by IFNγ -induced B7-H1 on APCs and is evolved toward Th17-mediated chronic inflammation by IFNγ - induced, APC-derived IL-1 and IL-23.

On the basis of studies in EAE, Dardalhon and co-workers (2008) propose a working model for the role of Th1 and Th17 cells in the induction of organ-specific autoimmune disease in which Th17 cells constitute the first wave of effector T cells migrating to the CNS and orchestrate the recruitment of further waves of effector T cells, particularly Th1 cells. Supporting the idea that both Th1 and Th17 cells are capable of inducing AI in human, Steinman (2008) suggests that no single molecule or Th pathway dominates and that there is no hierarchical scheme at all.

3.1.3. CD8+ cells in MS

The view on the role of CD4+ T cells was severely challenged when depletion of these cells in MS patients caused no improvement in relapse rates (van Oosten et al., 1997) and a global depletion of CD4+ T as well as CD8+ cells with an anti-CD52 monoclonal antibody led to reductions in relapses and new lesions, though with little improvement in long-term neurological deficits (Coles et al., 1999).

Now CD8+ T cells are considered as important players in MS pathogenesis. CD8+ as well as CD4+ T cells are found to invade and clonally expand in inflammatory central nervous system plaques. The pathology studies based on T cell receptor analysis indicate that CD8+ T cells may be the most common subset of T cells in the MS brain (Babbe et al., 2000). CD8+ T cells are present at the lesion edge as well as perivascular regions, whereas the CD4+ T cells are generally present only at the lesion edge.

In addition, a higher frequency of CD8+ T cells recognizing myelin proteins has been reported in patients with MS than in healthy controls (Crawford et al., 2004). IL-17+ cells are found to constitute a rather high proportion of CD8+ T cells in MS in contrast to EAE in which IL-17+ cells are reported to be mainly CD4+ (ie, Th17) (Langrish et al., 2005).

The potential of CD8 T cells to induce oligodendrocyte lysis in vivo as a likely consequence of direct Ag-recognition is recently demonstrated in EAE (Saxena et al 2008). With regard to human MS, autopsy studies demonstrate that CD8+ T cells are frequently associated with axonal injury in MS (Bitsch et al., 2000). Demyelination leads to the upregulation of MHC class I on axons and neurons within human MS lesions (Hoftberger et al., 2004), so the recognition of MHC class I on axons by CD8+ T cell might directly mediate axonal injury and neurologic deficit in MS (Howe et al., 2007). This idea is supported by data showing that CD8+ cells can, in some conditions, directly attach to and damage axons (Medana et al., 2001).

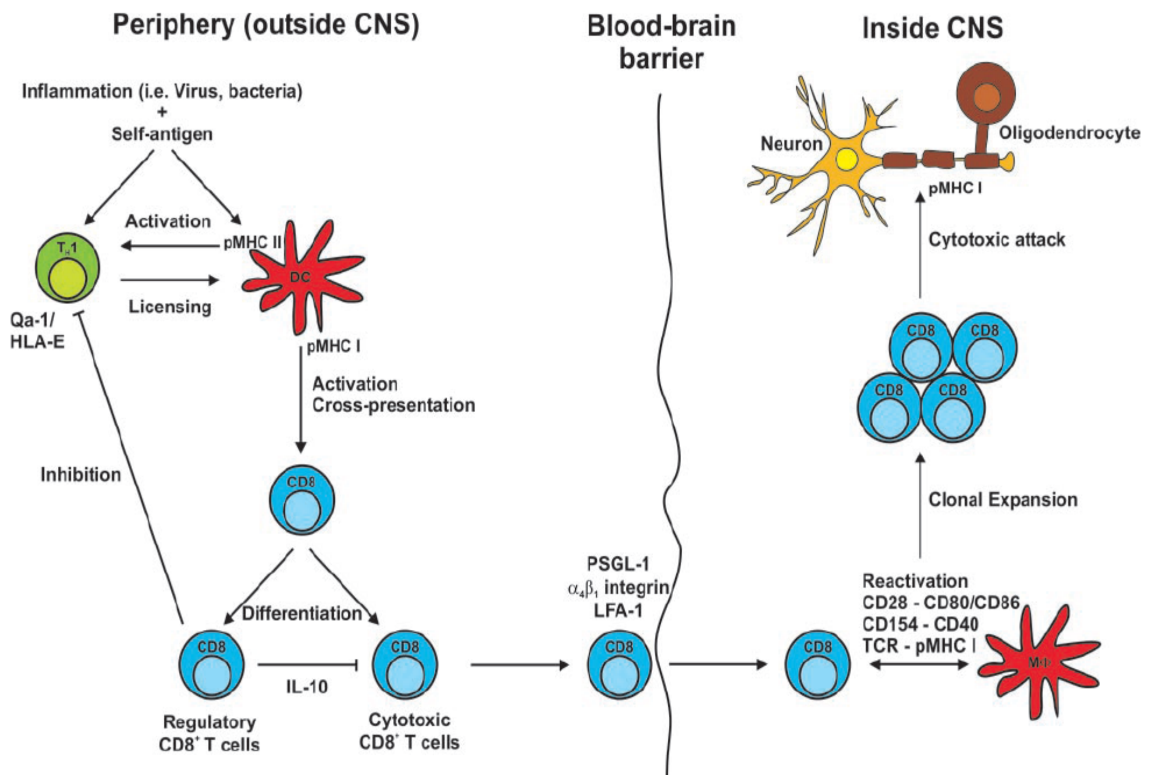

Figure 4. Activation invasion and clonal expansion of $CD8^{+}$ T cells in MS/ EAE (Friese & Fugger, 2005)

It can be speculated that the MS lesion is initiated by CD4+ T cells but the amplification and damage is mediated by CD8+ T cells (Friese and Fugger, 2005) (Figure 4). Established MS genetic associations with MHC class I alleles add strength to this model. Most recent data provide the evidence that directly implicated MHC class I alleles and CD8+ T cells in the pathogenesis of MS (Friese et al., 2008).

3.1.4. T-cell migration in MS

In MS and other neuroinflammatory processes, BBB dysfunction is associated with increased leukocyte trafficking into the CNS, resulting in intense perivascular leukocytic infiltration, a hallmark of MS lesions (Noseworthy et al., 2000). Therefore, an important therapeutic strategy for treating patients with MS is preventing the infiltration of lymphocytes into the CNS.

The attachments of blood-borne leukocytes to the vascular bed, followed by migration of immune cells across the endothelial barrier and migration into the brain parenchyma, are early events in CNS-directed immune responses. These steps are mediated by the interaction of leukocyte adhesion molecules with their cognate ligands on endothelial cells. Adhesion molecule ICAM-1 is expressed on the inflamed BBB in MS, while LFA-1 is expressed on infiltrating T cells, suggesting an important role for this pathway in T-cell migration in MS (Bo et al., 1996; Engelhardt, 2006).

Inhibition of interactions between integrin molecule a4β1 present on the surface of T cells with VCAM-1 present on endothelial cells of the BBB was shown to suppress the development of EAE (Yednock et al., 1992). Therapy with a4β1-integrin antibody (natalizumab) was successful in reducing the infiltration of lymphocytes into cerebrospinal fluid and ameliorated MS symptoms, however, effective blockade of CNS immunosurveillance has produced profound adverse effects, the development of progressive multifocal leukoencephalopathy (Stuve et al., 2006; Mareckova et al., 2007).

Recent data provide a new pharmacological target suggesting greater involvement of an adhesion molecule ALCAM than ICAM-1 in both lymphoid

(CD4+ or CD19+) and myeloid (Mac-1+ or CD14+) leukocyte diapedesis across CNS endothelium (Cayrol et al, 2008). ALCAM expression on BBB cells was upregulated in active MS and EAE lesions. Moreover, ALCAM blockade restricted the transmigration of CD4+ lymphocytes and monocytes across BBB endothelium *in vitro* and *in vivo* and reduced the severity and delayed the time of onset of experimental autoimmune encephalomyelitis.

3.1.5. T-cell activation and costimulation

The observations suggest that the B7-CD28-CTLA4 pathway is activated in MS and may play an important role in regulating disease activity.

Marked increase in the aberrant expression of CD80 and CD86 is seen on both CD4+ and CD8+ T cells from an MS patient with rapidly progressing disease compared to stable MS patients and controls (Mena et al., 1999). CD80 expression localized to B cells is increased during MS relapses, and treatment with IFNβ1b reduced the number of CD-80-expressing B cells but increased the number of CD-86 monocytes (Genc et al., 1997).

Dysregulation of CTLA-4 signaling may contribute to susceptibility to MS. Three CTLA-4 gene polymorphisms are found associated with MS (Ligers et al., 1999), however no association is found with disease course or severity (Masterman et al., 2002). The exon 1 A/G polymorphism is associated with the presence of oligoclonal bands in the CSF (Fukazawa et al., 1999).

Expression of both CD40 and CD154 are increased in lesions from post-mortem MS brains compared with controls, with CD40 found predominantly on macrophages and microglia, while CD154 colocalized with the CD4 T-cell marker (Gerritse et al., 1996). Expression of CD154 is found to be higher in peripheral blood monocytes isolated from SPMS compared with RRMS or healthy controls (Filion et al., 2003; Jensen et al., 2001), and was reduced by IFNβ treatment (Teleshova et al., 2000). PBMCs from SPMS patients produced more IL-12 and IFNγ when restimulated in vitro, compared with healthy controls. These data indicate that the CD40-CD154 pathway is important for the regulation of Th1 cytokine production in MS (Karni et al., 2002).

Programmed death-1 (PD-1) polymorphism is shown to be a genetic modifier of the progression of MS that may be related to PD-1-mediated inhibition of T-cell activation (Kroner et al., 2005).

In MS patients, CSF T-cell clones demonstrated reduced levels of Tim-3 and T-bet and secreted higher amounts of IFNγ than did those from control subjects, indicating that Tim-3 may represent an important regulator of Th1 responses in MS (Koguchi et al., 2006).

Thus, there is significant evidence that costimulatory molecules represent an important step in the control of T-cell activation in MS and are viable therapeutic targets.

3.1.6. T- cytokines in MS

Several studies demonstrate that a disturbed balance between pro- and anti inflammatory cytokines is associated with disease activity in MS patients. MS lesions contain increased levels of transcripts for genes encoding inflammatory cytokines and proteins involved in pathways downstream of these cytokines, as determined by microarray analysis (Lock et al., 2002).

Experimental evidences suggest that IFNγ plays a deleterious role in MS and its level in CSF correlates with disease severity (Balabanov, 2007). Interaction of IFNγ with oligodendrocytes is strongly implicated in the pathogenesis of EAE and MS. *In-vitro* studies show that pretreatment of oligodendrocytes with IFNγ results in severe upregulation of chemokines like CXCL10, MCP-1, MCP-1, and RANTES (Balabanov et al., 2007). These chemokines play an active role in enhancement of parenchymal infiltration, CNS inflammation and demyelination. However, the most pronounced effect of IFNγ is exerted on myelinating oligodendrocytes which undergo both apoptotic and necrotic death in response to IFNγ (Lin et al., 2007). On the other hand, optimal level of IFNγ may also have protective effect on disease pathogenesis such as preventing oligodendrocytes from oxidative stress (Lin et al., 2007). Moreover, IFNγ activates endoplasmic reticulum stress response in oligodendrocytes which attenuates severity of EAE. Nevertheless, the usage IFNγ in treatment led to relapse in individuals with MS, worsening the disease

(Panitch et al., 1987), in contrast to improvement seen in EAE (Billiau et al., 1988).

The production of IFNγ induces antigen presenting cells to secrete IL-12. Mice deficient for the IL-12p40 gene are resistant to EAE and neutralizing antibodies to IL-12 inhibit the in vivo development of EAE (Bright et al., 1998). Parallel to EAE, there are significant evidences that support the role of IL-12 in MS pathogenesis. Increased IL-12 production is observed when APCs are stimulated by T cells isolated from MS patients compared to T cells of normal subjects. Moreover, elevated level of IL-12 is found from serum of SPMS patients but not from control subjects (Gately et al., 1998)

Evidence that implicates TNFα in the underlying pathology MS includes: the observation that at autopsy MS patients have elevated TNFα levels at the site of active MS lesions (Hofman et al., 1989); the reports that CSF and serum TNFα levels in individuals with MS are elevated compared to unaffected individuals and TNFα levels correlate to the severity of the lesions (Sharief & Hentges, 1991; Beck et al., 1988; Maimone et al., 1991) and evidence that peripheral blood mononuclear cells from MS patients just prior to symptom exacerbation have increased TNFα secretion after stimulation compared to cells form the same patients during remission (Rieckmann et al., 1995; 1994). Paradoxically, blockade of TNFα is associated with worsening of MS (TNF neutralization in MS, 1999); in the context of the CNS, TNFα has neuroprotective properties (Turrin et al., 2006).

Interestingly, TNFα is produced by both and Th-17 cells cells after in vitro stimulation, suggesting that TNFα may play a role in both IL-12 and IL-23 linked immune responses (Langrish et al., 2005).

IL-17 mRNA is found more abundant in the blood and the mononuclear cells of cerebrospinal fluid in MS (Matusevicius et al., 1999), and it is also more abundant in the brain tissue of patients with MS (Lock et al., 2002). It is recently identified that not only T cells but different cell types (astrocytes and oligodendrocytes) in MS brains express and produce IL-17 and this is primarily restricted to the active areas of MS lesions (Tzartos et al, 2008). (More detailed description of the IL-17 functions is given in the part 3.1.2.)

TGFβ signaling in the brain leads to increased production of IL-6, which then enhances inflammation (Luo, 2007). TGFβ is elevated in chronic MS lesions, in which there is also intense production of IL-17 from astrocytes and oligodendroglia. Thus, TGFβ might promote IL-17 production during the chronic active phase of MS (Lock et al., 2002; Stromnes, et al. 2008).

IL-6 is found, together with other cytokines, at high levels in association with active MS lesions (Cannella & Raine, 1995; Lock et al., 2002). IL-6 is predominately produced by myeloid cells and is an important factor promoting inflammatory responses. The production of IL-6 by Th-17 cells but not Th1 cells may explain the hyperencephalogenicity of Th-17 cells (Langrish et al., 2005). In EAE IL-6 probably acts as a co-factor in demyelinating processes (Okuda et al., 1999; Samoilova et al., 1998). Increased levels of IL-6, with the lowest levels during acute relapses, is found in CSF from patients with relapsing–remitting MS (Malmeström et al., 2006). See more in part 6.3.

MS patients are characterized by a defect in IL-10 secretion (Astier et al., 2006). Numerous data have revealed the importance of IL-10 in regulating EAE. The neutralization of endogenous IL-10 increased the severity and incidence of SEB- or TNF-induced EAE relapse (Crisi et al., 1995) and the severity of the disease is more in IL-10 deficient mice than in wild-type (Bettelli et al., 1998). Mice transgenic for human IL-10 expressed under the control of the MHC class-II promoter were completely protected from induced EAE (Cua et al., 1999). In humans, an increased IL-10 production is associated with stable MS disease (Navikas et al., 1995). Alternatively, a decreased production of IL-10 associated with a significant increased production of IL-12p40 is detected in patients with SPMS (Balashov et al., 2000). IL-10 mRNA levels are found to be decreased before the occurrence of exacerbations and MRI activity (Rieckmann et al., 1994). Low amounts of IL-10 production are associated with higher disability and MRI lesion load in SPMS (Petereit et al., 2003).

The tissue damage and protection in MS are nuanced and are governed by multiple/redundant molecular interactions that involve many cytokines and other molecular cascades.

3.2. B cells in MS

Data suggesting that humoral immune components in the CNS may be involved in the development and perpetuation of MS have been accumulating for the decades. It is demonstrated that the cerebrospinal fluid and the inflammatory infiltrates in both active and chronic MS lesions contain B cells, plasma cells, antibodies and immunoglobulins. Although the pathological studies suggest that antibody-dependent mechanisms may be important only in subgroups of MS patients (type II pattern) (Lucchinetti et al., 2000), some authors believe this pattern is common to all plaques, reflecting a stage of development (Barnett & Prineas, 2004). Recently the therapeutic benefit of depleting B cells in mice and humans has refocused attention on B cells and their role in AI beyond autoantibody production.

3.2.1. Potential roles of antibodies in MS pathogenesis

The most consistent laboratory abnormality found in MS patients is increased intrathecal production of oligoclonal immunoglobulin (Ig), present in > 90% of persons with definite MS (Walsh, 1985). Several studies have correlated high levels of CSF Ig with worse prognosis (Avasarala et al., 2001; Izquierdo et al., 2002; Villar et al., 2002) meanwhile MS patients lacking CSF oligoclonal bands (OCBs) have a more benign course (Zeman et al., 1996).

Several CNS proteins are shown to be targeted by the immune system in MS (Figure 5), among them are MBP (Warren et al., 1995), myelin oligodendrocyte glycoprotein (Xiao et al., 1991), oligodendrocyte - specific protein (Bronstein et al., 1999), myelin-associated glycoprotein (Moller et al., 1989), 2,3-cyclic nucleotide-3-phosphodiesterase (Walsh & Murray, 1998) and ab-crystallin (Celet et al., 2000).

Clonal expansion and somatic hypermutation of B cells (Qin et al., 1998; Colombo et al., 2000, 2003) and also plasma cells (Ritchie et al., 2004) is observed in the CSF and in MS brain lesions (Owens et al., 1998) regardless of duration or the classification of disease, as expanded $CD19^+$ and $CD138^+$ clones are present in MS patients diagnosed with either primary-progressive

or secondary- progressive disease (Ritchie et al., 2004). B cell clonal expansion and intraclonal diversity is evident even in those patients that had been recently diagnosed with MS (Monson et al., 2005), suggesting that B-cell activation and clonal expansion are primary events in MS rather than a response to long-standing tissue damage.

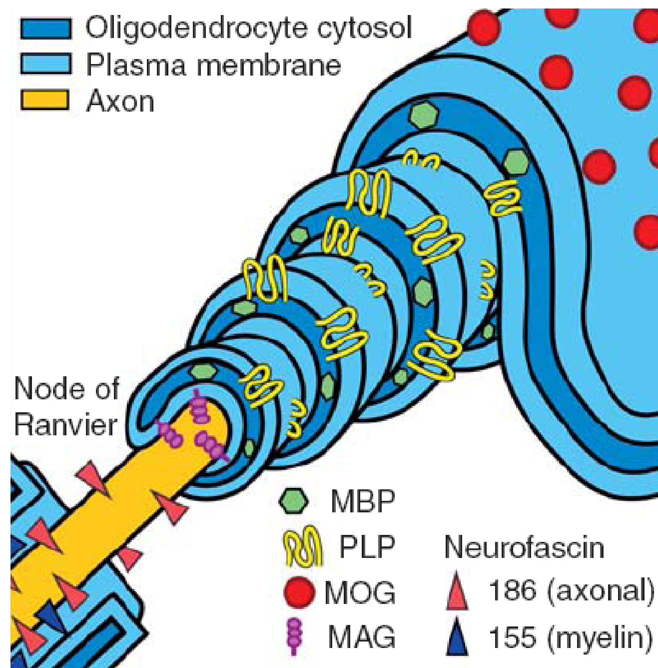

Figure 5. Locations of autoantibody targets in the CNS (McLaughlin & Wucherpfennig, 2008).

The major Ab-secreting cell type in MS CSF are considered CD138+ plasma blasts as there is a strong correlation between their numbers in MS CSF and intrathecal IgG synthesis. Sequencing of V-regions from CD138+ cells in MS CSF has revealed somatically mutated and expanded IgG clonotypes consistent with an Ag-targeted response, indicating a highly restricted B cell response in MS (Owens et al., 2007).

The ratio of B cells to T cells in cellular infiltrates correlates with clinical score in EAE. This suggests that the balance between B and T cells contributes to expression of clinical signs (Peterson et al., 2008). The cellular composition of the CSF of MS patients shows considerable interindividual variation, but the pattern tends to be stable within the same patient and a high B cell/monocyte ratio may be associated with more rapid disease progression (Cepok et al., 2001).

In addition to contributing to systemic AI, B cells may trigger autoimmune disease via molecular mimicry, the dual recognition of a non-self antigen molecule and a self-antigen molecule by a single B-cell receptor (Dalakas, 2006). Thus, antibodies may be a key component of the primary immune response responsible for initiation of tissue damage in MS, or may merely reflect tissue damage, future investigations will distinguish between these possibilities.

3.2.2. Ectopic B cell germinal centers in the CNS of MS patient

B cells show the increased trafficking across the BBB (Meinl & Hohfeld, 2006). *In vitro* human B cells migrate across the brain endothelium more rapidly than autologous T cells. Adhesion molecules, such as lymphocyte function-associated antigen-1 (LFA-1) and very-late antigen 4 (VLA-4 [α4β1-integrin]) enable lymphocyte extravasation at both sites. Stromal/follicular dendritic cells form a network within the follicles and secrete the B-cell homing chemokine CXCL13, upregulated in MS (Krumbholz et al., 2006).

The evidence indicates that B cells, plasma blasts or plasma cells migrating to the CNS of MS patients can persist there for many years, perhaps lifelong. Searching for these molecular mechanisms Serafini and co-workers (2004) identified ectopic lymphoid follicles that were enriched with B cells and plasma cells in the meninges of a subset of patients with MS (Figure 6).

The presence of proliferating B cells in the follicles is suggestive of the formation of a germinal centre (Aloisi & Pujol-Borrell, 2006). B cell follicle is described in the meninges of 41.4% patients with SPMS, but not in PPMS cases. The SPMS cases with follicles significantly differ from those without

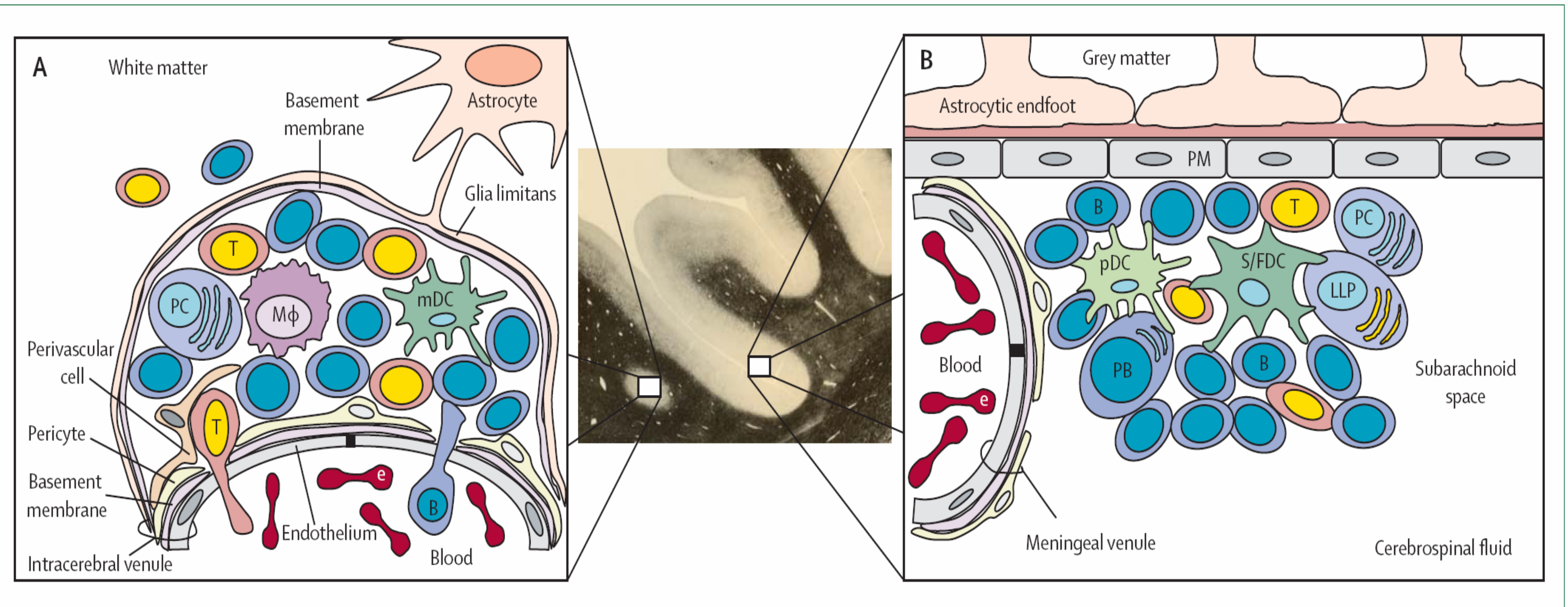

Figure 6 . Inflammatory cell infiltrates in the brain of a patient with MS (Franciotta, 2008)

A. Immune cells mainly accumulate in perivascular regions (Virchow–Robin spaces) in white matter lesions.
B. Ectopic lymphoid follicles are usually located close to meningeal venules and adjoin the pial membrane inside the cerebral sulci.

PC=plasma cell. T=T cell. B=B cell. Mφ=macrophage. e=erythrocyte. mDC=myeloid dendritic cell. pDC=plasmacytoid dendritic cell. PM=pial membrane. S/FDC=stromal/follicular dendritic cell. PB=plasmablast. LLP=long-lived plasma cell.

with respect to a younger age at MS onset, irreversible disability and death and more formation of a germinal centre (Aloisi & Pujol-Borrell, 2006). B cell follicle is described in the meninges of 41.4% patients with SPMS, but not in PPMS cases. The SPMS cases with follicles significantly differ from those without with respect to a younger age at MS onset, irreversible disability and death and more pronounced demyelination, microglia activation and loss of neurites in the cerebral cortex. Cortical demyelination in these SPMS cases is also more severe than in PPMS cases. Notably, all meningeal B-cell follicles are found adjacent to large subpial cortical lesions, suggesting that soluble factors diffusing from these structures have a pathogenic role (Magliozzi et al., 2007). The presence of lymphoid follicle-like structures in the cerebral meninges of some MS patients indicates that B-cell maturation can be sustained locally within the CNS andcontributes to the establishment of a compartmentalized humoral immune response. This long-term persistence of B cells might be promoted by local production of the B cell survival factor BAFF by astrocytes (Krumbholz et al., 2005). In perivenular and meningeal areas, B cells or plasma cells that are infected with Epstein–Barr virus might become the target of cytotoxic CD8+ T cells (Serafini et al., 2007).

3.2.3. B cells as antigen presenting cells in MS

A recent trial of Rituximab, a monoclonal antibody specific for CD20, has produced the unexpected finding of a rapid reduction in acute disease activity as assessed by MRI (Hauser et al., 2007) providing new knowledge on the function of B cells in the acute lesion. Because plasma cells are preserved and autoantibody titers do not decrease in all patients following B cell depletion, the beneficial effects of Rituximab in MS suggest that other B cell functions are critical, such as antigen presentation to T cells with a matching antigen specificity and/or cytokine and chemokine production.

Unlike other professional APCs, activated B cells have the unique capability to efficiently capture even minute amounts of antigen through the B cell receptor thus being the most efficient APCs for T cells with the same antigen specificity (Lanzavecchia, 1985). The interaction between B and T

cells resulting in antigen-specific B-cell and T-cell amplified proliferation, may underlie the autoimmune response against myelin basic protein in MS as autoantibodies and T cells from patients with this disease have been shown to have similar myelin basic protein epitope specificity (Wucherpfennig et al., 1997). Furthermore, T cell–B cell collaboration can result in the production of a distinct antibody type, conformation-sensitive antibodies, which can induce demyelination (von Budingen et al., 2004).

In addition to directly presenting antigens to autoreactive T cells, live B cells can also transfer antigen to macrophages by cell–cell contact (Harvey et al., 2007).

3.2.4. B cells as a source of cytokines in MS

There is substantial evidence that B cells both amplify and suppress immune responses by mechanisms that do not involve antibody (Sanz et al., 2007) (Figure 7). Effector B cell populations produce cytokines such as IL-2, IL-4, IL-13, TNFα, IL-6 (Be-2 cells) or IFNγ, IL-12 and TNFα (Be-1 cells), while regulatory B cells (Bregs) are distinguished by their ability to secrete IL-10 or TGFβ (Lund et al., 2005).

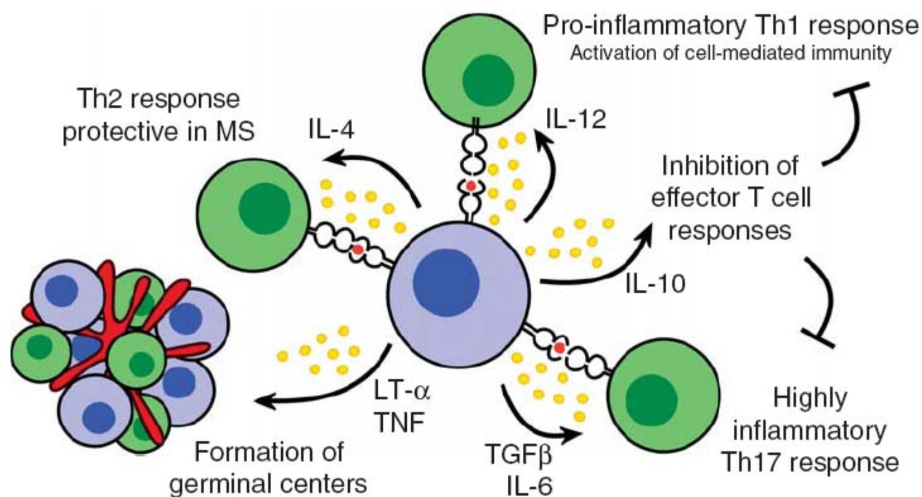

Figure 7. B cell-derived cytokines direct the ensuing immune response (McLaughlin&Wucherpfennig, 2008).

Cytokine production by normal activated human B cells is context-dependent; this involves reciprocal regulation of proinflammatory and anti-inflammatory cytokines. This cytokine network is dysregulated in patients with MS, whose B cells make significantly less IL-10 than B cells from healthy individuals (Duddy et al., 2007).

Interestingly, the alteration in the proinflammatory to anti-inflammatory cytokine ratio is reversed in MS patients treated with either Mitoxantrone or Rituximab (Duddy et al., 2007). This change in B cell cytokine production correlates with a significant reduction in the number of memory B cells in the treated patients. Consistent with this result, CD27+ memory B cells produce inflammatory cytokines like IL-12, LTa, and TNFα, while CD27- naïve B cells make IL-10 (Duddy et al., 2007). Thus, one of the major impacts of B cell depletion therapy is that the ratio of memory "effector" B cells to naïve "regulatory" B cells is reversed in favor of the naïve B cells, leading to a more suppressive or tolerogenic cytokine profile. Secretion of type 2 cytokines is promoted by approved MS therapies, including interferon-B and glatiramer acetate (Sospedra & Martin, 2005).

B cells can produce brain-derived neurotrophic factor, a pleotropic cytokine which can be proinflammatory but equally may promote regeneration in the CNS (Kerschensteiner et al., 1999). Similarly, rather than causing injury, antibodies may promote remyelination in the appropriate setting (Rodriguez & Lennon, 1990).

Effector B cell cytokines may contribute to disease independently of antibody secretion. TNFα itself has been shown to be toxic to oligodendrocytes (Jurewicz et al., 2003). LTa-expressing B cells regulate the formation of ectopic lymphoid tissue in autoimmune target organs (Weyand, et al. 2005).

In conclusion, B cells are involved in antigen capture and presentation to T cells, cytokine production, antibody secretion, demyelination, tissue damage, and remyelination in MS.

3.3. NK and γδ T cells in MS

Both NKT and γδ T cells have also been reported to participate in MS pathology (Gausling et al., 2001).

Ligand-activated NKT cells rapidly producing immunoregulatory cytokines can modulate autoimmune responses (Hammond & Kronenberg, 2003). A role for NKT cells in MS is supported by EAE data. EAE can be suppressed by selective IL-4 induction through binding of a synthetic glycolipid to CD1d that stimulates mouse and human NKT cells (Miyamoto et al., 2001). Altered numbers and functions of NKT cells are reported in MS (Demoulins et al., 2003). CD4+ NKT line cells expanded from MS patients in remission produce a larger amount of IL-4 than those from healthy controls or from MS in relapse that suggests an immunoregulatory role for these cells *in vivo* (Araki et al., 2003).

The exact way in which γδ T cells are involved in MS has not yet been discerned. In EAE they have been shown to both contribute and protect from disease. However, in human studies their role seems more consistent with contributing to disease: they are more numerous in the cerebrospinal fluid (Stinissen et al., 1995), concentrated around active demyelinating lesions (Wucherpfennig et al., 1992) and are potent producers of inflammatory cytokines (Hintzen & Polman, 1997). Limited TCR heterogeneity of CSF-infiltrating γδ T cells in MS suggests common antigen reactivity (Nick et al., 1995; Stinissen et al., 1995). The fraction Vγ2, representing the majority of peripheral blood γδ T cells, infiltrates chronic lesions and is detected in the CSF of MS patients (Battistini et al., 1995). Interestingly, oligodendrocytes selectively stimulate the expansion of this subtype of γδ T cells (Freedman et al., 1997). Human γδ T cells can lyse oligodendrocytes via perforin without the need for APCs, possibly through recognition of heat shock proteins (Battistini, et al. 1995). These findings, together with EAE studies in which γδ T cells appear to be important early mediators of damage (Rajan et al., 1996), support a role for γδ T cells in MS pathogenesis.

3.4. Dendritic cells in the context of MS

Dendritic cells (DCs) have been proposed to be pivotal in the development and maintenance of CNS AI and inflammation (Greter et al., 2005). In the context of EAE, DCs can exhibit tolerogenic (Kleindienst et al., 2005) or immunogenic (Dittel et al., 1999) properties in CNS. It is suggested that CNS-derived DCs could be linked to Treg cell expansion (Figure 8) thereby contributing to the resolution of CNS inflammation (Zozulya & Wiendl, 2008); however, this concept has not yet been proven experimentally.

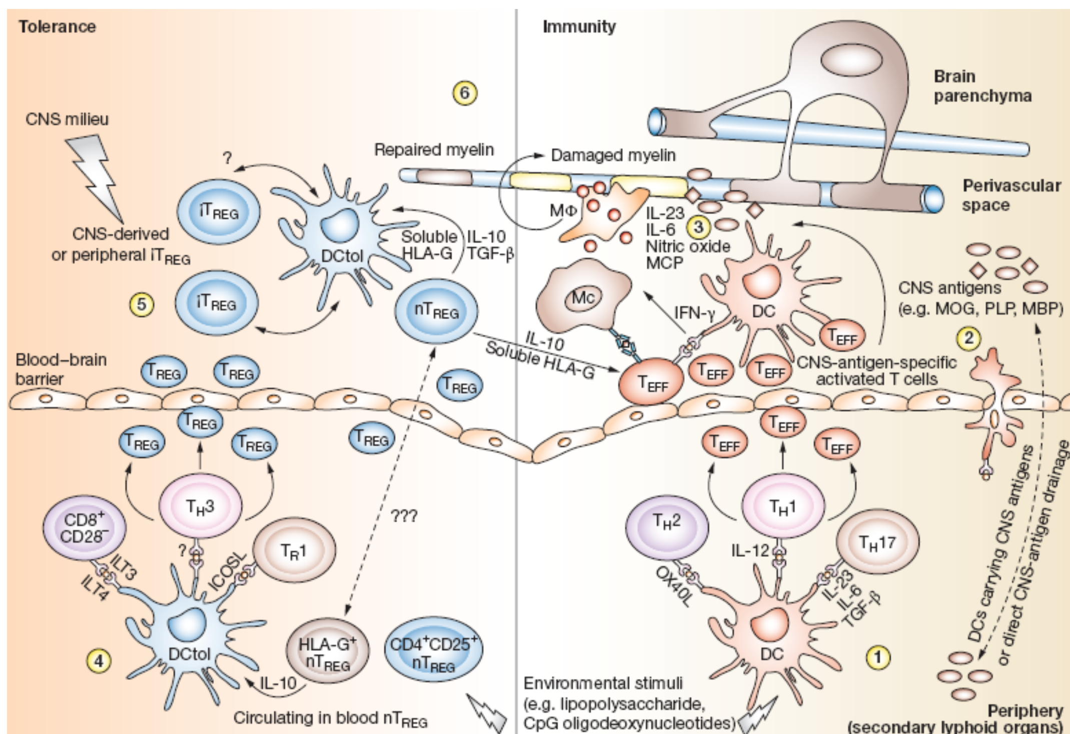

Figure 8. Balance between immunogenic and tolerogenic mechanisms in MS – a hypothesis (Zozulya & Wiendl, 2008)

To date, only a few studies have assessed the role of DCs in MS. The active participation of DCs in the pathophysiology of MS is supported by their increased presence and activation in patients with MS. Huang and coworkers

(1999) reported elevated numbers of peripheral blood DCs in patients with MS. Myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDcs) also infiltrate the MS brain, both mDCs and pDcs have been found in the CSF of such patients (Pashenkov et al., 2001). In addition, several investigations have identified DCs within demyelinating lesions of MS patients. Plumb and coworkers (2003) were able to detect rare mature CD83+ DCs within perivascular cuffs that were not present in control autopsy samples. Serafini and coworkers (2000, 2006) showed that DCs are present in CNS specimens from patients with MS preferentially localizing in the perivascular space and can be found in early active and chronic MS lesions. mDC have a major role in antigen presentation and T-cell activation, whereas pDC produce large amounts of the antiviral cytokine type-1 interferon (Franciotta et al., 2008).

The frequency and phenotype of circulating mDCs and pDCs in the blood of patients with primary progressive MS suggests that DCs are in an impaired maturation state in this condition (Lopez et al., 2006). Dysfunctional interactions between DCs with an altered phenotype and Treg cells have been described in patients with MS (Navarro et al., 2006; Stasiolek et al., 2006).

The cellular mechanism of action of DC-produced IL-23 in AI is the expansion of self-reactive IL-17, IL-17F, TNFα, and IL-6–producing T cells. It was confirmed that these IL-23–dependent CD4+ T cells are highly pathogenic and essential for the establishment of organ-specific inflammation associated with central nervous system AI (Langrish et al., 2005). Expression of IL-23 is higher in mDCs from patients with MS than in healthy controls and DCs from such patients produce more IL-23, which affects IL-10 production, suggesting a mechanism for expanding Th-17 cell populations in these patients (Vaknin-Dembinsky et al., 2006). It is interesting that IFNβ therapy increased IL-10 and decreased IL-23 expression independently of any Th1 or Th2 cytokines (Krakauer et al., 2008).

IL-27 produced by DC is known to orchestrate effective immunity by coordinating Th1, Th17, and Tregs cytokine production. It is highly upregulated at the peak of the EAE disease in CNS and the expression levels of this cytokine seem to correlate with the EAE disease severity (Fitzgerald et

al., 2007a; Li et al., 2005). IL-27 is unique in that while it induces Th1 differentiation, the same cytokine suppresses immune responses. *Il27r*$^{-/-}$ mice are hyper-susceptible to EAE. Furthermore, treatment with recombinant IL-27 significantly suppressed the effector phase of disease, which was associated with a decreased number of IL-17 producing cells (Fitzgerald et al., 2007b). This evidence favors a role for DCs during the initiation of MS as well as during the propagation of autoreactive T cells in situ within the CNS.

3.5. Role of complement in MS

A contribution from complement has long been suspected, based on the results of pathological and functional studies which have demonstrated complement activation products in MS brain and biological fluids (Ingram et al., 2008). Plasmapheresis has been found as efficient therapeutic approach in patients with type of demyelination associated with the deposition of antibodies and the activation of complement. However, the extent and nature of complement activation and its contribution to disease phenotype and long-term outcome remain unclear. Recent findings from studies using a mouse model of MS suggest that complement activation plays a proinflammatory role in the acute phase of MS but may also protect oligodendrocytes from apoptosis (Cudrici et al., 2006).

Complement binding to myelin has been reported as a source of demyelination, astrocytes may be a major CNS source for complement (Vanguri & Shin, 1986). Also complement activation has been shown to result in oligodendrocyte lysis and macrophage chemoattraction in MS (Johns & Bernard, 1997; Piddlesden & Morgan, 1993).

3.6. Is MS a disease of defective immune regulation?

The hypothesis that MS, as well as other autoimmune diseases, is closely linked to defects in immune regulation is being investigated

extensively. The alterations in the function of several populations of regulatory cells and regulatory pathways have been demonstrated in MS.

3.6.1. CD4+CD25+ Foxp3+ T cells

Much attention has focused on the CD4+CD25+ Foxp3+ T regulatory subset (Tregs) that play a critical role in controlling immune responses.

Research in EAE has provided strong evidence that a breach in Treg-mediated control of immunity in the CNS plays a major role in disease development. Depletion of Tregs heightens disease severity (Montero, 2004), while the presence of Tregs in the CNS is associated with recovery and remission. Adoptive transfer of polyclonal (Hori et al., 2002) or Ag specific (Yu et al., 2005) Tregs can prevent disease progression.

Unfortunately, much less is known about these cells in patients with MS. Tregs most studied are those isolated from the circulation of patients.

The majority of reports in patients found that despite normal numbers of Tregs in the peripheral blood (Viglietta et al., 2004; Haas et al., 2005; Venken et al., 2006) and normal or even elevated numbers in the CNS (Haas et al., 2005; Feger et al., 2007; Huan et al., 2005), these cells are dysfunctional in vitro (Viglietta et al., 2004; Haas et al., 2005; Haas et al., 2007; Huan et al., 2005).

Vandenbark and coworkers, however, describe decreases in Foxp3 levels in Tregs isolated from patients with MS and found that this decrease correlated with Treg loss of function (Huan et al., 2005). Additionally, Venken and coworkers report that RR (but not SP) MS patients express lower levels of Foxp3 than healthy controls (2006). These authors also found that the CD25high T cells of MS patients expressed significantly less Foxp3, but not CD25, than those of controls, highlighting the importance of using Foxp3 rather than CD25 to track Tregs (Venken et al., 2008).

These data suggest that the impaired suppression of autoantigen-specific and general T cell responses rather than a reduced survival of Treg or a resistance of responder T cells in MS patients may account for the persistent defects in the effector function of CD4+CD25+ Treg cells in MS patients.

It is also becoming evident that the Foxp3+ Treg compartment is heterogeneous and that minor but functionally important subsets may be altered in patients. For example, CD31+Foxp3+ Tregs, which are contained almost exclusively in the naive CD45RA+ fraction, are found to be decreased in MS patients (Haas et al., 2007).

In MS, it is not apparent if Treg cells exert their effects in the periphery or at the site of disease in the target organ. In EAE animals, it has been recently shown, that myelin-specific functional Tregs accumulate in the CNS but fail to control autoimmune inflammation (Korn et al., 2007).

3.6.2. B regulatory cells in MS

Under various conditions, B cells are tolerogenic rather than immunogenic (Raimondi et al., 2006). Regulatory mechanisms of B cells in immune responses (Figure 9) include : 1) the production of IL-10 that restores Th1/Th2 balance (1a) and directly inhibits inflammatory cascades (1b);

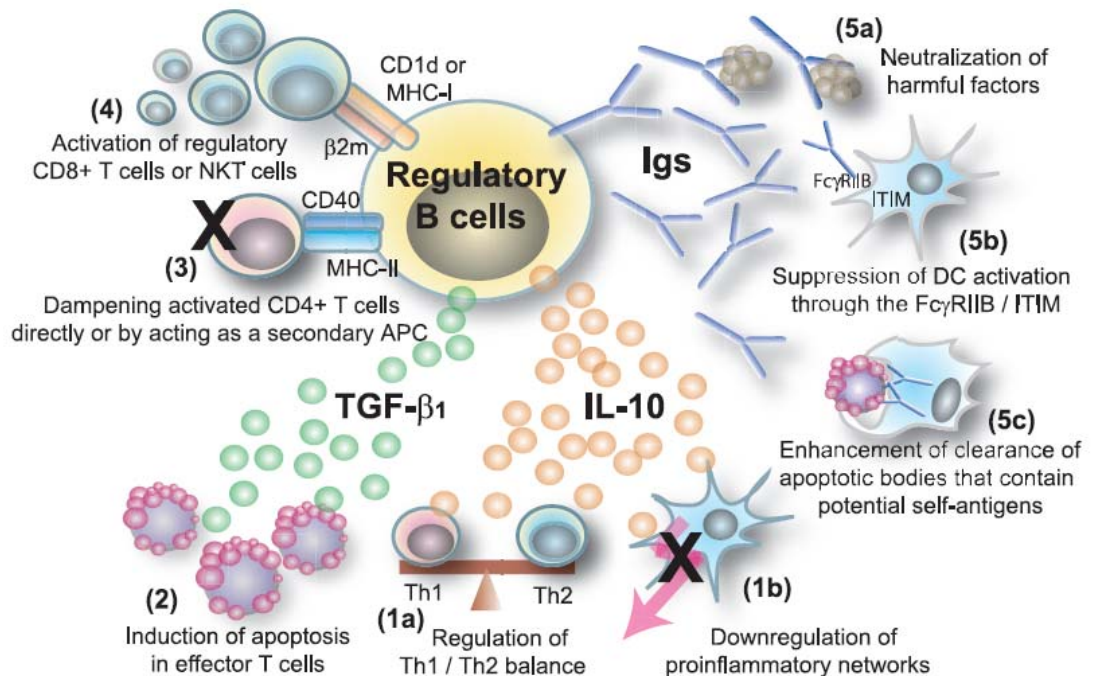

Figure 9. Regulatory mechanisms of B cells (Mizoguchi & Bhan, 2006).

2) the production of TGF-β1 that induces apoptosis of effector T cells; 3) the ability to dampen activated CD4+ T cells directly or by acting as secondary APC; 4) the recruitment of Treg subsets (CD8+ T cells and NKT cells) in a α2-microglobulin-dependnet fashion (MHC class I and CD1d); 5) the production of IgG and IgA that neutralize harmful soluble factors (5a), dampen DC/macrophage activation through the IgG/FcγRIIB interaction (5b), and enhance the clearance of apoptotic cells that are potential source of self-Ags for activating self-reactive T cells (5c) (Mizoguchi& Bhan, 2006).

B-cell regulatory subsets producing IL-10 have been described in an experimental autoimmune encephalomyelitis model (Fillatreau et al., 2002). A recent study reports an essential role of B cells in recruiting nTregs to the CNS that suppress EAE via an IL-10-dependent mechanism within a critical window in the EAE process, facilitating disease resolution before irreversible tissue damage occur (Mann et al., 2007).

Studies in EAE show that demyelinating lesions can be induced in B-cell-deficient mice (Hjelmstrom et al., 1998) and the recovery is incomplete and heterogeneous, suggesting that B cells may have immuneregulatory effects or play a neuroprotective role in EAE.

3.6.3. Other regulatory cells and pathways

Functional abnormalities in Tr1 -cell populations in patients with MS, specifically CD46-mediated Tr1 regulatory cells, are observed in a large proportion of patients with RRMS (Astier et al., 2006). This is characterized by a defect in IL-10 secretion, specifically upon CD46 activation as IL-10 secretion upon CD28 is normal in these patients. Increasing strength of stimulation by stronger TCR stimulation or enhanced IL-2 concentrations do not restore IL-10 production. The deficit in IL-10 secretion is specific as the concentrations of IFNγ secreted by CD46-activated T cells are not affected. In MS recent reports it have been also identified reduced IL-10 production by B-cells and nTreg cells, although the mechanism underlying the IL-10 deficiencies in these studies was unclear (Duddy et al., 2007; Costantino et al., 2008). Meanwhile Martinez-Forero et al (2008) study in Tr1 cells suggest

that the critical step impairing the response to IL-10 in MS patients is the lack of phosphorylation of STAT-3 which is main mediator of the IL-10 response.

T helper type 3 (Th3) cells, possible central mediators of peripheral immune tolerance through direct effects and indirectly by the induction of Foxp3+ Tregs (Carrier et al., 2007). Th3 cells produce high amounts of TGF-beta, low amounts of IL-4 and IL-10, and no IFNγ or IL-2 upon TCR ligation. They appear to mediate the suppression of EAE induced with either myelin basic protein or proteolipid protein by oral administration of MBP (Chen et al., 1994).

Regulatory functions of CD8+ T cells are described. It is shown that regulatory CD8+CD28− T cells contribute to natural resistance to EAE in the absence of CD28 costimulation (Najafian et al., 2003). Deficiency in the CD8+CD28- subset of suppressor cells has been demonstrated in MS patients (Crucian et al., 1995). Moreover, increases in b-adrenergic receptor density on CD8+CD28- cells were found in MS compared with controls (Karaszewski et al., 1991).

Recent studies demonstrated that CD8+ regulatory T cells (CD8+ Treg) can be considered relevant players in the immunopathogenesis of MS (Figure 10). Correale and Villa (2008) found CD8+ Treg specific clones recognizing and lysing activated myelin-reactive CD4+ T cells to be decreased in MS patients during exacerbations, especially in the CSF compartment. They also shown that upregulation of CD94/NKG2 receptors on natural killer and CD8+ T cells seemed to be responsible for the impairment of cytolytic activity during exacerbations, as their blocking could restore the cytolytic activity of CD8+ Treg from MS patients. This suggests an importance of HLA-E-restricted CD8+ Treg in the control of autoimmune disease. A population of CD8+ T cells that can kill CD4+ T cells has been found in patients treated with glatiramer acetate (Tennakoon et al., 2006). The killing seems to be specific for glatiramer acetate and may involve presentation through HLA-E.

The additional regulatory cell type that has been noted in daclizumab-treated patients with MS belongs to the natural killer lineage and is characterized as “CD56 bright” (Bielekova et al., 2006). The reduction in

disease activity indicated by contrast-enhancing MRI lesions has a strong correlation with the abundance of CD56-bright natural killer cells.

These data collectively indicate that patients with MS do suffer a regulatory defect, which is due to impaired function of regulatory cells, but the mechanism of this functional defect has not yet determined.

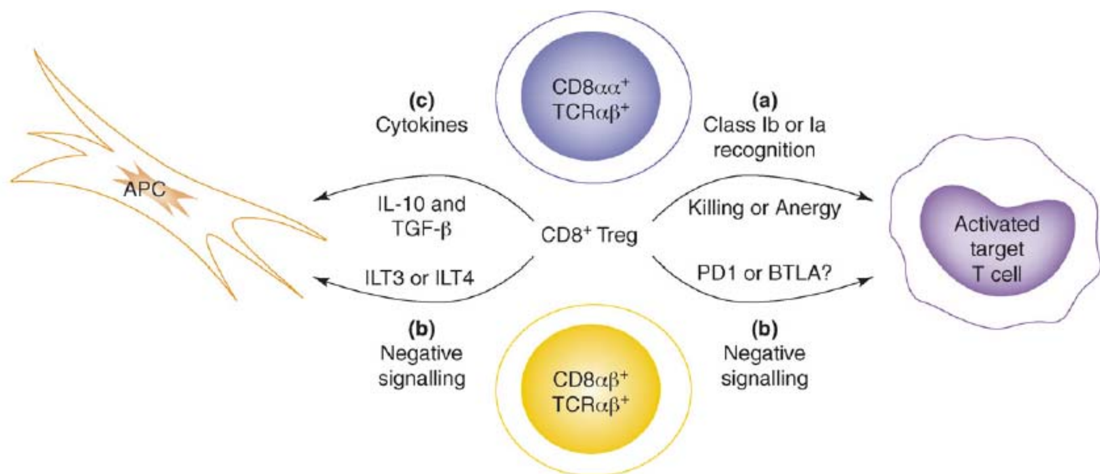

Figure 10. Mechanisms of suppression of CD8+TCRab+ regulatory T cells (Smith et al., 2008).

In addition to the regulatory mechanisms associated with specific populations of regulatory cells, the regulation of gene expression at the site of inflammation may contribute to this process. An example is the association between expression of osteopontin and the degree of destruction in EAE lesions. Mice deficient in osteopontin have a milder course of disease (Hur et al., 2007) and it is abundantly expressed in MS lesions (Chabas et al., 2001). The mechanism is not known with certainty, but evidence indicates that osteopontin promotes the survival of activated T cells (Hur et al., 2007).

A regulatory pathway involving the catabolism of tryptophan by indoleamine 2,3-dioxygenase has been linked to autoimmune diseases such

as EAE and possibly MS (Platten et al., 2005; Sakurai et al., 2002). Tryptophan catabolites (kynurenines) produce downregulation of Th1 cytokines, leading to a shift in the Th1-Th2 phenotype.

Other regulatory pathways have been suggested to contribute to disease activity in MS, including a defect in apoptotic pathways (Julià et al., 2006), but evidence for this remains indirect.

3.7. Axonal damage

MS has historically been considered a disease of myelin and the primary focus of therapeutic investigation has centered on the oligodendrocyte and mechanisms of preventing demyelination or inducing remyelination. However, while demyelination is an underlying etiological event, evidence has started to suggest that the clinically relevant locus of functional disability is injury to the axon (Howe et al., 2005). It is now generally accepted that in addition to the acute inflammatory component, a degenerative process also exists and contributes substantially to the progression of disability. The degenerative processes include loss of axons in lesions (Figure 11), diffuse damage to white

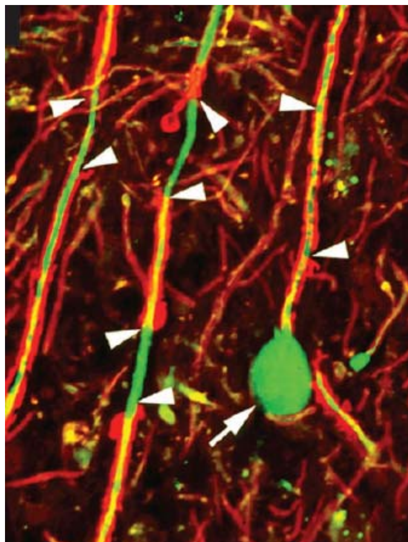

Figure 11. Axons are transected during inflammatory demyelination (Trapp, 2008).

matter distant from areas shown to be involved by histopathology or magnetic resonance imaging, and involvement of both deep and cortical gray matter (McFarland & Martin, 2007).

A neurodegenerative model of MS posits that demyelination is a permissive factor that creates an environment in which the axon becomes susceptible to injury mediated either by loss of axoglial trophic interactions (Wilkins & Compston, 2005) or by immune-mediated attack of the denuded axon (Howe & Rodriguez, 2005). Recent observations of axonal and functional preservation in chronically demyelinated mice genetically deficient in perforin (Howe et al., 2006) and the finding that genetic disruption of MHC class I function preserves motor function (Rivera-Quinones et al., 1998) and axon integrity (Ure & Rodriguez, 2002) during chronic demyelination, argue strongly in favor of the immune-mediated injury model.

Different mechanisms contribute to axonal damage, including the direct effects of pro-inflammatory cytokines, complement fixation, apoptosis, cell-mediated cytotoxicity and neurodegeneration. Pathological findings suggest that the relative contribution of each one of these processes in disease progression differs in each patient (Lucchinetti et al. , 2004).

Axonal damage usually regarded as a secondary event, but grey matter lesions and axonal damage without the formation of lesions do also occur early in disease development (Neuhaus et al., 2003; Tsunoda & Fujinami, 2002). In recent years it has been described that axonal damage may actually precede the myelin degradation, as widespread axonal loss occurs at the earliest clinical stage of MS (Filippi et al., 2003).

3.8. The contribution of epigenetics to the pathogenesis

Genetic studies and genome-wide profiling arrays have contributed to the identification of genes that may exert a combinatorial effect in affecting the susceptibility of individuals to develop the disease. However, it is becoming increasingly evident that gene expression based on DNA sequence alterations

is not sufficient to explain the variety of manifestations observed in disease (Lu et al., 2006). In the last decade, there have been several findings implying the significance of epigenetic modifications as causal factors for multifactorial diseases like MS.

Several lines of evidence raise the possibility of epigenetic mechanisms in MS. Genetic-epidemiological studies have demonstrated several findings implicating epigenetic influences. The concordance rate in monozygotic (MZ) twins demonstrates that genetic factors are important, but most MZ twins are discordant, and concordant pairs are almost entirely female (Sadovnick, 1993). The female excess characterizes the total MS population, despite regional variation. In Canada, the female-to-male ratio has been increasing for at least 60 years and now surpasses 3.2:1, whereas in Scotland, this ratio was around 1:1 almost five decades ago, currently matches or exceeds Canada's gender ratio (Orton et al., 2006). The gender of the affected parent influences the risk for subsequent children to develop MS, and this risk is increased when the mother is affected (Sadovnick et al., 2000). Furthermore, maternal half-siblings (2.35%) are at a greater risk of developing MS when compared with paternal half-siblings (1.31%) (Ebers et al., 2004). These observations indicated a maternal effect in MS susceptibility (Herrera et al., 2007). Results of recent study suggest that epigenetic modifications differentiate among human leukocyte antigen class II risk haplotypes and are involved in the determination of the gender bias in MS (Chao et al., 2009).

The response to X-linked self-antigens could be influenced by X-chromosome inactivation and contribute to the gender bias observed in autoimmune disorders. The X-inactivation pattern studied recently (Knudsen et al., 2007) did not explain the female predominance of MS patients in general, altough the comparison of the X-inactivation pattern between patients grouped according to disease course indicated a possible difference in degree of skewing between patients with a progressive versus a relapsing course.

Traditionally above mentioned differences in susceptibility to disease have been attributed to exposure to environment. At the same time, it is becoming

evident that the effect of environmental factors is exerted by modulating the epigenome of the cells, the higher-order, inheritable set of instructions determining gene expression including modifications of chromatin components (Petronis, 2006). Epigenetic regulators such as histone deacetylases (HDACs) and histone acetyltransferases (HATs) are increasingly being implicated as direct or indirect components in the regulation of expression of neuronal, immune and other tissue specific genes. A role of HDAC/HAT imbalance in inflammation and neurodegeneration is supposed to influence MS mechanisms (Gray & Dangond, 2006). As one example, the regulation of expression of the HLA-II genes has been shown to be intimately linked to both HATs and HDACs (Zika & Ting, 2005).

The importance of sex hormones in MS has been well studied (Tomassini & Pozzilli, 2006), it is important to stress the ability of sex steroids to epigenetically modulate gene expression by affecting chromatin components and transcriptional complexes (Kaminsky et al., 2006).

The epigenetic processes are suggested to participate in myelin repair and that the inefficient epigenetic modulation of the oligodendrocyte differentiation program contributes to the age-dependent decline in remyelination efficiency (Popko, 2008; Shen et al., 2008). The loss of the mechanisms of epigenetic regulation of gene expression in the myelinating cells may contribute to the pathogenesis of MS by affecting the repair process and by modulating the levels of enzymes involved in neo-epitope formation. More specifically, there is evidence in support of aberrant methylation of the promoter of PAD2, a specific enzyme involved in deimination of myelin basic protein, as potential mechanism affecting the generation of neo-epitopes (Casaccia-Bonnefil et al., 2008). The altered post-translational modifications of nucleosomal histones and DNA methylation in white matter oligodendroglial cells are considered relevant for therapeutic intervention in MS (Casaccia-Bonnefil et al., 2008).

Thus, epigenetic factors may affect MS induction and clinical severity by modulating diverse biological processes.

4. EPIDEMIOLOGY OF MS

Epidemiological studies provide strong evidence for both environmental and genetic causes of MS.

4.1. Environmental risk factors for MS

MS is relatively common, affecting over 2 million people worldwide. The uneven geographical distribution of MS is central to understanding the role of environment. Within regions of temperate climate, MS incidence and prevalence increase with latitude, both north and south of the equator. It is rare in the tropics and subtropics in all continents. Suggestions that geographical distribution are entirely explained (Poser, 1994) by regional variation in the frequency of genetic risk factors, reflecting the past migration of higher risk groups such as northern Europeans seem improbable to other authors that consider the gradients like reflection of gene-environment interactions (Ebers, 2008).

An alternative explanation for the latitude effect on MS is that sun exposure protects against MS by raising body levels of vitamin D (Islam et al., 2007). Ultraviolet radiation is the principal catalyst for endogenous vitamin D3 synthesis in humans, and low levels of vitamin D3 are common at high latitudes - especially during winter months when sun exposure is limited. Interestingly, residential or occupational exposure to sunlight might be associated with lower mortality from MS, and a loss of immunoregulatory effects of vitamin D3 might explain how its deficiency could increase the risk for MS (Freedman et al., 2000; van der Mei et al., 2001). However MS risk is not related to sunshine alone, the patterns of MS incidence in different zones suggests possible interactions between diet and sunlight (Kampman et al., 2007).

Recent study further implicates vitamin D as a strong environmental candidate in MS as the sequence analysis localised a single MHC vitamin D response element to the promoter region of HLA-DRB1 (Ramagopalan et al.,

2009). It is absolutely conserved on HLA-DRB1*15 haplotypes, in contrast, there is striking variation among non-MS-associated haplotypes. The direct functional interaction of this response element with the major locus determining genetic susceptibility was shown by several methods. These findings support a connection between the main epidemiological and genetic features of this disease.

Differences in susceptibility related to differences in place of birth and subsequent domicile that found in migration studies suggested that MS risk can be altered by exposure to the putative environmental triggers up to early adulthood (between 5 and 15 years of age)(Hammond et al., 2000).

There is convincing evidence that environment affects outcome and clinical features (Sadovnick et al., 2007) but only few studies have examined differences in long-term outcome by geographic location.

Infection was suggested as possible explanation of geographic variations and migrant data. In this context, two general hypotheses were originally proposed. A common aspect to both is that a widespread microbe, rather than a rare pathogen, causes MS. The first hypotheses postulates that there is a virus that increases the risk for MS if acquired in late childhood or adulthood, but is less harmful and confers protective immunity if acquired in infancy (Poskanzer et al., 1975). The second postulates that MS is caused by a pathogen that is more common in regions of high MS prevalence (Kurtzke, 1993). According to authors this pathogen is widespread and in most individuals causes an asymptomatic persistent infection; only rarely, and years after the primary infection, does this agent cause neurological symptoms (ie, MS).

4.2. Role of virus infection in MS

Immune-mediated tissue damage in MS can result from viral infections in which the host immune response is directed to viral rather than self proteins, or as a consequence of nonspecific (bystander) immune responses that change the local cytokine environment (McCoy et al., 2006). Increasing

evidence suggests that poorly controlled host immune responses account for much of the tissue damage in chronic infections, a similar mechanism may underlie many chronic diseases with features suggestive of an infectious causative factor, including MS (Tortorella et al., 2000).

A virus is suggested the most likely cause of MS (Gilden, 2005) because more than 90% of patients with MS have high concentrations of IgG, manifest as oligoclonal bands, in the brain and CSF. It is interesting in this relation that most chronic inflammatory CNS disorders are infectious.

Virus may remain dormant in the central nervous system but then becomes activated in adulthood and provoke direct lysis of oligodendrocytes, as in progressive multifocal leucoencephalopathy, or could initiate immunopathology (Buchmeier & Lane, 1999). The data from study Barnett and Prineas (2004) points to the possible viral cause of MS as oligodendrocyte death accompanied by microglial activation is found to be the primary event in new MS lesion formation, rather than lymphocyte infiltration.

Susceptibility to persistent infection and its consequent demyelination is likely dependent upon the balance between viral persistence and host defense immunity. Differential interactions between dendritic cells and virus are critical in determining resistance versus susceptibility in the Theiler murine encephalomyelitis virus–induced demyelinating disease model of MS. Although protective prior to viral infection, higher levels of type I interferons (IFNs) and IFNγ produced by virus-infected DCs from susceptible mice further contribute to the differential inhibition of DC development and function (Hou et al., 2007).

In almost all viral infections of the CNS the ratio of cases to infections is low, for example, poliomyelitis occurs in one person per 500–2000 people infected. The risk of acquiring MS in an environment shared with adopted (non-biological) relatives would be expected to be the same as in the general population (1 in 1000) because the disorder to infection ratio of MS is likely to be low and the period over which MS is acquired is probably decades. Accordingly, the frequency of MS in adopted relatives would not be expected

to rise above that in the general population if the disease were caused by a virus (Gilden, 2005).

A viral trigger involved in MS was suggested more than 100 years ago and an extensive list of candidate viruses has emerged since then. The predominant viruses with putative positive associations are measles, mumps, varicella-zoster, Epstein–Barr virus (EBV), parainfluenza and canine distemper virus. For every implicated virus there are also papers refuted a connection. Advances in molecular biology have allowed identifying viral nucleic acids in MS tissue samples and have increased the number of candidate viruses to include endogenous retroviruses, coronaviruses, several new human herpesviruses and the bacterium *Chlamydia pneumoniae* (Opsahl & Kennedy, 2005). Nevertheless, in spite of the new techniques most implicated viruses sport numerous papers that refute a connection with MS in almost equal proportion to those that show an association. However the absence of identifiable infectious agent, especially viral, does not rule out its presence at a certain time - point and the concomitant long term triggering of an autoimmune cascade of events thereafter (Grigoriadis & Hadjigeorgiou, 2006).

The epidemiological and laboratory studies have provided most strong support for an important role of the ubiquitous herpesviruses – EBV, human herpes virus 6 (HHV-6) and MS-associated human endogenous retroviruses (HERV).

4.2.1. Altered immune response to EBV in patients with MS

EBV is intimately involved with the regulation of the human immune system, which makes it extraordinary difficult to prove whether an altered regulation of or an altered immune response to EBV are consequences of an unspecific immune dysregulation due to the autoimmune disease state or specifically involved in disease progression.

Epidemiologic studies. Nearly 100% of patients with MS are seropositive for EBV compared with 90% of healthy people (Banwell et al., 2007). The

difference in the seroprevalence rate is more prominent in children with MS. A Canadian study reported that 83% of pediatric MS patients were seropostive for MS compared with 42% in age-matched healthy subjects, with no signs for a recent EBV infection in the MS cohort (Alotaibi et al., 2004). A more recent German study found a near-complete seropositivity for EBV antibodies in children with MS (98.6% vs 72.1% in age-matched healthy controls), again with no signs of a recent EBV infection (Pohl et al., 2006).

Besides seroprevalence rates, EBV-specific antibody titers are reported to be increased in patients with MS showing an age-dependent relationship between the rise of titers and the onset of MS (Levin et al., 2005). Elevations of antibody titers to the EBNA1 (the dominant EBV-encoded antigen for CD4+ T cells) among MS cases first occurred between 15 to 20 years before the onset of symptoms and persisted thereafter (DeLorenze et al., 2006).

The later age at infection with EBV increases the odds of development of MS at least 20-fold despite sharing a similar childhood environment (Thacker et al., 2006).

Nevertheless, a well established feature of MS epidemiology, the marked reduction in MS risk among migrants (up to the age of 15 and even in adulthood) from high to low-risk areas is not easily explained by differences in age at EBV infection. This suggests a role of other infectious or noninfectious agents that may be at play (Asherio & Munger, 2007a).

A recent study showed that incidence of EBV infection, as measured by EBV seropositivity, is linked to HLA haplotype (De Jager et al., 2008)

Experimental studies. Analysis of CSF-derived antibodies and EBV-specific T cells indicates that the immune response to EBV is altered at both the humoral and cellular level. EBV-targeting antibodies are part of the oligoclonal bands. Cepok and coworkers (2005) reported that the two most frequent MS-specific and high-affinity epitopes identified are both derived from EBV. A qualitatively and quantitatively distinct EBNA1-specific T-cell repertoire has been found in patients with MS despite normal viral loads (Lünemann et al., 2006). Wucherpfennig and Strominger (1995) identified several virus-derived peptide sequences characterized by molecular mimics to

immunodominant myelin-derived T-cell epitopes; one of the mimics with strong agonistic activity was derived from the DNA polymerase protein of EBV.

Tregs in MS patients predominantly neutralize effector memory cells specific for epitopes different from dominant EBNA1 epitop (amino acids 473–508) in healthy individuals (Voo et al., 2005), thereby not preventing potentially crossreactive and harmful effector T-cell responses.

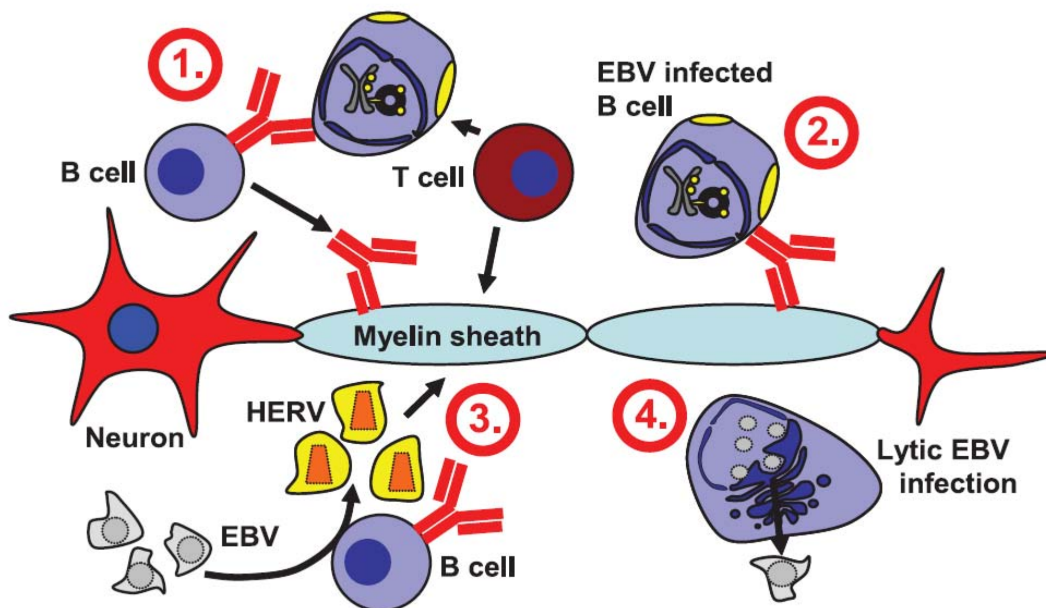

Figure 12. Potential mechanisms responsible for the association of EBV infection with MS (Lünemann, 2007).

(1) EBV-specific T cells or antibodies could cross-react with autoantigens expressed in the CNS and attack the myelin sheath of axons.
(2) Latent EBV antigens could sustain the survival of autoreactive B cells.
(3) EBV infection transactivates retroviral elements such as HERVs, which in turn mediate cell death of oligodendrocytes.
(4) Autoreactive B-cell activation could initiate EBV replication and in turn augment EBV-specific T- and B-cell responses

Recent findings indicate that substantial numbers of B cells that are infected with EBV accumulate in the intrameningeal follicles and in white matter lesions and are probably the target of a cytotoxic immune response (Aloisi, 2007).

Thus, in genetically predisposed individuals, and probably with the contribution of additional environmental factors, including other viruses, EBV infection can not be optimally controlled by the host's immune system, resulting in expansion of infected B cells in extralymphatic niches (Fig 12).

4.2.2. Role of HHV-6 in MS

The combination of the following studies enhances the viral hypothesis and makes HHV-6 a credible virus for involvement in MS.

Primary infection with HHV-6 can cause CNS complications such as encephalitis (Yoshikawa et al., 2000). In addition, in vitro infection of immature dendritic cells induces functional and phenotypic alterations, suggesting an ability to affect immune function (Kakimoto et al., 2002). There is also evidence to suggest that HHV-6 infection may result in oligodendrocyte cell death (Kong et al., 2003) or a decrease in cell proliferation rate (Dietrich et al., 2004), and could initiate demyelination as the virus encodes a protein capable of generating cross-reactivity with myelin basic protein (Cirone et al., 2002; Tejada-Simon et al., 2003).

The presence of HHV-6 DNA and viral proteins is demonstrated in patient lesions (Challoner et al., 1995). Subsequent studies have demonstrated the presence of the HHV-6 genome in oligodendrocytes, lymphocytes and microglia of lesional tissues (Blumberg et al., 2000; Goodman et al., 2003). It is worth to underlie that in most studies, HHV-6 is found in normal control samples, and is frequently absent in some of MS samples (Moore and Wolfson, 2002).

A prospective European study reported increased titers to HHV-6 and measles virus to be associated with the development of MS (Sundstrom et al., 2004).

4.2.3. MS-associated human endogenous retroviruses

Exogenous human retroviruses HTLV-1 and HIV may cause diseases with similarities to MS, underlining the relevance of retroviruses in several neurological diseases (Voisset et al., 2008). At present, human endogenous retroviruses (HERVs) seem to be of greater relevance for MS.

HERVs represent both putative susceptibility genes and putative pathogenic viruses in MS. HERV can cause gene polymorphism and influence the disease predisposition through (lack of) interaction with cellular gene product. One of the MS susceptibility regions, discovered in the Scandinavian study, is 2q24-32, where at least two HERV-H Env ORFs are localised (Christiansen, 2005).

Gammaretroviral HERV sequences are found in reverse transcriptase-positive virions produced by cultured mononuclear cells from MS patients, and they have been isolated from MS samples of plasma, serum and CSF, and characterised to some extent at the nucleotide, protein/enzyme, virion and immunogenic level (Christensen et al., 2003; Antony et al., 2004).

Increased antibody reactivity to specific gammaretroviral HERV epitopes is found in MS serum and CSF, and cell-mediated immune responses have also been reported. Further, HERV-encoded proteins can have neuropathogenic effects (Christiansen, 2005).

Several HERVs are activated by inflammatory events in MS and some of the proteins (such as syncytin-1) may themselves have inflammatory properties and contribute to pathogenesis. In addition, several groups have shown that HERVs are activated by infection with other viruses either in immune cells or in the CNS (Brudek et al., 2004, 2007; Nellåker et al., 2006; Lee et al., 2003; Ruprecht et al., 2006). These include common infections that have been independently linked with MS, including human herpesvirus 6, herpes simplex virus type 1, EBV, and influenza virus but not CMV. These studies could bring together several disparate findings into one general model implicating viruses and HERVs in MS.

The proposed mechanisms (Colmegna & Garry, 2006) by which expression of defective retroviruses might lead to the dysregulation of the

immune response include: transcriptional activation, production of neo-antigens by modification of cellular components, molecular mimicry, epitope spreading, activation of innate immunity through pattern recognition receptors and coding of endogenous superantigen motifs that bypass the normal MHC restrictive process of T-cell stimulation.

4.3. Genetic epidemiology

Evidence for genetic factors in the disease has been demonstrated in numerous population and family-based studies. These studies assess population prevalence, familial aggregation, recurrence risk, and segregation analysis in an attempt to identify and elucidate the genetic contribution to the disease. It seems that the environment is important in setting thresholds for genetic penetrance.

4.3.1. Population prevalence of MS

The susceptibility to MS is illustrated by a higher risk of MS in Caucasians as compared to other ethnic groups. High frequency rates of MS are found in Scandinavia, Iceland, the British Isles, and North America (~1–2 in1,000), New Zealand, whereas, with exception of Sardinia, lower frequencies are distinctive of southern European populations (Rosati, 2001; Sotgiu et al., 2003). MS is rare in Asia, black Africans and indigenous populations of the Americas, New Zealand and Australia (Pugliatti et al., 2002).

The prevalence of MS in Spain is medium to high, between 50 and 80 patients per 100,000 inhabitants (Fernandez et al., 1994; Uría et al., 1997; Ares et al., 2007).

Incidence of MS is low in childhood, increases rapidly after age 18, reaches a peak between 25 and 35 years and then slowly declines, becoming rare at age 50 and older. Risk is greater in women than in men; the female-to-male ratios are between 1.5 and 2.5 in most populations, with a trend toward greater values in the most recent studies (Orton et al., 2006). In high-risk populations, the lifetime risk for MS is about 1 in 200 for women (Hernán

et al., 1999) however the role of female gender in terms of genetic transmission became clear only recently when "maternal parent-of-origin" effect in MS susceptibility has been confirmed (Herrera et al., 2008).

4.3.2. Familial aggregation and recurrence risk

The risk of MS is higher in family members of MS patients than in the general population. Rigorous epidemiologic studies have measured disease risk in different types of relatives of MS patients including MZ twins, first degree relatives including dizygotic twins, siblings, parents, and children; and second degree relatives such as half siblings, aunts, uncles, nephews, and nieces.

A commonly used measure in these studies is recurrence risk ratio (λ)—a value generated by comparing recurrence rates in the relatives of MS patients to the disease prevalence for the general population. Numerous familial aggregation studies have shown that this recurrence risk ratio for MS decreases with the degree of relationship between individuals. For example, studies have reported an increased relative risk of 100–190 in identical twins, 20–40 in full siblings, 7–13 in half siblings, and 5.5 in the offspring of an affected parent with MS in Canada (Willer et al., 1993). Compared to the general population, these elevated risks suggest a strong but non-Mendelian inheritance of MS susceptibility. Twin studies in several populations indicate increased concordance rates among MZ (25–30%) compared to dizygotic (2–5%) twins with MS (Willer et al., 1993, Mumford et al., 1994) although the 95% confidence intervals in all studies are relatively wide. Estimating the risk of MS in a population-based cohort Nielsen and coworkers (2005) found a sevenfold increased risk of MS for first-degree relatives compared with the background population. While all these data also provide evidence of a strong genetic component in the disease, a MZ twin concordance rate significantly less than 100% also highlights the contribution of gene–environment interactions to MS disease susceptibility.

The familial risk of MS could depend on either shared genes or shared environmental factors such as diet, lifestyle, or infection. Evidence for genetically determined familial aggregation is also seen in adoption studies documenting an increased risk of MS only in biological relatives of adopted probands (Ebers et al., 1995). An additional source of genetic evidence for MS is found in segregation analysis, which tests for both the existence and mode of inheritance of a disease gene analizing familial aggregation of MS (Dyment et al., 2002).

A variety of genetic models with multiple genes, dominant inheritance, and synergistic interaction between risk genes are consistent with the observed familial recurrence rates in MS (Lindsey, 2005). The nonlinear relationship between familial recurrence risk and the degree of relatedness, as well as the available genetic data, permit Yang and coworkers (2005) to suggest that between 20 and 100 common variants, each increasing risk by only a modest factor of 1.2–1.5, would be sufficient to account for the prevalence and heritability of MS. On the other hand, many hundreds if not thousands of rare variants would be required to explain MS susceptibility, even if each of them produce a 10–20 times higher relative risk.

4.3.3. Clustering MS with other autoimmune diseases

MS exhibits several characteristics common to autoimmune disorders—including polygenic inheritance, partial susceptibility conferred by a human leukocyte antigen (HLA)-associated gene and evidence of environmental exposure.

Becker and coworkers (1998) comparing of the linkage results from 23 published autoimmune or immune-mediated disease genome-wide scans discoved that a majority (approximately 65%) of the human positive linkages map nonrandomly into 18 distinct clusters (Figure 13). Overlapping of susceptibility loci occurs between different human immune diseases and by comparing conserved regions with experimental autoimmune/immune disease models. This nonrandom clustering supports a hypothesis that, in some cases,

clinically distinct autoimmune diseases may be controlled by a common set of susceptibility genes.

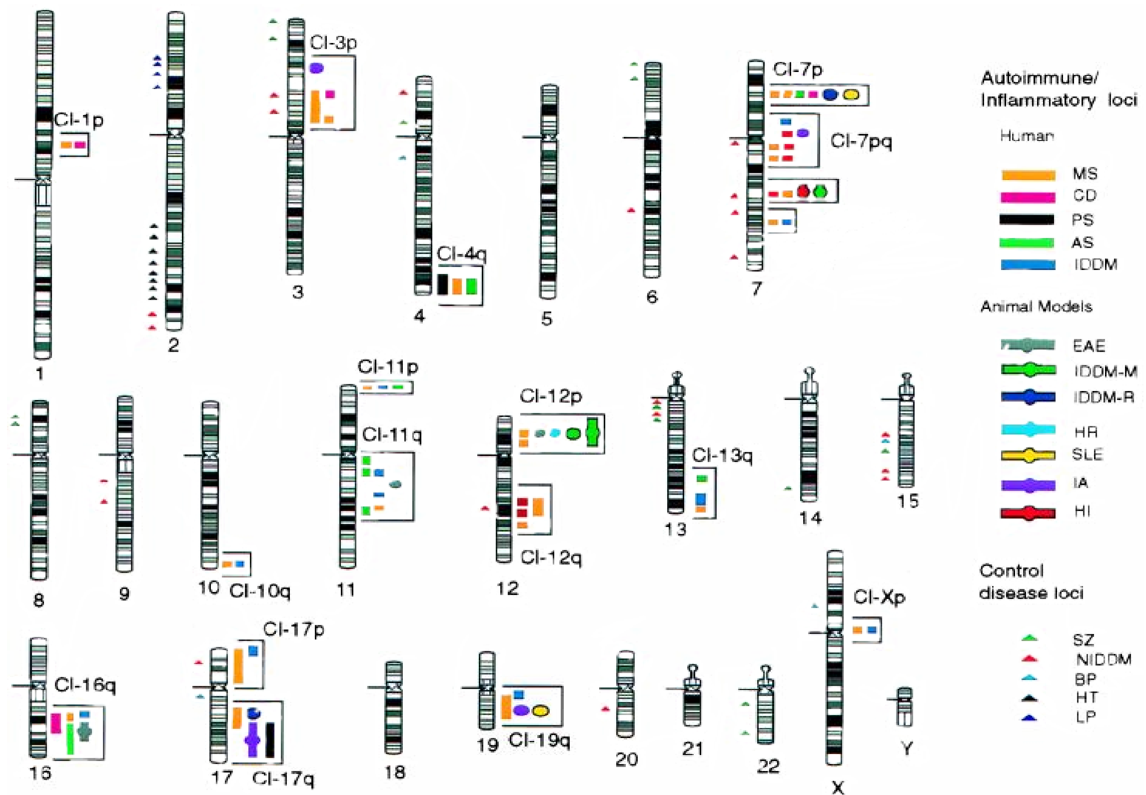

Figure 13. Clustering of autoimmune candidate loci (Becker et al., 1998)

MS, multiple sclerosis; CD, Crohn disease; EAE, experimental autoimmune encephalomyelitis; PS, familial psoriasis; AS, asthma; IDDM, insulin-dependent diabetes (human; M, mouse; R, rat); HR, *B. pertussis*-induced histamine sensitization; SLE, murine lupus; IA, rat inflammatory arthritis; HI, humoral immunity; SZ, schizophrenia; NIDDM, non-insulin-dependent diabetes (type II); BP, bipolar disorder; LP, leptin-associated obesity; and HT, hypertension.

The question on a genetically determined predisposition in (some) patients with MS, which favours the development of AI is addressed in several studies.

Barcellos and coworkers (2006a) report an increased prevalence for other autoimmune diseases in families in whom multiple members had the disease compared with historic epidemiological data from the general population. Such an increased prevalence was not only seen in patients with MS, but also in their first-degree relatives.The authors reported also an association between an increased risk for AI in MS families and a common genetic variant of CTLA4 gene implicated in the regulation of AI. These data provide evidence that in certain MS families there is an increased risk for development of AI in general and that this risk could be genetically determined.

Several more epidemiological studies have demonstrated the concurrence of MS and other AID by analyzing the data from population-based studies with standardised ascertainment methods.

A Danish study investigated patients with multiple sclerosis, patients T1D and their respective first-degree relatives using population-based disease registers. Although *HLA* patterns of T1D and MS are considered mutually exclusive patients with diabetes had an increased risk of developing MS, which persisted after stratification by sex. The relative risk of T1D was increased in first degree relatives of people with MS (Nielsen et al., 2006). Gupta and co-workers use a population-based dataset in the UK to assess the association between inflammatory bowel disease and demyelinating diseases, including MS and isolated optic neuritis (2005). Patients with Crohn's disease and ulcerative colitis have an increased risk of incident and prevalent demyelinating disease, as assessed in cohort and case–control studies. Likewise, Bernstein and co-workers use population-based administrative databases to investigate immune-mediated disorders associated with IBD. They find significantly increased risk of MS in people with ulcerative colitis (2005). The risk of MS is significantly increases also in families with systemic lupus erythematosus comparing with the general population (Corporaal et al., 2002). The population-based study on hospitalized patients observes a familial risk for MS of about 6.0 in first-degree relatives of patients and it was independent of the type and sex and the proband (Hemminki et al., 2009).

However, Canadian Collaborative Project on Genetic Susceptibility to MS investigating comorbid AI in MS on the basis of patient reports found similar the overall risk of AID in people with MS and in their first degree relatives (Ramagopalan et al., 2007). The results of this study make an important point about the need for standardised methods for the ascertainment of data to

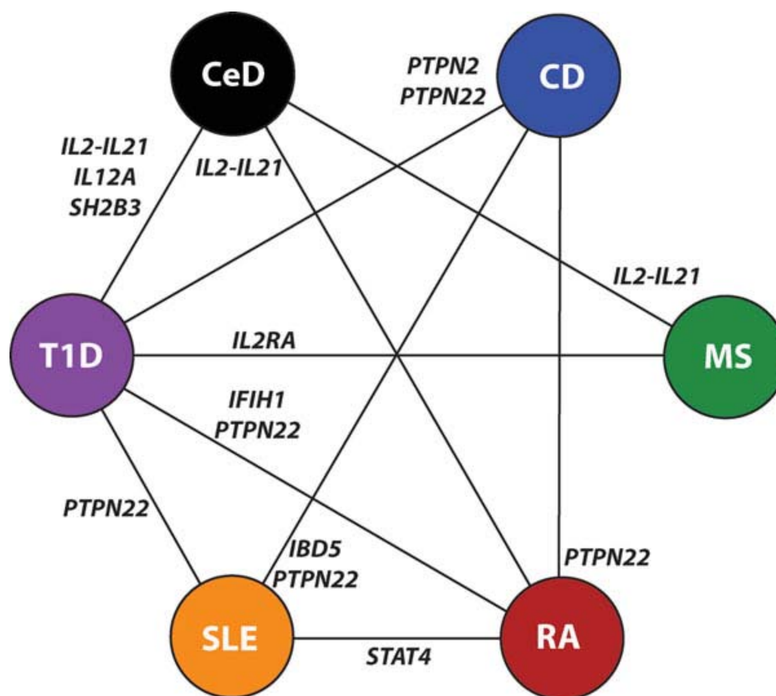

Figure 14. An overlap in the genetic risk loci for CeD, CD, MS, RA, SLE and T1D (Lettre & Rioux, 2008).

avoid bias. The validity of self-reported medical disorders can be affected by several factors, including age, sex, education etc. The choice of controls is also important question. Ramagopalan and co-workers use spousal controls, whereas the other population-based studies used population controls, and the conclusions are different.

In summary, an overlap in the genetic risk loci for CeD, CD, MS, RA, SLE and T1D (Figure 14) has been determined in candidate gene and genome-

wide association studies that suggest common mechanisms leading to the development of autoimmune diseases (Lettre & Rioux, 2008).

Taken together, these epidemiological studies provide overwhelming evidence in support of a strong genetic component in MS. The data also suggests that, like most common complex diseases, MS susceptibility is the result of multiple genes acting either independently or interactively in their contribution to overall risk.

5. GENETIC SUSCEPTIBILITY TO MS

5.1. Approaches toward gene identification in MS

To date, geneticists have used two tools to dissect the genetic basis of MS: linkage scans and association studies.

5.1.1. Linkage genome screens

Linkage scans rely on statistics to identify cosegregation of polymorphic markers and disease state to identify genomic regions containing susceptibility loci. This serves to narrow the scope of studies and allow a subset of the genome to be studied by association analysis with a higher density of assayed markers.

Since 1996, several genomic linkage screens of MS families have been undertaken in a number of populations. Each of these studies used microsatellite markers to trace the inheritance of segments of the genome in families with multiple affected individuals. This resulted in the identification of numerous regions with “nominal” or “suggestive” linkage.

The most recent screen, in which 2692 individuals were genotyped for 4506 markers, uncovered genome-wide significant linkage to the HLA on chromosome 6p21, as well as suggestive linkage to loci on chromosomes 17q23 and 5q33 (Sauser et al., 2005).

Nevertheless, the individual studies, while useful in pointing out regions that may be linked to complex diseases, do not give a complete picture of all the genetic factors or their relative risks involved in MS. To overcome these limitations meta-analysis approaches are employed to combine the results from the various studies. Such analysis also provides a more complete exclusion map of genomic segments thought unlikely to contribute to MS susceptibility. Several meta-analyses of these screens have been published. Each analysis used a different methodology to combine the previous studies. Wise and colleagues (1999) used a non-parametric ranking method to analyze the results of the first four screens; they found support for five regions: 6p, 19q, 5p, 17q and 2p. The Transatlantic Multiple Sclerosis Genetics Cooperative (2001) combined the raw data from the first three screens in a global mega-analysis and reported eight regions with moderate support: 17q11, 6p, 5q, 17q22, 16p, 3p, 12p and 6q.

Meta-analysis of all to date 10 genome-wide linkage studies for multiple sclerosis, using an extended GSMA method (Hermanowski et al., 2007) presented once more the strongest evidence for linkage on chromosome 6p (HLA region), 6q, 10q and 18p. Aside from 6p, all these regions do not show strong evidence for linkage in individual studies or in pooled analyses.

5.1.2. Candidate gene studies

Association studies, also called the candidate gene approach, rely on statistics to detect association of polymorphic genetic markers located within the gene (including the promoter region) to disease phenotype and therefore these alleles must either be causal or in strong LD with the causal variant. Compared to linkage analysis, association studies have been shown to have much higher statistical power to detect loci of modest effect (i.e. incompletely penetrant) that it is expected for common, complex diseases (Risch, 2000). In association studies patients and controls must be unrelated and belong to the same ethnic group to avoid stratification.

Single nucleotide polymorphisms (SNPs), are now increasingly used as genetic marker and non-synonymous SNPs causing amino acid differences in the final gene product are considered most informative because of their possible strongest effect on gene function. However, even synonymous SNPs may influence the final gene product. Most recently, the possibility to use deletions–insertions as markers is being considered.

The strategies which have been applied for selection of the genes in candidate gene studies were based on location within a linkage peak, known function or combaning different methods to identify and prioritize susceptibility genes.

Because MS is characterized by demyelination of the CNS with strong autoimmune component, functional candidate genes with potential biological relevance to a disease such as those coding for immunoglobulin, cytokines, chemokines, TCRs, HLA, and myelin antigens have been investigated. Nevertheless, despite reports of numerous genes with significant results, most candidates (outside the HLA) have failed to be replicated in independent datasets. For example, an obvious candidate for MS, MBP, yielded both positive linkage and association results in a genetically isolated population in Finland (Tienari et al., 1992). However, other research groups have failed to replicate this result in non-Finnish populations (Rose et al., 1993; Eoli et al., 1994). Meanwhile, there are several exceptions, between them *IL2Ra* gene, described for the first time by our group (Matesanz et al., 2007) or *IL7Ra* (Lundmark et al., 2007) whose associations with MS being discovered by candide gene strategy are confirmed now in genome-wide association studies (GWASs) in MS.

Candidate genes identified through functional studies using the EAE have also yielded inconsistent results. Several reasons may be postulated for the disappointing results in MS genetic studies. First, the genes may confer such a low increase in risk that many thousands of families or case-control cohort would be necessary to attain enough power to identify linkage or association. Second, heterogeneity may exist between families or individuals in the same study or across studies performed in different populations. Third, the power of

studies can be substantially reduced by incomplete genetic information from genotyping errors and missing genotypes.

5.1.3. Genome-wide association study

The goal of GWAS is to detect phenotypic associations of modest effect that would have eluded previous linkage and candidate gene approaches. A GWAS typically involves genotyping thousands of clinically well-defined patients and matched controls with hundreds of thousands of SNPs selected without any previous hypothesis. Although genotyping at a density of a SNP per 5-10 kb represents only a small proportion of the total number of known SNPs, it captures the majority of all common genetic variation, due to the extensive correlation between SNPs (LD) (Ardlie et al., 2002). Recent studies have shown that LD stretches across long segments of the genome permit use a small subset of variants (haplotype-tagging SNPs) that identify all common haplotypes within a block (Daly et al., 2001). Recently, the completion of the HapMap project (International Hapmap Consortium, 2005), together with the development of new genotyping technologies, has given the tools necessary to comprehensively and in an unbiased manner search for DNA polymorphisms associated with many autoimmune diseases. Two more key features of the GWAS are: very stringent quality-control criteria is used to process genotype and phenotype data and replications are done in equally large and well-designed cohorts using different genotyping platforms. The advent of GWAS approaches has resulted in the impressive addition of new common DNA sequence variants that influence risk of developing of many autoimmune diseases (Figure 15).

Utilizing new genotyping technologies and genomic resources such as the HapMap (International Hapmap Consortium, 2005), GWASs in MS have been identified several potential disease markers. The first report (IMSGC, 2007) includes family trios and case-control groups. Using a two-step procedure, transmission disequilibrium test with 334,923 SNPs in the initial screen, a total of 110 SNPs were selected for the second stage confirmatory analysis. Of these SNPs, two within the interleukin 2 receptor α gene *(IL2Rα)*

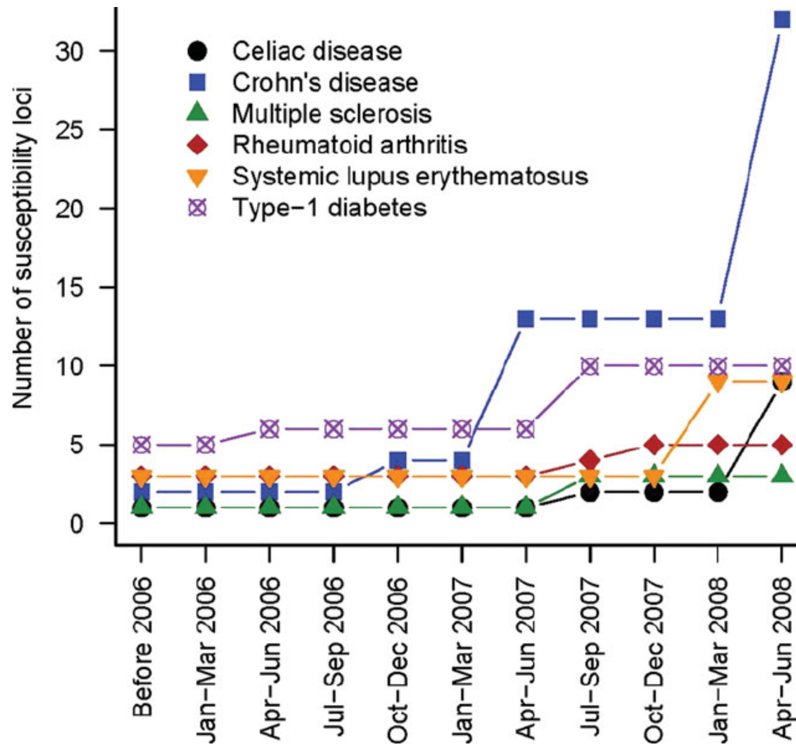

Figure 15. An increase in the rate of discovery of risk loci for many autoimmune diseases following the emergence of GWAS in 2006 (Lettre & Rioux, 2008).

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on chromosome 10p15, a non-synonymous SNP in the interleukin 7 receptor α gene (*IL7Rα*) on chromosome 5p13, and multiple SNPs in the *HLA-DR* locus on chromosome 6 are found strongly associated with MS.

MS group was also included in the large association scan of 14,500 non-synonymous SNPs reported by the Wellcome Tust Case Control Consortium (WTCC, 2007), which, apart from HLA, suggested association between MS and SNPs in nine different genes (*SLC4A5, FJL10902, FJL10204, IL7R, INNPP5A*, *LRP5, ZNF45, GIPR*, and *SAP102*) of which *IL7R* now has been confirmed (Weber et al., 2008) while the others need confirmation in studies of independent data.

The last GWAS in MS has been realized by Baranzini and coworkers (2008), analizing more than 550.000 SNPs in case-control study in Caucasians of European origin. Gene ontology-based analysis of it results shows a functional dichotomy between genes involved in the susceptibility pathway and those affecting the clinical phenotype. It is interesting to emphasize that there is no big coincidence between the loci of risk detected in these studies with the exception of the *HLA*, *IL2Ra* and *IL7Ra*. The reasons of low replication of the detected loci are explained by multitest character of GWAS and low Odds Ratios of these associations that produce a big number of false positives. Very big populations need to confirm or to discard the detected associations.

5.2. HLA and MS

Given the strong and reproducible linkage findings of the chromosome 6p region containing the HLA, this region has been the focus of many association studies in MS.

The classical HLA encompasses approximately 3.6 megabasepairs (Mb) on 6p21.3 and is divided into three subregions: the telomeric class I, class III, and the centromeric class II regions. HLA class I and class II encoded molecules are highly polymorphic cell surface glycoproteins playing a fundamental role in self/non-self immune recognition. Recently the concept of the extended HLA (xHLA), spanning about 7.6 Mb of the genome, has been established by the finding that LD and HLA -related genes exists outside the classically defined locus (Horton et al., 2004).

Of the 421 genes within the xHLA, 60% are expressed and approximately 22% have putative immunoregulatory function (Horton et al., 2004). Upon determination of the physical size of the region, it appeared that LD extended more than 2 Mb in some cases. In difference with other genome regions, there is a higher amount of LD observed between segments of strong LD (Miretti, 2005). Such tight segment-to-segment LD poses an important obstacle in HLA research.

This super-locus contains several genes that influence susceptibility to MS (Dyment et al., 2004). The association with *HLA- DRB1*1501* locus is observed across virtually all populations, and in both primary progressive and relapsing–remitting patients, suggesting that HLA-related mechanisms are contributing to both phenotypes (McDonnell et al., 1999; Barcellos et al., 2006b). The only exceptions are an association with *HLA–DRB1*0301* and *HLA–DRB1*0405* in patients with MS from Sardinia (Marrosu et al., 2001).

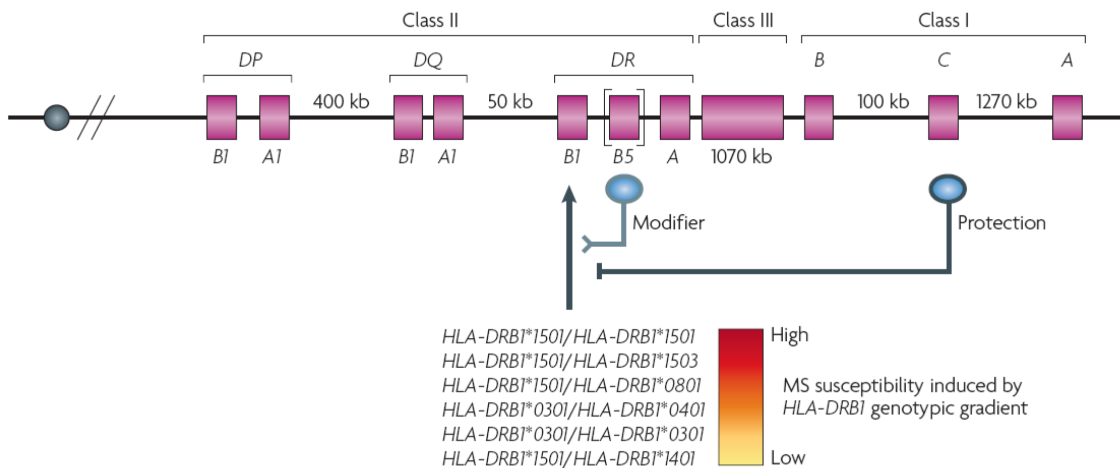

Figure 16. The 6p21–6p23 chromosomal region and MS (Oksenberg et al., 2008).

The association signal segregates often with the HLA class II extended haplotype *HLA-DQB1**0602, HLA-DQA1**0102, HLA-DRB1**1501, HLA-DRB5**0101* (Fogdell et al., 1995). A comprehensive analysis of *HLA-DRB1* and *HLA-DQB1* alleles and haplotypes in an African American MS cohort show a selective genetic association with *HLA-DRB1* and suggests a primary role for the *HLA-DRB1* gene in MS susceptibility that is independent of *HLA-DQB1* (Oksenberg et al., 2004).

Several epistatic interactions were uncovered between MHC alleles or haplotypes, fundamental in determining susceptibility to MS (Figure 16). These had remained occult over the three decades since the MHC association

was first described because they had to be specifically sought. _HLA-DRB1_ allelic copy number and cis and/or trans epistatic effects (Barcellos et al., 2003; Barcellos et al., 2006, Dyment et al. , 2005) suggest a disease-association gradient, from high vulnerability (_HLA-DRB1*1501_ homozygotes and HLA- _DRB1*1501/HLA-DRB1*0801_ and _DRB1*1501/HLA-DRB1*0801_ heterozygotes) to moderate susceptibility (_HLA-DRB1*03/HLA-DRB1*04_ heterozygotes and HLA-DRB1*0301 homozygotes) and resistance (_HLA-DRB1*1501/ HLA-DRB1*1401_ heterozygotes; in this genotypic configuration, the presence of _HLA-DRB1*1401_ nullifies the susceptibility effect of _HLA-DRB1*1501_).

HLA-DRB1*14-, HLA-DRB1*11-, HLA-DRB1*01-, and _HLA-DRB1*10_-bearing haplotypes are protective overall but they appear to operate by different mechanisms. The first type of resistance allele is characterised by _HLA-DRB1*14_ and _HLA-DRB1*11_. Each shows a multiplicative mode of inheritance indicating a broadly acting suppression of risk, but a different degree of protection. In contrast, a second type is exemplified by _HLA-DRB1*10_ and _HLA-DRB1*01_. These alleles are significantly protective when they interact specifically in "trans" with _HLA-DRB1*15_-bearing haplotypes. _HLA-DRB1*01_ and _HLA-DRB1*10_ do not interact with _HLA-DRB1*17_, implying that several mechanisms may be operative in major histocompatibility complex-associated MS susceptibility, perhaps analogous to the resistance alleles (Ramagopalan et al., 2007).

Emerging data also suggest a modifier role for _HLA-DRB5_ and HLA-class I alleles. Thus, EAE data using triple _DRB1/DRB5/hTCR_ transgenic mice demonstrate epistasis within the MHC and strongly favor a disease-modifying effect for _DRB5_ (Gregersen et al., 2006) (Figure 16).

Variation in the _HLA-C_ gene is found to influence susceptibility to MS independently of any effect attributable to the nearby _HLA-DRB1_ gene. Specifically, the _HLA-C*05_ allele, or a variant in tight linkage disequilibrium with it, appears to exert a protective effect (Yeo et al., 2007) (Figure 16).

The mechanisms underlying HLA association in autoimmune disease are not clearly understood. One long-held view suggests a breakdown in

immunological tolerance to self-antigens through aberrant class II presentation of self or foreign peptides to autoreactive T lymphocytes. It is suggested that specific HLA class II alleles determine the targeting of particular autoantigens resulting in disease-specific associations (Smith et al., 1998; Stratmann et al*.,* 2003; Wucherpfennig, 2005). But in light of studies on inheritance of resistant HLA alleles the restriction of antigen presentation by *HLA-DRB1*15* seems an improbably simple mechanism of major histocompatibility complex-associated susceptibility (Ebers, 2008).

Yet again, the phenomenon of LD is strong in the HLA complex and definitive mapping factors important for the risk of MS require full assessment of all polymorphic genes in the HLA complex in a very large sample size.

5.3. Genetic influences on the clinical phenotype

Concordance in families for early and late clinical features indicates that genes influence age of onset, disease course and other aspects of the clinical phenotype in addition to susceptibility.

Several studies have investigated the role of HLA alleles on disease outcome which is vary considerably among patients, however, the results have been unclear and conflicting (de la Concha et al., 1997; Weinshenker et al., 1998; Barcellos et al., 2003).

Evidence has been reported that *HLA-DR2*-associated MS is characterized by earlier age at onset (Oturai et al. 2004; Smestad et al. 2007) than MS without DR2. Sellebjerg and coworkers (2000) found that *DRB1*1501*-positive cases are associated with higher levels of CSF inflammation as assessed by IgG synthesis levels and higher levels of matrix metalloproteinase-9 activity. *DRB1*1501*-positive patients also have a lower percentage of T-cells in CSF expressing *HLA-DR* and CD25.

Recently, genotyping sets of benign and malignant MS patients show that HLA-*DRB1*01* is significantly underrepresented in malignant compared with benign cases. This allele appears to attenuate the progressive disability that characterizes MS in the long term (De Luca et al., 2007). Authors conclude

that *HLA-DRB1*01*, previously implicated in disease resistance, acts as an independent modifier of disease progression. Zivadinov and coworkers (2007) reveal a robust relationship between alleles *HLA-DRB1*1501, -DQB1*0301, -DQB1*0302, -DQB1*0602, and -DQB1*0603* and more severe damage on inflammatory / neurodegenerative MRI measures by high-resolution HLA genotyping analysis. *HLA-DRB1*15* allele is found to modulate the course of MS for RR onset patients likely by precipitating the SP phase (Cournu-Rebeix et al., 2008).

6. GENES SELECTED FOR THE STUDY

The selection of the MS susceptibility candidate genes for this thesis has been done combining different criteria to identify and prioritaze the genes:

- Selection based on previously determined location within a linkage peak or associations in GWAS studies performed in MS.
- Selection according known function identified in autoimmunity through functional studies in experimental autoimmune encephalomyelitis
- Selection based on the association with other autoimmune diseases.
- Selection on the basis of important function in type I interferon system.

6.1. Interleukin 2 and IL2 receptor

Interleukin 2 (IL-2) is the first cytokine to be cloned (Taniguchi et al, 1983). IL-2 is a 15-18 kDa α-helical glycosylated prootein belonging to a family of short-chain, four-helix bundle cytokines, including IL-4, IL-7, IL-9, and IL-15, that all share a common receptor chain for signal transduction and have overlapping and complex roles in immunity. It is secreted predominately by activated CD4+ and in a lesser extent by CD8+ T cells. Activated DCs, NK

cells, and NKT cells also produce IL-2 (Granucci et al., 2005; Jiang et al., 2005), but the biological relevance of IL-2 from these cells remains unclear.

6.1.1. *IL2* gene structure and expression

The *IL2* gene exists in a single copy per haploid human genome (Fujita et al., 1983). It is localized on 4q27 chromosome. The gene contains four exons. Alternative splicing is used to produce splice variants of human *IL2*, called *IL2d2* and *IL2d3*, which lack second or third exons. Their nucleotide sequences are otherwise identical to *IL2*. Unlike neither rhIL-2, neither rhIL-2d2 nor rhIL-2d3 are effective costimulators of T cell proliferation. In contrast, both show dose-dependent inhibition of IL-2 costimulation of T cell proliferation as competitive inhibitors of IL-2 (Tsytsikov et al., 1996).

The regulatory regions of the human *IL2* promoter have been studied in detail and it was initially established that -300 nt upstream from the start of transcription was sufficient for maximal activity in transient transfection assays in Jurkat T cells (Durand et al., 1987). Within this minimal promoter region, two major TCR responsive regions were identified in the human gene and termed Ag-responsive elements (ARRE), which are controled by the binding of AP-1, NF-AT and Oct transcription factors. Another important site within the *IL2* minimal promoter is a region that responds to CD28 costimulation, known as the CD28 response region. This region contains a CD28 response element as well as NFAT protein and AP-1 transcription factor-binding site that functions cooperatively with the CD28RE. The architectural protein, HMGA1 also binds to the CD28RE. Furthermore, this binding has differential effects on NF-kB, NF-AT, and AP-1 interactions at this region playing an important regulatory role in the formation of transcription factor complexes (Attema et al., 2002).

Expression of *IL2* is rapidly and transiently produced upon engaging the TCR and costimulatory molecules such as CD28 on naive T cells; the precise combination of nuclear factors, forming an enhanceosome at the minimal promoter in response to specific activating signals, governs expression of the *IL2* gene (Figure 17).

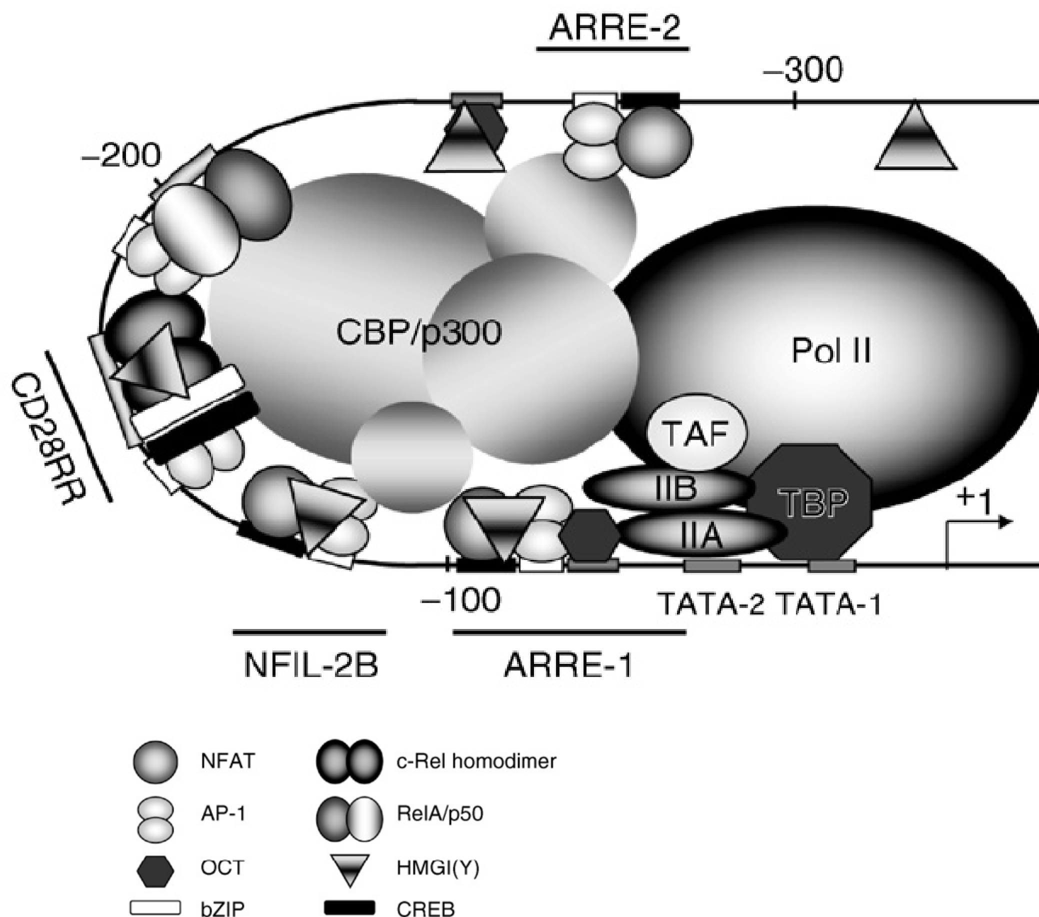

Figure 17. Schematic representation of the putative IL2 enhanceosome assembled at the proximal promoter following T cell activation (Bunting et al, 2006).

Control of *IL2* gene expression during an immune response is mediated by the coordinated binding of transcription factor complexes to the *IL2* promoter to initiate transcription and by the signal-dependent binding of negative regulators which function to suppress *IL2* in a tissue specific manner. The balance between activators and repressors needs to be critically maintained in order to achieve correct temporal and cell-specific expression of *IL2* (Bunting et al. , 2006). The transient nature of IL-2 secretion depends on transcriptional induction by TCR signals and stabilization of IL2 mRNA by

costimulatory signals, followed by transcriptional silencing of the *IL2* gene and rapid degradation of IL2 mRNA (Jain et al., 1995).

A classical auto-regulatory feedback loop has recently been described in which IL-2 inhibits its own production. This loop depends on activation of STAT5 and IL-2-dependent induction of the transcriptional repressor Blimp-1 (B lymphocyte maturation protein-1) (Villarino et al., 2007; Gong & Malek, 2007).

6.1.2. IL-2 functions

The first function attributed to IL-2 was a potent capacity to enhance *in vitro* T-cell proliferation and differentiation (Smith, 1988). Subsequent to initial descriptions of the function of IL-2, numerous studies have highlighted many more seemingly contradictory functions of this cytokine.

With respect to immune-enhancing functions, IL-2 has a role in supporting proliferation, survival (Blattman et al., 2003) of T cells, and differentiation of naive T cells into effector and memory cells (Ke et al, 1998). Recent evidence indicates that IL-2 is also an important factor that allows the generation of memory T cells, which are able to undergo secondary expansion when they re-encounter an antigen (Williams et al., 2006). Furthermore, IL-2 has the ability to overcome the proliferation block of anergic cells generated *in vitro* and *in vivo* (Powell et al., 1998).

In opposition to these immune-enhancing functions, IL-2 can promote activation-induced cell death of T cells (Lenardo, 1991) and was therefore implicated in downregulating antigen-specific T-cell numbers after the clonal expansion phase of an immune response (Refaeli et al., 1998). IL-2 also has anti-inflammatory properties (Figure 18).

IL-2 is demonstrated to influence several endogenous mechanisms of B cell tolerance including those of developmental arrest, follicular exclusion, and apoptosis (Wrenshall et al., 2007). In a similar process to IFNγ, which exerts anti-inflammatory properties by suppressing T-helper 17 cells, IL-2 can constrain IL-17 production (Laurence et al, 2007). The biological activity of IL-2 in vivo is greatly increased by association with IL-2 mAbs (Boyman, 2006).

Thus, the main non redundant role of IL-2 in vivo is concidered the maintenance of peripheral T cell tolerance; it plays a critical role in Treg homeostasis promoting the thymic development, peripheral homeostasis and suppressive function of Treg which constitute a fundamental part of immunological self-tolerance and immune regulation (Malek, 2008).

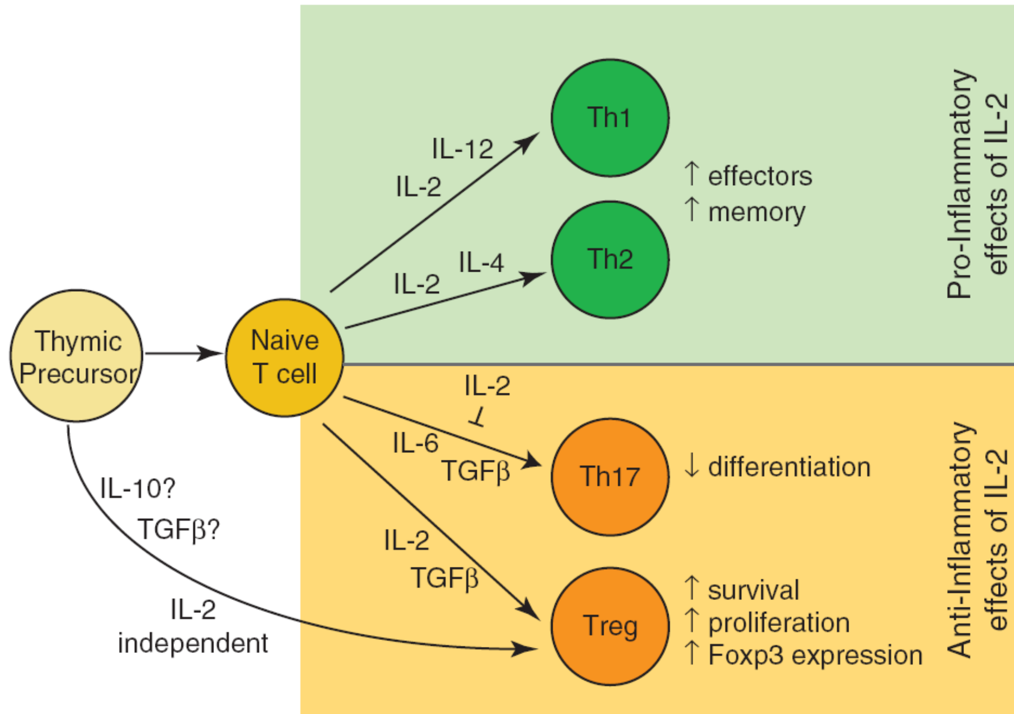

Figure 18. Opposing roles of IL-2 in inflammatory immune responses (Hoyer et al., 2008)

6.1.3. IL-2R

To exert its biologic effects, IL-2 must interact with a specific membrane IL-2 receptor (Figure 19). The high-affinity receptor for IL-2 is a heterotrimeric membrane protein complex consisting of an IL-2-specific α-subunit (IL-2Rα, CD25), a β-subunit (IL-2Rβ, CD122) and the common cytokine receptor γ-chain (γc, CD132). The γ chain is shared by other

receptors belonging to this family (IL-4R, IL-7R, IL-9R, IL-15R, IL-21R, and so on). The heterodimeric IL-2Rβγ complex is expressed constitutively on resting T cells.

The IL-2Rα chain alone binds to IL-2 with low affinity (dissociation constant (*K*d) = ~10^{-8} M), it has a short cytoplasmic tail that is not involved in recruitment of cytoplasmic signal transduction molecules. In combination with the IL2Rβ and γc chains, the affinity of the receptor for IL-2 is increased by three orders of magnitude (*K*d = ~10−11 M) (Lin & Lenard, 1997).

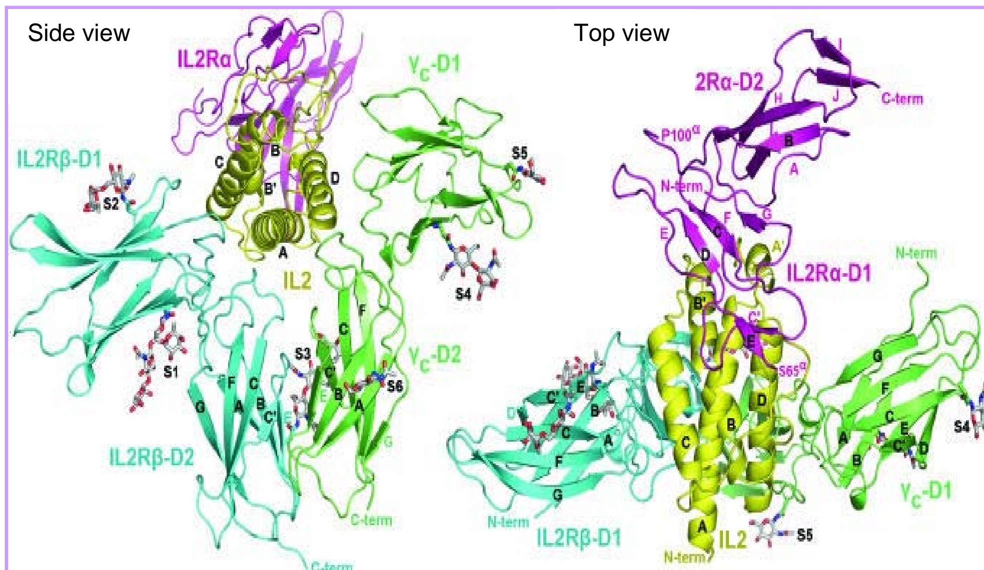

Figure 19. The quaternary IL-2 signaling assembly (Stauberet al., 2006)
IL-2 binds to the elbow regions of IL-2Rβ and γc. IL-2Rα docks on top of this assembly without forming any contacts with the other two receptor subunits.

The crystal structure of IL-2 bound to the IL-2R reveals that each receptor subunit contacts IL-2, with most contacts at the IL-2/CD25 interface, and that significant interactions occur between CD122 and γc, leading to a stable quaternary complex of IL-2, CD25, CD122, and γc (Stauber et al.,

2006). The cytoplasmic domains of the IL-2R β and γ chains are responsible for signal transduction through activation of the Janus kinase 3 (JAK3)/ signal transducer and activator of transcription 5 (STAT5) and AKT dependent signalling pathways, and the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, respectively (Figure 20).

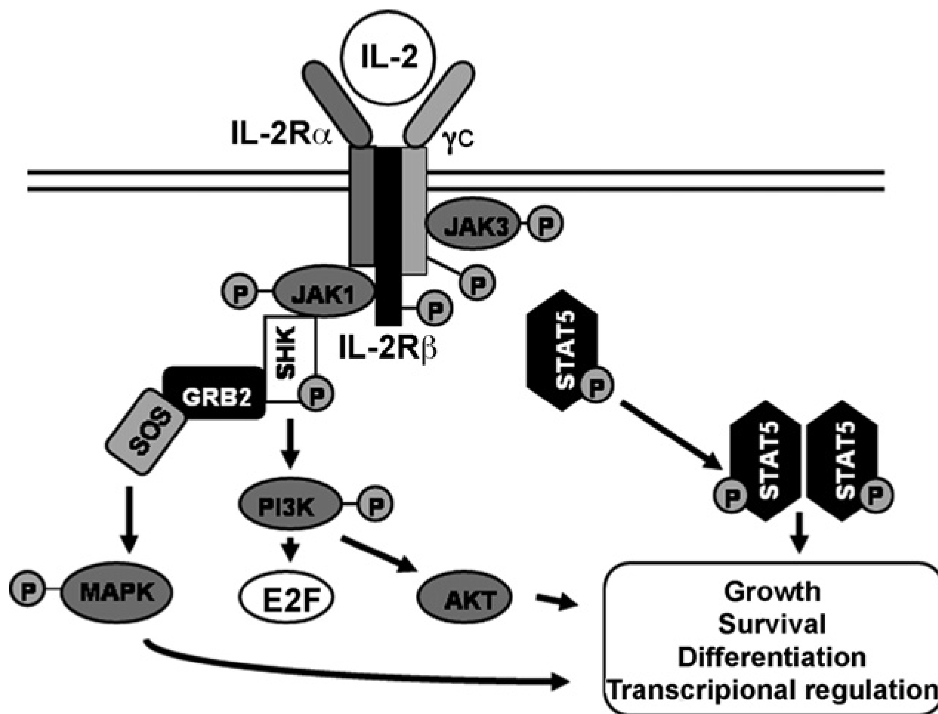

Figure 20. IL2 signaling (Chistiakov et al., 2008).

STAT5 is the main IL-2-induced STAT in activated T cells that regulates genes important for effector function and T cell growth. Although activation of the MAPK, PI3K, and STAT5 pathways are important for conventional activated T-cells, STAT5 is the main pathway by which IL-2R contributes to Treg cell production and maintenance (Malek, 2008).

Expression of the IL-2Rα gene is tightly regulated at the transcriptional level. This gene is induced not only by antigen, but also by mitogens and

lectins that mimic antigenic stimulation as well as a range of other stimuli. These include cytokines such as IL-1, IL-2, IL-7, IL-12, IL-15, TNFα, and TGF-β, and also several viral proteins such as Tax of the human T-cell lymphotrophic virus type I, EBNA-1 (EBV nuclear antigen-1) and EBNA-2 of EBV in human B lymphoma cells. In addition to viral proteins, IFNα that is produced in the course of an immune response to viral infections can induce IL-2Rα expression in human T cells. IL-16, which is a natural, soluble ligand for CD4, can also induce the expression of IL-2Rα and β chains (Kim et al. , 2006).

IL-2Rα expression is undetectable on resting T cells but is induced by their exposure to antigen. Concomitantly, antigens also induce the secretion of IL-2, which can in turn increase and prolong IL-2Rα expression, thus acting as a positive feedback regulator for the expression of its own high-affinity receptor. This positive auto-regulatory loop of the IL-2/IL-2R system plays a major role in controlling the magnitude and duration of the T cell immune response (Kim et al., 2006). Up-regulation of IL-2Rα after activation is a feature of many cell types (for example, NK cells, B cells, monocytes).

Soluble IL-2Rα is produced by a membrane proteolytic mechanism (Rubin & Nelson, 1990). Cleavage of IL-2Rα is observed in parallel with activation of T cells, and *in vitro* activated human T cells release soluble IL-2Rα (sIL-2Rα) in culture supernatants. The released form of IL-2R, which is 10kDa smaller due to lack of transmembrane and intracytoplasmic domains, still retains the ability to bind IL-2, although it is approximately 100- to 1 000-fold lower than the high-affinity IL-2R complex. The ability of this molecule to bind to IL-2 suggests a potential role in the regulation of IL-2-dependent cell function by competing with cellular IL-2R for the growth factor IL-2, thus down-regulating the immune response (Caruso et al., 1993). sIL-2R acts as a binding protein, effectively prolonging the half-life of IL-2, with low-affinity slL-2R delivering IL-2 to high-affinity membrane receptors and/or serving to locally confine the effects of IL-2, hence, keeping immune responses local (Rubin & Nelson, 1990).

6.1.4. IL-2 and the genetic basis of autoimmunity

The phenotype of *IL2*$^{-}$ and *CD25*$^{-}$ deficient mice is very similar. Adult mice develop massive enlargement of peripheral lymphoid organs associated with polyclonal T and B cell expansion, which, for T cells, is correlated with impaired activation-induced cell death in vivo. Older animals develop autoimmune disorders, including hemolytic anemia and inflammatory bowel disease (Sadlack et al., 1995). IL-2Rα is found essential for regulation of both the size and content of the peripheral lymphoid compartment (Willerford et al., 1995). Mice lacking the IL2Rβ chain also exhibit multi-organ autoimmune disease resulting in early death (Suzuki et al., 1995).

The discovery of novel T cell subset characterized by high level IL2Rα expression CD4+CD25+, ‘‘naturally occurring’’ Treg cells in conjunction with the observation that *IL2*−/− and *IL2Rβ*−/− mice lacked CD4+CD25+ T cells, permited to suggest that AI associated with IL-2/IL-2R deficiency is primarily due to failed production of Treg cells (Malek et al., 2002). Later, after the finding of new marker, transcriptional regulator FoxP3 as a key regulatory gene of CD4+CD25+ Treg (Hori et al., 2003) it was shown that phenotype of Foxp3-deficient mice lacking Treg cells is very simillar to that of IL-2/IL2R deficient mice, consistent with the genetic basis for *IL2/IL2R* in immune tolerance at the level of Treg cells. Even though IL-2 and IL-15 share common receptor signaling subunits, i.e., CD122 and γc, IL-15- and IL-15Rα- deficient mice are autoimmune-free and contain a normal number of Foxp3+ Treg cells, firmly establishing that IL-2 is the critical cytokine for Treg cells. IL-2- and IL-2R-deficient mice are not completely devoid of Foxp3+ cells, but rather lack population of mature CD4+CD25+Foxp3high Treg cells and contain few immature CD4+CD25-Foxp3 low T cells (Malek, 2008).

6.1.5. Role of IL2 in the development and homeostasis of CD4+CD25+Foxp3+ Tregs

Besides confirming a growth/survival role, IL-2 provides two additional and important functions during Treg development in thymus: induction of CD25 and heightened levels of Foxp3. These two activities likely represent

critical essential maturation steps in Treg cell development. Induction of CD25 is critical for high-affinity IL-2 binding by Treg cells to promote responsiveness to limiting amount of IL-2 in vivo during development/maturation and peripheral homeostasis. IL-2 is implicated in establishing the Treg cell functional program by fixing and maintaining Foxp3 levels (Gavin et al., 2007). The presence of STAT5-binding sites within the promoter region of the Foxp3 gene is consistent with a direct function for IL-2R signaling as it readily activates STAT5 (Zorn et al., 2006). This programming begins in the thymus and likely continues in the periphery. IL-2 plays the predominant role in Treg development, but in its absence the IL-7Rα and IL-15Rα chains are up-regulated and allow for IL-7 and IL-15 to partially compensate for loss of IL-2 (Vang et al., 2008).

Considerable evidence has been accumulated supporting an important role for IL-2 in Treg cell homeostasis. Upon exiting the thymus during neonatal life, IL-2 is responsible for rapid amplification of the number of Treg cells in peripheral lymph nodes to insure suppression of autoreactive T cells that escape negative selection, thereby maintaining tolerance. Accordingly, after treatment with anti-IL-2, Treg cell numbers are substantially reduced in lymph nodes of neonatal mice (Bayer et al., 2005). IL-2-dependent control of peripheral Treg cells is not just limited to newborn mice. Young BALB/c and NOD mice develop autoimmune disease after treatment with anti- IL-2 (Setoguchi et al., 2005). The homeostasis of Treg cells in mature immunocompetent mice also depends on IL-2. In comparison to wild-type peripheral Treg cells, the IL-2R β-defective Treg cells exhibit lower growth and negligible turnover rates (Bayer, 2007). Using Foxp3-deficient mice simultaneously bearing the IL2-/-, totally lacking Tregs, it was recently demonstrated a heretofore unrecognized novel function of IL-2 in the Treg-controlled autoimmune response in that it regulates the organ-specific (toward lung and skin but not liver) AI in Sf mice (Zheng et al., 2007).

IL-2 couples the functional state of Treg cells to the intensity of the immune reaction; the delicate balance of this cytokine is further affected by

reciprocal cytokine composition in the environment and their relative rates of proliferation. High IL-2 concentrations sensitize Teff cells to apoptosis, while no such effect parallels its influences between cytotoxic and suppressive cells in situ. IL-2 mediates several feedback regulatory loops within the communication between the Teff cells (as targets of suppression) and Treg cells (as effectors of suppression). Since Treg cells require higher concentrations of IL-2 than Teff cells (Fehervari et al., 2006) accumulation of IL-2 at high concentrations within the local inflammatory infiltrate is expected to induce the generation of adaptive Treg cells and activate innate Treg cells. This activation includes induction of Treg cell proliferation and induction–maintenance–enhancement of their suppressive activity. According to this scenario, the onset of cytotoxic cell activity creates an ambient environment for Treg cell function as a coupling mechanism of immune homeostasis (Figure 21).

IL-2-mediated coupling also imposes differential sensitivity to AICD. Under inflammatory conditions, the sensitivity of Treg cells is generally lower than that of Teff cells; however, it is significantly affected by the nature of TCR engagement, the cytokine composition in the environment and their relative rates of proliferation. High IL-2 concentrations sensitize Teff cells to apoptosis, while no such effect parallels its influence on Treg cells indicating a survival advantage oover Teff cells (Yarkoni et al., 2008).

All these data support a model where Treg cell development is blocked without IL-2, leading to production of a reduced number of immature CD4+ Foxp3lowCD25- T cells. These cells remain competent to seed the periphery but are unable to suppress autoreactive T cells.

6.1.6. Implication of IL2/IL2Ra system in MS and other autoimmune diseases

Studies in EAE gave evidence for the implication of *IL2* locus in the susceptibility to and severity of this disease. A genetic linkage analysis using a cross between susceptible and resistant mice revealed a narrowly defined locus affecting EAE severity which contains the *IL2* gene (Encinas et al, 1999).

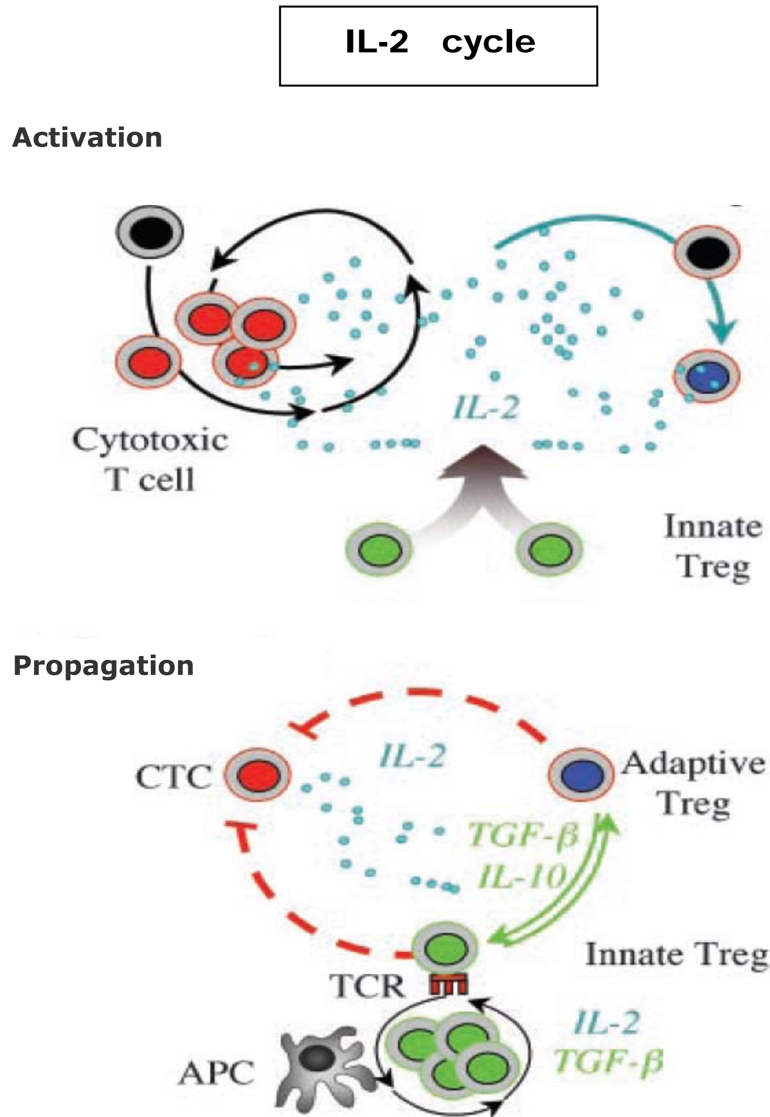

Figure 21. IL-2-related cycles of communication between effector and regulatory T cells at the onset of the immune reaction (activation) and under inflammatory conditions (propagation) (Yarkoni et al., 2008).

Vulnerability to EAE is markedly reduced in C57BL/ 6 mice lacking *IL2*, as well as its clinical severity in *IL2+/-* heterozygous mice, suggesting that this cytokine may play a critical role in autoimmune processes of the central nervous system (Petitto et al., 2000). The constitutive expression of *IL2* can

result in organ-specific AI (Heath et al., 1992), although conversely lower intrinsic IL-2 production on the NOD genetic background increases diabetes susceptibility (Yamanouchi et al., 2007). For naive T cells, enhanced IL-2 signaling can convert what is otherwise a tolerogenic response into one that includes the destruction of self-tissues (Waithman et al, 2008). The polymorphisms upstream of the minimal *il2* promoter in mice, that affect the transcriptional activity (del Rio et al., 2008), are suggested as candidates for a shared autoimmune disease susceptibility locus in EAE, autoimmune ovarian dysgenesis and insulin-dependent diabetes mellitus.

Similarly to findings in several autoimmune diseases as T1D (Roncarolo et al., 1988) and Sjörgen´s syndrome (Miyasaka et al., 1984), the IL2 secretion in PBL after stimulation was found significantly selectively diminished in MS patients with relapse and in remission (Selmaj et al., 1988; Wandinger et al., 1997). Studies on cytokine patterns within postmortem CNS specimens however revealed high levels of IL2 expression in acute MS lesions (Canella Raine, 1995). The mean percentage of CD3 positive T cells producing IL-2 is significantly higher in SPMS than in RRMS (Nguyen et al., 2001).

Potent effects of IL-2 in CNS have been described, including activities related to cell growth and survival, transmitter and hormone release and modulation of bioelectric activities (Hanisch & Quirion, 1995). IL-2 may be involved in regulation of sleep and arousal, memory functions, locomotion and the modulation of neuroendocrine axis. Mainly oligodendrocytes and neurons have been shown to respond to the cytokine (Hanisch et al., 1996; Benveniste & Merrill, 1986; Otero & Merrill, 1997). On the other hand, marked neurotoxicity as the neuroendocrine consequence of chronically elevated CNS levels of IL-2 has been observed (Hanisch et al., 1996).

As a result of GWAS several polymorphisms in the chromosome 4q27 region have been recently involved in susceptibility to multiple autoimmune and inflammatory diseases such as T1D and Graves´ disease (Todd et al., 2007), celiac disease and RA (van Heel et al., 2007), psoriasis (Liu et al., 2008). Wellcome Trust Case Control Consortium study reports SNPs (rs17388568 and rs3136534) from 4q27 *TENR-IL2-IL21* region associated

with T1D and Graves´ disease (Todd et al., 2007). Recent meta-analysis of data from three GWAS in T1D obtained further support for 4q27 *IL2-IL21* region (P = 1.9 x10^{-8}) (Cooper et al., 2009). After discovery and replication of highly significant associations from GWAS conducted in celiac disease, van Heel and co-workers (2007) indicate that rs6822844, from the same *TENR-IL2-IL21* block with high degree of LD, has the strongest association in three ethnic cohorts. *TENR-IL2-IL21* region includes three genes. *TENR* gene has testis-specific expression and unlikely could be implicated in MS etiology, while the *IL2* and *IL21* genes are two strong positional and functional candidates. An etiological variant(s) controlling susceptibility to autoimmune disorders on chromosome 4q27 needs to be identified.

The *IL2Ra* gene resides on chromosome 10p15.1, in the vicinity to the T1D susceptibility locus IDDM10, which was mapped to chromosome 10p14-q11 and consistently showed a strong linkage to disease in several whole-genome scans (Concannon et al., 1998; Cox et al., 2001; Concannon et al., 2005). Using a tag -SNP approach, Vella et al. (2005) showed association between the *IL2Ra* gene and T1D in a large-scale case–control study providing highly significant p-value and then replicated it in the independent family-based study. The results of Vella and coworkers (2005) have been confirmed by Qu and coworkers (2007) who analyzed a Canadian family dataset and found two SNPs showing the highest association with T1D. Both marker located in the 5′ end of the long intron 1 of the *IL2* gene within 3 kb of each other, are in high LD and confer similar odds ratios. Although the data of Qu and coworkers provided a strong evidence for the involvement of the *IL2* genetic variants in susceptibility to T1D, the causal variant was not found.

6.2. IL6 and its role in MS

Proinflammatory cytokines are known to play an important role in disease progression and tissue damage of autoimmune diseases such as MS,

rheumatoid arthritis, and systemic lupus erythematosus. IL-6 is considered one of the major proinflammatory cytokines.

IL-6 is a pleiotropic cytokine expressed by APCs such as DC, macrophages and B cells among other cells of the hematopietic system but is also produced by a variety of non-hematopoietic cells including astrocytes (Kamimura et al., 2003). IL-6 binds to a receptor complex consisting of the specific IL6R and the common signaling component gp130 which is also shared by other cytokines of the IL-6 family. Jak1 is thought to be most relevant for IL-6 signaling although Jak2 and Tyk2 also transduce some of the IL-6 signals (Heinrich et al., 2003). STAT 3, to a lesser extent, STAT1 and the transcription factor CCAAT/enhancer binding protein have been involved in IL-6 signaling (Gerhartz et al., 1996). Lastly, phosphatidyl-inositol 3-kinase has been described as a signal transducer of IL-6 triggering the activation of Akt and subsequently promoting survival in many cell types (Chen et al., 1999).

Although IL-6 has been initially described as B cell growth factor and has long been known to play a role in antibody production this might be a mostly indirect effect through its upregulation of IL-21 production in CD4 T cells (Dienz & Rincon, 2009). IL-6 is now considered as an important mediator of the immune response especially by directly acting on CD4 T cells and determining their effector functions. The invasion of CD4 T cells into the CNS is thought to play a significant role in the pathology of EAE as well as MS in humans.

IL-6 exerts its effects on cytokine production through a diverse set of key molecules. Upregulation of SOCS1 expression inhibits IFNγ signals thereby diminishing additional IFNγ production. IL-4 production is mediated through the induction of NFATc2 and c-maf expression. STAT3 directly regulates IL-6 induced IL-21 expression, which therefore precedes expression of the other cytokines. Simultaneous stimulation through IL-6 and TGFβ induces high level expression of retinoic-acid receptor related orphan nuclear receptor (RORγt) which mediates IL-17 production (Dienz & Rincon, 2009).

IL-6 contribute to T helper cell differentiation and subsequent cytokine production by various T cell subsets (Figure 22). IL-6 exerts inhibitory activity

towards Th1 and Treg differentiation/ function, upregulating RORγt expression and downregulates Foxp3 thereby shifting the balance from Treg towards Th17 differentiation. IL-6 present during antigen stimulation of CD4 T cells promotes autocrine IL-4 production which further enhances Th2 differentiation through an autofeedback loop, modulating the Th1/Th2 balance (Rincon et al., 1997). IL-6 promotes Th17 differentiation either alone (Th2) or together with TGFβ (Th17). The role of IL-6 in T follicular helper (Tfh) cell differentiation is suggested due to its specific upregulation of IL-21 in naïve CD4 T cells (King et al., 2008).

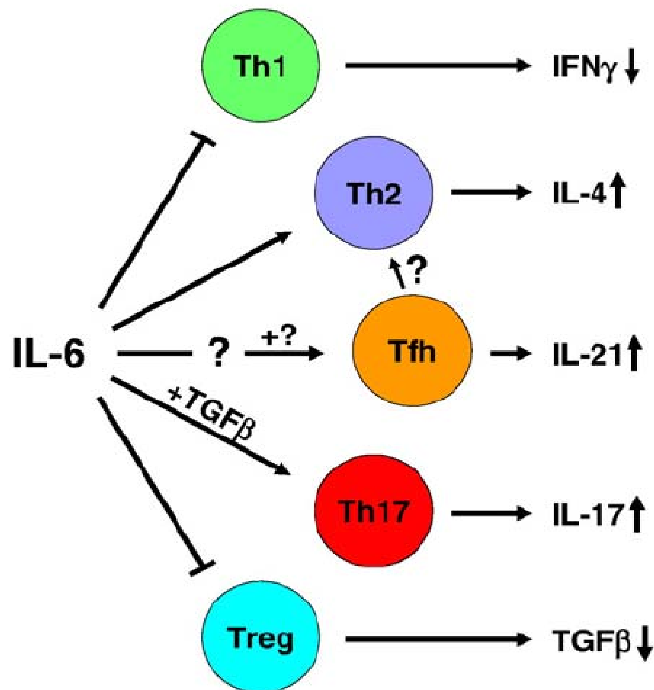

Figure 22. Contribution of IL-6 to T helper cell differentiation and subsequent cytokine production by various Tcell subsets (Dienz & Rincon, 2009).

The IL-6 blockade in EAE produces protective effect and is likely to be mediated via the inhibition of the development of MOG-peptide-specific Th17 cells, considering a key event in the pathogenesis of EAE, and Th1 cells that in turn leads to reduced infiltration of T cells into the CNS (Serada et al., 2008).

There has been a growing appreciation of the destructive potential of elevated levels of IL-6 in the CNS. IL-6 potentiates neural injury in Alzheimer disease, Parkinson disease, HIV encephalopathy, MS, depression, and cognitive impairment (Gruol & Nelson, 1997; Frei et al., 1991). Neutralization of CNS IL-6 attenuates traumatic spinal cord injury in rats and is associated with reduced iNOS activity (Tuna et al., 2001). Augmented levels of IL-6 in serum of MS patients have also been reported (Frei et al., 1991). Mononuclear cells in CSF and in blood of MS patients have expressed high levels of *IL-6* mRNA (Navikas et al., 1996) and people with the disease have higher levels of IL-6 secreting DC than healthy subjects.

IL-6 has been implicated in the etiology of EAE in transgenic animals. *IL6* knockout mice have been shown to be highly resistant to the development of EAE (Okuda et al., 1998). The histological examination revealed that no infiltration of inflammatory cells was observed in the central nervous system of *IL6*-deficient mice. Furthermore, Samoilova and coworkers (1998) established that the resistance to EAE in *IL6*-deficient mice was associated with a deficiency of MOG-specific T cells to differentiate into either Th1 or Th2 type effector cells in vivo. High levels of *IL6* mRNA-expressing cells are present in the CNS in protracted relapsing-EAE, while almost absent in acute EAE (Diab et al., 1997).

In MS lesions *IL6* expression is up-regulated in active lesions relative to inactive lesions (Baranzini et al., 2000; Mycko et al., 2004) and could contribute to axonal destruction. The *IL6* gene maps to chromosome 7p21. It does not contain any common polymorphism within coding regions, and attention has consequently focused on promoter polymorphisms. A SNP at position *-174 G>C* (rs1800795) in gene promoter was found to influence *IL6* expression in vitro (Fishman et al., 1998), it is also functional in vivo with an increased inflammatory response associated with the *G* allele (Bennermo et al., 2004).

The potential modifying role of *IL6* gene on disease course in EAE and the association with other autoimmune diseases prompted us to investigate the role this gene may have in MS disease in humans.

6.3. FcRL3 and autoimmunity

The chromosomal region 1q21-q23 (Figure 23) contains a gene cluster of Fc-like receptors, *FCRL1-FCRL5*, and a family of Fcγ receptor (FcγR) II/III genes (Davis et al., 2001). The cluster spans around 300 kb, it also includes two LD blocks and the longest of those has a 150-kb length that includes all the above genes except for *FCRL5*.

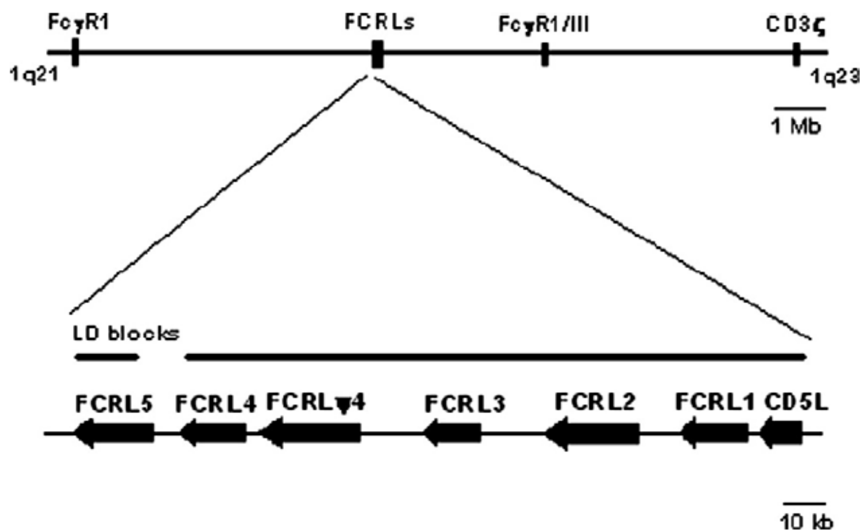

Figure 23. Structure of the FcRLγ gene cluster (Chistiakov & Chistiakov, 2007)

FCRLs exhibit high structural homology with classical receptors for the Fc portion of IgG, FcγRs (Miller et al., 2002). FcγRs play a critical role in the modulation of immune response (Figure 24). Autoreactive antibodies in MS can mediate their effector function through binding to members of this family. Upon interaction with IgG, these receptors initiate phagocytosis, antibody-dependent cellular cytotoxicity, transcription of cytokine genes, and release of inflammatory mediators (Dijstelbloem et al., 2001).

The genomic sequence of *FCRL3* consisting of 16 exons and 15 introns spans 24.3 kb. Transcription of the *FCRL3* gene results in the full-length isoform and four alternatively spliced products (Xu et al., 2002).

FCRL3 is mainly expressed in secondary lymphoid organs although its exact function and the nature of its ligands remain unknown (Miller et al., 2002; Kochi et al., 2005). The expression of this immune receptor is mainly restricted to CD19+ cells, which represent the B-cell population, but could be also detected at low levels in CD4+ and CD8+ lymphocytes (Kochi et al., 2005). *FCRL3* encodes a glycoprotein that is a member of the immunoglobulin receptor superfamily.

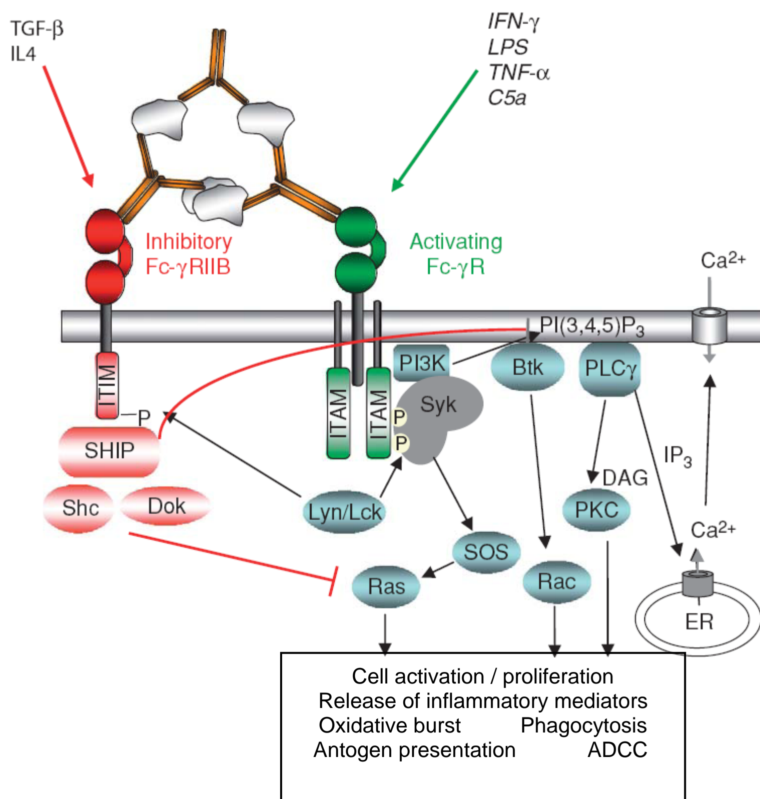

Figure 24. Coregulation of activating and inhibitory FcR signalling (Nimmerjahn & Ravetch, 2007)

Although its precise function remains unknown it contains immunoreceptor tyrosine-based activation and inhibition motifs in its cytoplasmic domain, suggesting that it plays a role in immune cell regulation (Figure 24) (Ravetch & Bolland, 2001).

The chromosome 1q21-q23 was found to harbor a susceptibility locus for several AID; however, because of LD within the gene cluster, it is difficult to resolve a true disease-associated gene variant(s) in this genomic region. A highly significant association between four single SNPs located within the *FCRL3* gene and RA was reported in the Japanese population (Kochi et al., 2005). These four markers showed the strongest association with disease among 41 SNPs across the *FCRL* gene cluster tested in that study. They are fcrl3_3, fcrl3_4, fcrl3_5, and fcrl3_6 SNPs corresponding to the *-169C/T, -110 A/G, +358 C/G*, and *+1381 A/G* dimorphic markers. The first two markers are situated at the 5′ promoter region, whereas the *+358 C/G* and *+1381 A/G* SNPs are located in exon 2 and intron 3 of the *FCRL3* gene, respectively. The four markers are in strong LD with each other except for the fcrl3_4 SNP that shows relatively weak LD with the other three SNPs.

The *FCRL3 −169CT* variant has been shown to affect the binding affinity of the nuclear factor-kappa-B and the level of expression of the gene *in vitro*. *In vivo*, the *FCRL3 −169C* allele was also associated with an increased level of expression of the gene, and an increased autoantibody production (Kochi et al., 2005).

6. 4. The involvement of IL-7R signaling in disease

The IL-7 receptor (IL-7R) contains a unique 75 kd α chain and a γ chain shared with IL-2, 4, 7, 9, 15, and 21 (Mazzucchelli, 2007). *IL7Ra* gene has two common mRNA isoforms, one encoding full-length protein, which is membrane-bound, and the other with exon 6 spliced out to encode soluble receptor (McKay et al., 2008)

Binding IL-7 to its cognate receptor activates multiple pathways that regulate lymphocyte survival, glucose uptake, proliferation and differentiation. While IL-7 shares pro-survival and proliferative capacities with related interleukin family members, it also plays non-redundant roles in T and B cell development and homeostasis (Fry & Mckall, 2005). IL-7 is one of the signals

crucial to drive the innate like differentiation program of human iNKT cells in fetal life (de Lalla et al., 2008). DC development, maturation and antigen presentation are partly controlled by IL-7 (Varas et al., 1998). IL-7 preferentially induces DC1 and Th1 polarization and increases IL-2 and IFNγ production (Gringhuis et al., 1997; Borger et al., 1996).

IL-7Rα is also expressed on DC and monocytes, suggesting a possible role for IL-7 in multiple hematopoietic lineages (Reche et al., 2001). In recent study, a low level of IL-7Rα was shown to be an excellent marker for Foxp3 expression and human T reg cells, which in most instances up-regulate IL-2R while remaining IL-7R$^{lo/-}$ (Liu et al., 2006).

Major signaling cascades activated by the binding of IL-7 to IL-7R include the Jak-STAT and PI3K-Akt pathways (Jiang et al., 2005). In lymphocytes, IL-7R signaling results in survival, proliferation and differentiation, depending on the developmental stage of the lymphocyte. In DC, IL-7R signaling has an immunomodulatory role, especially in the context of thymic stromal lymphopoietin (TSLP), which also signals through the IL-7Rα in a heterodimeric complex with the TSLP receptor. Recent data indicate that IL-7R signaling contributes to thymic Treg cell development and peripheral homeostasis; in states of diminishing thymic output with age and infection, IL-7 may be a more important factor preventing autoimmune disease at the level of Treg cells (Bayer et al., 2008).

Both IL-7 and IL-7Rα knockout mice have been studied. IL-7 knockout mice are deficient in T cells, B cells, NK cells, and NKT cells as well as intra-epithelial lymphocytes (von Freeden-Jeffry et al., 1995). Similarly, mice lacking IL-7Rα have a similar but more severe phenotype than IL-7 knockout mice (Peschon et al., 1994), possibly because TSLP signaling that triggers some unique signaling pathway is also abrogated in the *IL7Rα* knockout mice (Isaksen et al., 1999).

A mutation in the *IL7Rα* in humans leads to an unusual form of combined immunodeficiency disease characterized by severe T-cell defects but normal B cells and natural killer cells (Puel et al., 1998).

IL-7 is considered to be present in limiting amounts in the body and competition for IL-7 maintains mature T cell homeostasis by limiting the size of the surviving T cell population (Mahajan et al., 2005). Consequently, *IL7Rα* is under tight transcriptional control, and the cell type and stage-specific regulation of *IL7Rα* ensures optimal utilization of limited cytokine stores. According to the CD8 coreceptor tuning model there is a co-regulation of TCR and IL-7R induced signaling (Park et al., 2007) (Figure 25).

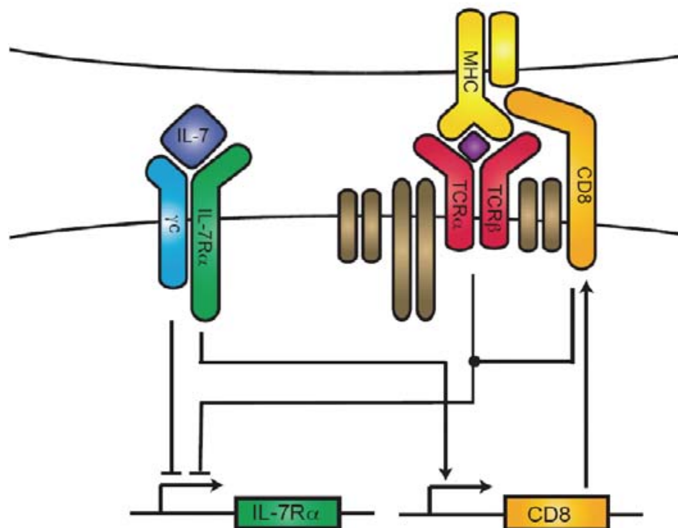

Figure 25. Influence of integrated gene regulation on signaling.
(Palmer, 2008)

IL-7 transcriptionally increases CD8α expression to promote TCR-self peptide-MHC engagement, but TCR signaling impairs IL-7R signaling. Exposure of CD8+ T cells to low levels of IL-7 *in vitro* for a few hours can pre-dispose them to an enhanced syngeneic mixed lymphocyte reaction with syngeneic DCs (Park et al., 2007). This phenomenon suggests that autoimmune phenotypes may involve deregulation of coreceptor tuning machinery.

Negative feedback to *IL7Rα* transcription, triggered by IL-7R signaling, is believed to maximize the size of the population in the presence of limiting

amounts of IL-7. It is suggested the 'altruistic' IL-7Rα downregulation by activated cells in their competition with naïve cells for ligand (Park et al., 2004) that prevents depletion of available IL-7 by the expanding population of activated cells, which is required for naïve T cell survival. Conversely, other network control mechanisms such as positive feedback loops that amplify the IL-7R signal have also been observed. IL-7R signaling contributes to signal amplification and sharp response thresholds. In developing B cells, IL-7R signaling causes upregulation of the transcription factors EBF and E2A, which in turn upregulate IL-7Rα, leading to a self-sustaining positive feedback loop (Medina & Singh, 2005).

The involvement of IL-7R signaling in many diseases is not completely understood. A likely causal single nucleotide polymorphism (rs6897932) in the *IL7Rα* gene is recently identified in a population of MS patients (Gregory et al., 2007). It results in aberrant expression of a soluble isoform of the IL-7Rα by putatively disrupting an exonic splicing silencer. Simultaneously, the level of the normal membrane-bound IL-7Rα is reduced. This may have an impact on the pathogenesis of MS by altering the dynamics of the IL-7 or TSLP signaling network in multiple cell types. Notably, TSLP-activated DCs are involved in CD4+ T cell homeostasis and Treg cell development in the thymus (Watanabe et al., 2005). The effect of the *IL7Rα* marker appears to be HLA-independent as in a data subset from US, the association is the same in both*HLA-DRB1*1501/1503* carriers and non-carriers (Gregory et al., 2007).

6.5. The importance of *OAS1* in antiviral response

Initially identified as IFN-induced proteins that generate low-molecular-weight inhibitors of cell-free protein synthesis, the 2,5-oligoadenylate synthetases (OAS) are distinguished by their capacity to synthesize 2′,5′-linked phosphodiester bonds to polymerize ATP into unique 2′,5′-oligomers of adenosine (Rebouillat & Hovanessian, 1999), which are an important component of an antiviral RNA decay pathway.

Epidemiological findings suggest that MS is triggered by unknown environmental factor in genetically susceptible individuals. Several cases of virus-induced demyelinating encephalomyelitis in human beings and in experimental models as well as the presence of IgG oligoclonal bands in the cerebrospinal fluid indicate that the causative factor may be viral. Recently EBV is showed to be present within B cells that infiltrate the meninges and white matter in progressive MS cases (Serafini et al., 2004). The CSF of MS patients contains clonotypes that are reactive against EBV sequences (Cepok et al., 2005). Viral peptides activate human T cell clones specific for MBP (Wucherpfennig & Strominger, 1995). Also MS relapses are often associated with common viral infections. Thus genes associated with IFN response and virus infectivity are reasonable candidates for studies in the context of MS susceptibility and progression.

The four *OAS* genes identified in humans, termed *OAS1*, *OAS2*, *OAS3* and *OASL* (OAS-like), have been mapped to chromosome 12q24.13 (Ghosh et al. , 1997; Hovanessian et al., 2007). They are clustered within a region of 130 kb chromosome, which represents the 2′–5′OAS locus. They share the same orientation of transcription and are arranged in the order centromere – 5′-*OAS1—OAS3—OAS2*-3′ telomere. These proteins have considerable homology to each other but it seems to be differential expression and induction of each form of the human OAS proteins. Also, each of the three functional OAS proteins has unique biological functions (Marie et al., 1990). The precise roles of the different OAS proteins, especially relating to RNaseL-independent antiviral effects, are still ill-defined.

The OAS1 has been shown to be an important part of the total constitutive activity of OAS enzymes. OAS1 protein accumulates in the cell cytoplasm as an inactive monomer (Figure 26). Following activation by viral double-stranded RNA (dsRNA), the catalytically active form of OAS1 enzyme oligomerizes to form a tetramer that synthesizes 2′, 5′-oligoadenylates that, in turn, activate the constitutively expressed inactive ribonuclease L (RnaseL) enzyme to cleave cellular and viral RNAs (Ghosh et al., 1997).

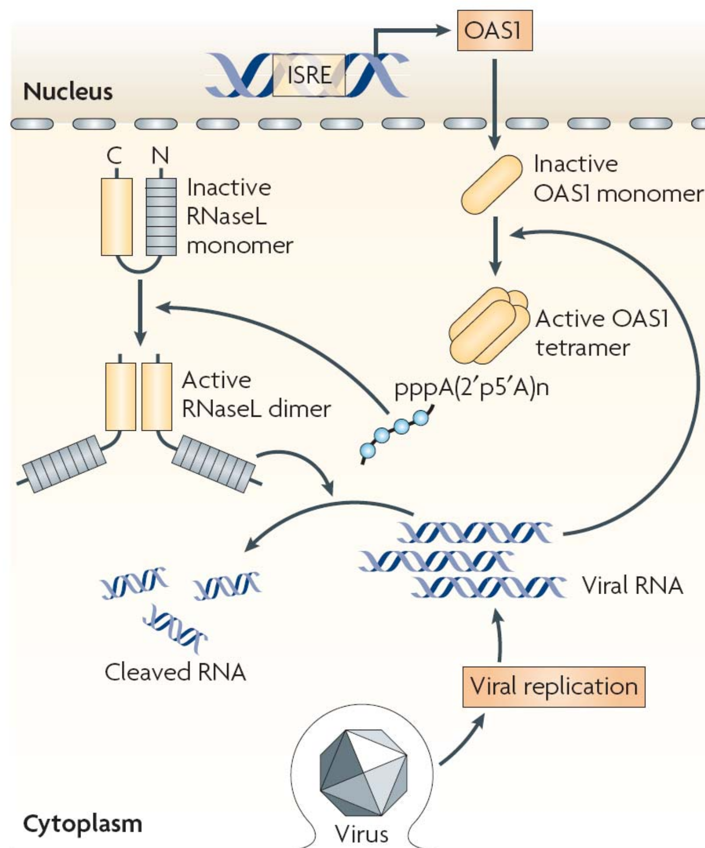

Figure 26. The OAS1–RNaseL antiviral pathway (Sadler&Williams, 2008)

As the OAS proteins are constitutively expressed at low levels, they can act not only as effector protein but also as double-stranded RNA-specific pattern-recognition receptors (PRRs) for the detection of viral dsRNA in the cytoplasm. RNA degraded by RNaseL can activate the other cytoplasmic PRRs, resulting in the induction of type I IFN gene expression (Malathi et al., 2007). This is an amplification mechanism for IFN production through activation of the OAS–RNaseL pathway.

The direct importance of OAS1 protein in the antiviral response in humans has been highlighted by genetic studies showing that polymorphisms within a splice-acceptor site of the *OAS1* gene (producing two isoforms of the enzyme

with different activities) significantly correlate with the antiviral response to the yellow fever vaccine in immunization clinical trials (Bonnevie-Nielsen et al., 2005).

The antiviral activity of *Oas1b*, the mouse ortholog of *OAS1*, is crucial in the inhibition of West Nile and other flavoviruses, both in vivo and in vitro (Mashimo et al., 2002; Perelygin et al., 2002; Lucas et al., 2003). SNPs at a splice enhancer site in *OASL* have been correlated with susceptibility to infection with WNV (Yakub et al., 2005).

A polymorphism identified in *OAS1* has also been associated with T1D (Field et al., 2005, Bonnevi-Nielsen et al., 2005). The A/G substitution at rs10774671 involves the last nucleotide of intron 6 of *OAS1*. *G*, the minor allele, retains the splice acceptor site with the consensus sequence *AG*, while the *A* allele ablates the splice site. This splicing SNP is shown to be associated with OAS1 enzyme activity change, the *G* allele being associated with higher enzyme activity (Bonnevie-Nielsen et al., 2005). Higher basal activity has also been reported in subjects with T1D compared to healthy siblings (Bonnevie-Nielsen et al., 2000).

Based on the hypothesis of common genetic risk determinants to autoimmune diseases and strong evidence of virus involvement in the MS pathogenesis (see part 3.2) we considered *OAS1* as interesting gene candidate for MS susceptibility and response to IFNβ treatment.

STUDY OBJECTIVES

III. STUDY OBJECTIVES

1. To investigate the role of *IL2/IL2Ra* system in susceptibility to MS.

- Analysis of an association with the locus 4q27 where *IL2* gene maps.
- Functional studies of *-330 IL2* gene promoter polymorphism association with MS.
- Analysis of MS associations with polymorphisms localized in *IL2Ra* gene regulatory regions.
- Comparative analysis of *IL2Ra* polymorphisms associated with MS and T1D and localization of causal SNPs.

2. To study the associations with MS of genes associated with other autoimmune diseases.

- Analysis of the *IL6* gene promoter polymorphism associated previously with RA
- Analysis of *FCRL3* gene promoter polymorphisms associated previously with RA, T1D, SLE.

3. Validation of MS asociation with *IL7Ra* gene polymorphism determined by GWAS.

4. Analysis of the associations with susceptibility to MS of polymorphisms in *OAS1* gene related to interferon response pathway and antiviral response in Humans.

SUBJECTS AND METHODS

IV. SUBJECTS AND METHODS

The samples collection of DNA and RNA has been obtained from a cohort of unrelated patients with clinically defined MS, according to Poser's criteria (Poser, 1983) and reached 800 cases nowdays. It has been collected in four public hospitals of Andalusia (Clinical Hospital San Cecilio and Hospital Virgin de las Nieves of Granada, Hospital Carlos Haya of Malaga and Hospital Virgin Macarena of Seville. The healthy control panel (n=800, year 2008) has been collected in blood banks of four provinces of Andalusia (Granada - Almeria, Malaga, Seville).

As genetic markers for association studies in this thesis we chose single nucleotide gene polymorphisms (SNPs) that presented the big source of variability between persons. We used preferably functional SNPs, most informative because of their possible effect on gene function: located in regulatory gene regions as promoters, 5´ and 3´ untranslated regions; non-synonymous SNPs, causing amino acid differences in the gene product; synonymous SNPs that also may influence the final gene product; intronic SNPs, that can affect splicing and sites of transcription factor union. We also took an account positional data from linkage studies, choosing the genes from previously associated chromosome area.

Two systems of polymorphism genotyping were used for the most SNPs. PCR-RFLP (Polymerase Chain Reaction - Restriction Fragment Length Polymorphism), based on differential restriction pattern of amplified product caused by presence of a SNP and Taqman Pre-designed SNP Genotyping Assay (Applied Biosystems). This assay uses the 5' nuclease activity of the polymerase together with two probes TaqMan to discriminate between two alleles of a SNP.

Several functional studies were performed to investigate the promoter polymorphism. They were realized by means of transient transfection with luciferase gene reporter and measurement of luciferase activity in cell extracts, relative allelic expression analysis using coding SNP as a marker and expression analysis by quantitative real-time PCR. Studies of

quantitative gene expression were performed using reverse transcription of RNA and real time PCR with SYBR Green.

Statistical analysis was performed with standard statistical package (Epi Info v. 6.02; World Health Organization, Geneva, Switzerland). Hardy Weinberg equilibrium was tested using χ^2 goodness-of-fit test. Differences in minor allele frequencies between cases and controls were tested using Pearson´s χ^2 test on 2x2 contingency tables. Comparisons between cases and controls were estimated by logistic regression. Haplotypes and linkage disequilibrium among markers (r^2) were estimated with Haploview (Barrett, 2005). Power analysis was made using QUANTO v. 1.1 software (Gauderman J. and Morrison)

PUBLICATIONS

V. PUBLISHED RESULTS

This thesis is based on the following publications, which will be referred in the text by their roman numerals.

I. Matesanz F, **Fedetz M**, Collado-Romero M, Fernández O, Guerrero M, Delgado C, Alcina A. Allelic expression and interleukin-2 polymorphisms in multiple sclerosis. J Neuroimmunol. 2001; 119: 101-5.

II. **Fedetz M**, Alcina A, Fernández O, Guerrero M, Delgado C, Matesanz F. Analysis of -631 and -475 interleukin-2 promoter single nucleotide polymorphisms in multiple sclerosis. Eur J Immunogenet. 2002; 29: 389-90.

III. **Fedetz M**, Ndagire D, Fernandez O, Leyva L, Guerrero M, Arnal C, Lucas M, Izquierdo G, Delgado C, Alcina A, Matesanz F. Multiple sclerosis association study with the *TENR-IL2-IL21* region in a Spanish population. Tissue antigens. In press

IV. Matesanz F *, **Fedetz M** *, Leyva L, Delgado C, Fernández O, Alcina A. Effects of the multiple sclerosis associated -330 promoter polymorphism in IL2 allelic expression. J Neuroimmunol. 2004; 148: 212-7.

* These authors contributed equally to the work

V. Matesanz F, Caro-Maldonado A, **Fedetz M**, Fernández O, Milne RL, Guerrero M, Delgado C, Alcina A. IL2RA/CD25 polymorphisms contribute to multiple sclerosis susceptibility. J Neurol. 2007; 254: 682-4.

VI. Alcina A, **Fedetz M,** Ndagire D, Fernández O, Leyva L, Guerrero M, Abad-Grau MM, Arnal C, Delgado C, Lucas M, Izquierdo G, Matesanz F. IL2RA/CD25 gene polymorphisms: diverse associations with multiple sclerosis and type 1 diabetes. PLoS ONE. 2009; 4(1):e4137. Epub 2009 Jan 6.

VII. **Fedetz M**, Matesanz F, Pascual M, Martín J, Fernández O, Guerrero M, Alcina A. The -174/-597 promoter polymorphisms in the interleukin-6 gene are not associated with susceptibility to multiple sclerosis. J Neurol Sci. 2001; 190: 69-72.

VIII. Matesanz F, Fernández O, Milne RL, **Fedetz M**, Leyva L, Guerrero M, Delgado C, Lucas M, Izquierdo G, Alcina A. The high producer variant of the Fc-receptor like-3 (FCRL3) gene is involved in protection against multiple sclerosis. JNeuroimmunol. 2008; 195: 146-50.

IX. Alcina A, **Fedetz M**, Ndagire D, Fernández O, Leyva L, Guerrero M, Arnal C, Delgado C, Matesanz F. The T244I variant of the interleukin-7 receptor-alpha gene and multiple sclerosis. Tissue Antigens. 2008; 72:158-61.

X. **Fedetz M**, Matesanz F, Caro-Maldonado A, Fernandez O, Tamayo JA, Guerrero M, Delgado C, López-Guerrero JA, Alcina A. OAS1 gene haplotype confers susceptibility to multiple sclerosis. Tissue Antigens. 2006; 68: 446-9.

ELSEVIER

Journal of Neuroimmunology 119 (2001) 101–105

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroin

Allelic expression and interleukin-2 polymorphisms in multiple sclerosis

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Received 29 November 2000; received in revised form 11 May 2001; accepted 31 May 2001

Abstract

We have investigated the association of two single nucleotide polymorphisms (SNPs) at positions −384 and 114 in the human interleukin-2 (hIL-2) with multiple sclerosis (MS). For two of the −384 genotypes (G/T, T/T), we observed an association with the susceptibility to secondary progressive (SP) course of MS ($P = 0.005$ and $P = 0.013$, respectively). Expression level differences of the IL-2 alleles (between one- and three-fold) were not attributable to the −384 promoter polymorphism. These data indicate for the first time the relevance of the *il-2* gene locus in human MS and its possible involvement in other autoimmune diseases. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Interleukin-2; Multiple sclerosis; Autoimmunity; Polymorphisms; Allelic expression; Detection

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder characterized by multi focal damage of the central nervous system (CNS). This is believed to be mediated by autoantigen-specific CD4+ T-cells and Th-1 cytokines affecting primarily oligodendrocytes (Raine, 1997; Noseworthy, 1999).

Interleukin-2 (IL-2) is an immunoregulatory cytokine, disregulation of the IL-2/IL-2 receptor system could lead to functional or pathological alterations in the immune system including autoimmunity (Horak et al., 1995; Sadlack et al., 1995; Matesanz and Alcina, 1999).

Potent effects of IL-2 in CNS have been described, including activities related to cell growth and survival, transmitter and hormone release and modulation of bioelectric activities (Hanisch and Quirion, 1995). IL-2 may be involved in regulation of sleep and arousal, memory functions, locomotion and the modulation of neuroendocrine axis. Mainly oligodendrocytes and neurons have been shown to respond to the cytokine (Hanisch et al., 1996; Benveniste and Merrill, 1986; Otero and Merrill, 1997). On the other hand, marked neurotoxicity as the neuroendocrine consequence of chronically elevated CNS levels of IL-2 has been observed (Hanisch et al., 1996).

Standard genetic epidemiological studies of multiplex families, twins, half-siblings and adoptees have provided evidence for genetic contribution to disease (Dyment et al., 1997; Oksenberg et al., 1993). Indirect evidences for genetic factors to susceptibility come from the recognition of resistance to developing MS in several ethnic populations (Noseworthy, 1999).

Recent findings (Reboul et al., 2000) suggest that the IL-2Rβ locus contributes to the genetic susceptibility in a subgroup of the MS patients. Experimentally, in mice IL-2 gene deletion produces a reduction in susceptibility to experimental autoimmune encephalomyelitis (EAE) (Petitto et al., 2000). Genetic analysis has found a narrowly defined locus of less than 0.15 cM that has effects in both EAE and diabetes and contains *il-2* gene (Encinas et al., 1999).

Abbreviations: hIL-2, human interleukin-2; MS, multiple sclerosis; RR, relapsing–remitting; RT-PCR, reverse transcriptase polymerase chain reaction; SP, secondary progressive; SNP, single nucleotide polymorphism.

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0165-5728/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0165-5728(01)00354-X

While mouse *il-2* is known to be highly polymorphic in the coding sequence that affects the activity of the different strain-specific allotypes (Matesanz et al., 1992, 1993; Matesanz and Alcina, 1996), only recently several human *il-2* polymorphisms have been identified, one at position −384 of the promoter region (John et al., 1998) and another, a silent change in the first exon, at position 114 from the initiation codon (Matesanz et al., 2000).

In the present study, we analyzed the influence of −384 and 114 polymorphisms on *il-2* allelic expression, their association with susceptibility to MS and the age of disease onset. In addition, based on the 114 polymorphism as a marker, a detection system has been set up for studying *il-2* expression in human T lymphocytes (Matesanz et al., 2000).

2. Methods

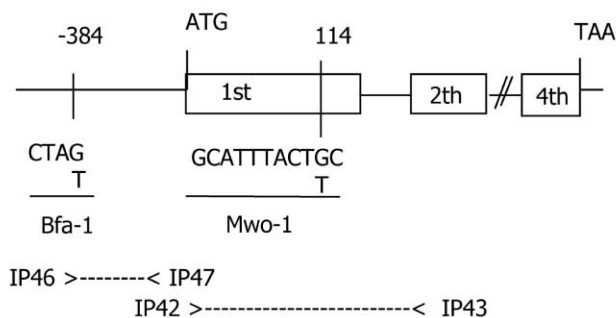
2.1. Study subject

DNA was collected from 173 patients affected by clinically definite MS (relapsing–remitting (RR), $n = 115$), secondary progressive (SP, $n = 58$) and 153 healthy controls with similar genetic background. All the patients and controls were Caucasians from Granada and Malaga, Spain.

2.2. PCR amplification and restriction enzyme digestion

The DNA material was extracted by standard procedures (Ausubel et al., 1990). For the 114 polymorphism determination, about 100 ng of DNA from each DNA sample was PCR amplified with oligonucleotides IP42 (ATGTACAGGATGCAACTCCT) corresponding to the first 20 coding nucleotides of the human *il-2*, and the oligonucleotide IP43 (TGGTGAGTTTGGGATTCTTG) complementary to bases 167–187 in the 5′ part of the human *il-2* second exon (Fig. 1). The PCR conditions were: 1 cycle at 94 °C for 2 min; 35 cycles: 94 °C for 20 s, 52 °C for 40 s, 72 °C for 20 s; and 1 cycle: 72 °C for 10 min. The amplification yielded a band of 262 bp. Each sample was digested for 1 h at 60 °C with 3 U/20 μl of the *Mwo-1* restriction enzyme (New England Biolabs). This digestion produces two fragments of 111 and 151 bp from the 114-G allele product.

The sequence containing −384 polymorphism was amplified with oligonucleotide IP46 (ATTCACATGTT-CAGTGTAG TTCT), which has been modified to create a restriction site for the *Bfa-1* enzyme with the G allele. The forward primer was IP47 (GTGATAGCTCTAATT-CATGC). The PCR amplification yielded a band of 131 bp. After its digestion with *Bfa-1* (New England Biolabs), the fragments of 110 and 21 bp were separated on 12% polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light.



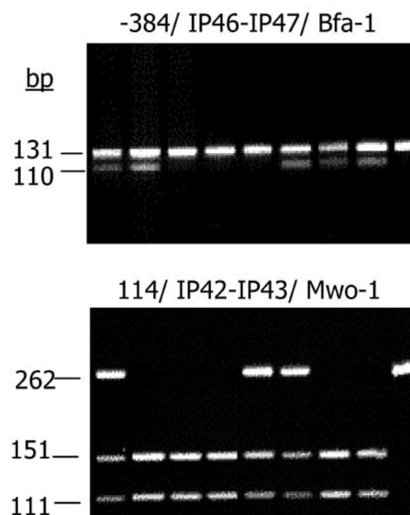


Fig. 1. Strategy for the detection of the IL-2 polymorphisms. (A) Scheme representing the human *il-2* gene and the localization of the oligonucleotides (arrows) utilized in the PCR amplification. The sequences for the restriction enzymes *Mwo-1* and *Bfa-1* and for the different IL-2 alleles originated by the polymorphisms at −384 and 114 are indicated. (B) Detection of *il-2* polymorphism in samples of MS patients. Ethidium bromide stained gels of the PCR-restriction enzyme digestion products of DNA from different MS patients. Top gel is an example of the result obtained detecting the −384 polymorphism. The bottom gel represents the samples from the same patients analyzed for the 114 polymorphism. The size of the resulting bands is indicated at the right.

2.3. Quantification of relative expression of the 114 alleles

Total RNA was isolated from 18 h activated T cells with (10 μg/ml) phytohemagglutinin (Difco) and (100 ng/ml) phorbol 12-myristate 13-acetate (PMA) (Sigma) (Flemming et al., 1993). Human interleukin-2 (hIL-2) cDNA was synthesized by MMLV reverse transcriptase (RT) (GibcoBRL) with oligonucleotide IP39 (TGG-GAAGCACTTAA TTATCA), complementary to the last 20 nucleotides of the hIL-2 coding sequence. The cDNA was PCR amplified with oligonucleotides IP39 and IP38 (TAACCTCAACT CCTGCCACA) corresponding to bases −20/−1 of the gene under the following conditions: 1

cycle at 94 °C for 2 min; 30 cycles at 94 °C for 20 s, 52 °C for 40 s and 72 °C for 25 s; 1 cycle at 72 °C for 10 min. A nested PCR was performed with oligonucleotides IP42 and IP41 (GTTTCAGATCCCTTTAGTTC) sequence bridge between the last 10 bases of the 3rd exon and the first 10 bases of the 4th exon under the following conditions: 1 cycle at 94 °C for 2 min; 35 cycles at 94 °C for 20 s, 52 °C for 40 s and 72 °C for 20 s; 1 cycle at 72 °C for 10 min. It yielded a band of 172 bp. After the *Mwo-1* digestion, the amplified bands from the allele 114-G produce two fragments of 111 and 61 bp. The fragments were separated on 12% polyacrylamide gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light. The fluorescence intensity of each band was quantified by light densitometry digitalization of the image with the NIH image software.

2.4. Statistical analysis

Comparisons of genotype frequencies between healthy controls and total MS patients, RR–MS and SP–MS subjects, ere calculated by the χ^2 test for independent variables using the Stalcalc program (EpiInfo, version 6.0; Stone Mountain, GA). Mean age at disease onset was compared by the *t*-test.

3. Results and discussion

3.1. IL-2 polymorphisms

A marked reduction in susceptibility to EAE in *il-2*-knockout mice and trends to latency in developing signs of EAE as well as its less clinical severity in *il-2* heterozygous mice (Petitto et al., 2000) present important arguments supporting the candidacy of *il-2* in MS.

We analyzed two recently described human *il-2* polymorphisms, one located in the promoter region, at nucleotide −384 (John et al., 1998) 5′ of the start codon (ATG), and the another in the first exon, at position 114 downstream of the ATG (Matesanz et al., 2000). Because this last polymorphism is silent, it does not change the encoded amino acid. Detection system based on PCR and restriction enzymes digestion was set up for each polymorphism as indicated in Fig. 1.

3.2. Distribution of IL-2 −384 or 114 polymorphisms between MS patients and healthy controls

We examined the genomic DNA of 153 healthy controls and 173 multiple sclerosis (MS) patients to determine the frequency of the −384 and 114 polymorphisms of the IL-2 gene as reflected in Fig. 1. Linkage disequilibrium was observed between the −384 and 114 polymorphisms since we found six different genotype combinations of nine theoretically possible. All those homozygous for allele G at position −384 were homozygous for allele G at 114 and we did not obtained any combination of alleles with G at position −384 and T at 114 (results not shown). Therefore, those heterozygous at position 114 must be linked to the genotypes (−384 T/114 T:−384 T/114 G) or (−384 T/114 T:−384 G/114 T).

We observed an association in the distribution of the −384 IL-2 genotypes (G/T and T/T) with SP course of MS ($P = 0.005$ and 0.013) as indicated in Table 1. The increased frequency of these genotypes in this group of patients may imply higher susceptibility to the develop-

Table 1
Distribution of −384 *il-2* promoter genotypes in MS patients and healthy controls[a]

	MS course[b]		Controls
	RR[c]	SP[c]	
(−384)			
(G/G)	12 (10.4)	4 (6.9)	14 (9.1)
(G/T)	46 (40.0)	32 (55.2)**	50 (32.7)
(T/T)	57 (49.6)	22 (37.9)*	89 (58.2)
Total	115	58	153

[a] Genotype distribution analysed by χ^2 test with Yates corrections: (**) $P = 0.005$; (*) $P = 0.013$.
[b] Number (%) of individuals are presented.
[c] RR: relapsing–remitting; SP: secondary progressive.

Table 2
Distribution of 114 *il-2* genotypes in MS patients and healthy controls[a]

	MS course[b]		Controls
	RR[c]	SP[c]	
(114)			
(G/G)	59 (51.3)	29 (50.0)	71 (46.4)
(G/T)	41 (35.7)	25 (43.1)	63 (41.2)
(T/T)	15 (13.0)	4 (6.9)	19 (12.4)
Total	115	58	153

[a] Genotype distribution analysed by χ^2 test.
[b] Number (%) of individuals are presented.
[c] RR: relapsing–remitting; SP: secondary progressive.

Table 3
Age of multiple sclerosis onset in relation to genotypes[a]

	(−384)		
	GG	TG	TT
(114)			
GG	28.2 ± 12.6 (15)	27.3 ± 10.0 (32)	24.5 ± 6.9 (25)
TG	ND[b]	29.4 ± 10.9 (29)	29.0 ± 9.5 (31)
TT	ND	ND	27.7 ± 9.3 (15)

[a] Mean age ± standard deviation (number of MS patients) are indicated.
[b] ND: this genotype combination was not detected.

ment of multiple sclerosis. The distribution of −384 IL-2 genotypes in RR patients and the 114 IL-2 genotypes in both SP and RR groups were not significantly different from the control group as reflected in Tables 1 and 2.

In the control group, the allele frequencies showed little or no difference from the previously reported distribution among healthy UK Caucasians (Reynard et al., 2000). Our data are not consistent with those obtained using separate polymorphic microsatellite markers to analyze association *il-2* with susceptibility to MS (McDonell et al., 2000). They found no significant differences in allele frequencies between MS group and controls.

3.3. *il-2 polymorphisms and onset of disease*

The key role of the IL-2 in the maintenance of the self-tolerance (Horak et al., 1995; Sadlack et al., 1995; Matesanz and Alcina, 1999), its activities in the CNS and variations of the expression levels could influence the onset of an inflammatory and autoimmune disease as the MS. When frequencies of IL-2 genotypes were calculated and correlated to the age of disease onset, no differences were found (Table 3).

3.4. *Relative expression level of the il-2 alleles in individual samples is not controlled by the −384 polymorphism*

Since variation in *il-2* expression could contribute to susceptibility to MS, we determined the influence of the −384 polymorphism on the *il-2* transcription level in six MS patients and five healthy blood donors. We took advantage of the 114 polymorphism in heterozygous individuals to quantify the relative amount of allele 1 (114-T) versus allele 2 (114-G) and to study their linkage with the −384 polymorphism at the promoter region. As seen from Table 4, there were some differences in the relative expression of the *il-2* alleles (paternal and maternal chromosome) of each sample both in MS patients and in the controls. However, these different proportions of allelic expression were not specifically attributable to the presence of the G/T or T/T nucleotides in the −384 promoter position. It is possible that another regulatory unknown polymorphism exists which may affect the transcription of the *il-2* gene. Nevertheless, this method of quantification of the relative expression of the *il-2* alleles based on the 114 polymorphism (Matesanz et al., 2000) could be useful for studying the effect of polymorphisms on the *il-2* expression in cells without the need of any genetic manipulation.

Table 4
Promoter polymorphism and relative allelic expression (RAE)[a] of *il-2*

	Genotype		RAE
	(−384)	(114)	114-T/114-G
Controls	G/T	G/T	1.34
	T/T	G/T	1.04
	G/T	G/T	1.52
	T/T	G/T	1.52
MS patients	G/T	G/T	1.11
	G/T	G/T	0.80
	T/T	G/T	1.0
	T/T	G/T	1.85
	T/T	G/T	0.83
	T/T	G/T	1.14

[a] RAE was obtained by gel lane densitometry quantification of the indicated 114 polymorphism-heterozygous individuals (see Methods).

4. Concluding remarks

Many studies support an important genetic component of susceptibility to MS (Dyment et al., 1997). Extensive efforts have been made to identify the major genes influencing disease susceptibility (Kuokkanen et al., 1996) as well as the cytotoxic products targeting to oligodendrocytes (Raine, 1997). Major histocompatibility complex (MHC) genes are important, although estimations indicate that MHC genes contribute no more than 10% of the genetic susceptibility (Risch, 1987).

Our results showed that two of the −384 IL-2 genotypes (G/T and T/T) were associated with susceptibility to MS (group with SP course of disease). However, none of the tested *il-2* polymorphisms has a statistically significant effect on determining the age of disease onset.

It is worth noting that the −384 promoter polymorphism was not related with the amount of allelic transcription and, therefore, it might be possible that observed differences in the relative expression of the *il-2* alleles are dependent on another polymorphism till now undiscovered.

Mouse *il-2* gene located in a genome fragment of chromosome 3 has been associated with the susceptibility to EAE and to diabetes type-1 (Encinas et al., 1999; Lyons et al., 2000). These results, together with our data, suggest that an equivalent locus in human may contain genetic elements determining susceptibility to MS. However, clarification of these elements in the human will require a fine mapping of all the single nucleotide polymorphisms (SNPs) in *il-2* and application to large patient collections.

Acknowledgements

This work was supported by grant number 1FD97/0596 from Comisión Interministerial de Ciencia y Tecnología-Comisión Europea and PM 99/0101 from Ministerio de Ciencia y Tecnología to A. Alcina. M. Fedetz is a holder of Agencia Española de Cooperación Internacional (A.E.C.I.) fellowship.

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Analysis of −631 and −475 interleukin-2 promoter single nucleotide polymorphisms in multiple sclerosis

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Summary

We have analysed the interleukin-2 (IL-2) promoter single nucleotide polymorphisms −475 A/T and −631 G/A, relative to the initiation codon, in patients with multiple sclerosis (MS) and in healthy controls. Both groups showed a very low frequency of T at −475 and A at −631. Our results suggest that these polymorphisms do not contribute to MS susceptibility.

Introduction

Recent data indicated the possible relevance of interleukin-2 (IL-2) and interleukin-2 receptor beta (IL-2Rβ) gene loci in multiple sclerosis (MS) (Reboul *et al*., 2000; Matesanz *et al*., 2001). In a mouse model of experimental autoimmune encephalomyelitis (EAE), *il-2* gene deletion produced a reduction in susceptibility to EAE (Petitto *et al*., 2000). In addition, genetic analysis in nonobese diabetic (NOD) mice has defined a locus of less than 0.15 cM that is important in both EAE and diabetes susceptibility and contains the *il-2* gene (Encinas *et al*., 1999).

Genetic markers in the form of single nucleotide polymorphisms (SNPs) (more than 1 million) are now available in the 'Human Genome Resources' SNP database at the National Center for Biotechnology Information (NCBI) for use in genotype–phenotype studies. In the 800-bp 5′ upstream region of the human *il-2* gene, three SNPs have been described at positions −384 (John *et al*., 1998), and −475 and −631 obtained from Seattle SNPs (University of Washington, Fred Hutchison Cancer Research Center; web address http://pga.mbt.washington.edu). We have recently shown that the −384 polymorphism is associated with the secondary progressive course of MS (Matesanz *et al*., 2001). In the present work we have studied the associations of the −475 A/T and −631 G/A SNPs with MS in a Caucasian group from southern Spain.

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Received 11 November 2001; accepted 8 April 2002

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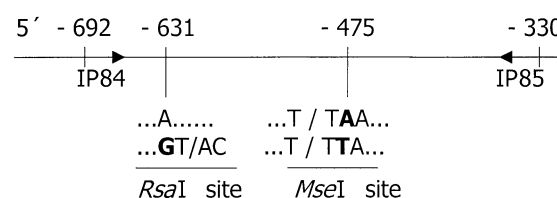
Materials and methods

Two PCR primers were designed to amplify the region from −692 to −360: forward primer IP84 (5′-ATAGA CATTAAGAGACTTAAAC-3′) and reverse primer IP85 (5′-GTAACTCAGAAAATTTTCTTTG-3′) (Fig. 1a). PCR-RFLP was carried out as follows: 50 ng of genomic DNA was amplified in a 30-μl final volume containing 75 mM Tris HCl (pH 9), 2 mM $MgCl_2$, 150 mM KCl, 2 mM $(NH_4)SO_4$, 200 μM dNTP (Amersham, Buckinghamshire, UK), 6 pmol of each primer and 1.5 U of Taq (Biotools,

(a) *il-2* promoter



(b)

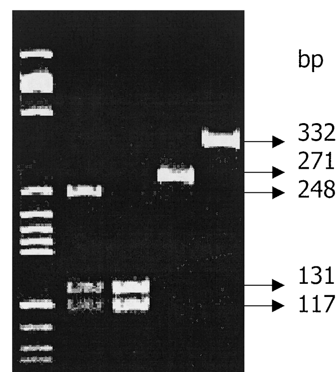


Figure 1. Strategy for the detection of *il-2* promoter SNPs. (a) Scheme representing the *il-2* promoter and the localization of the oligonucleotides (arrows) used in the PCR amplification, the restriction sequences for *Rsa*I and *Mse*I and the different alleles produced by the −475 and −631 polymorphisms. (b) PCR-RFLP analysis of the −631 and −475 polymorphisms in ethidium bromide stained polyacrylamide gel. 'U' represents uncut PCR product and 'M' molecular size markers.

Madrid, SP). PCR conditions were: 1 cycle at 94 °C for 2 min; 35 cycles each of 94 °C for 20 s, 50 °C for 40 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min.

The PCR product yielding a band of 332 bp was divided into two tubes and each aliquot was digested with *Mse*I at 65 °C or *Rsa*I at 37 °C (both from Fermentas, Vilnius, Lithuania). Digestion with *Rsa*I resulted in two fragments of 61 and 271 bp for the –631/G allele. Digestion with *Mse*I yielded different patterns for the −475/T allele (seven fragments of 248, 30, 21,11, 9, 8 and 5 bp) and the −475/A allele (eight fragments of 131, 117, 30, 21, 11, 9, 8 and 5 bp). The digestion products were separated on 10% polyacrylamide gels and visualized using ethidium bromide staining (Fig. 1b).

Results and Discussion

DNA samples from 98 healthy Caucasian controls from southern Spain and 127 MS patients of the same ethnic origin were analysed for –631 and −475 IL-2 promoter polymorphisms. All the samples studied for the –631 SNP had the G/G genotype. For the −475 site, only one heterozygous individual in the control group was found, the rest of the samples carrying the A/A genotype.

The frequencies of the –631 A allele (0.01) and the −475 T allele (0.03) given in the SNP database (Seattle SNPs) are higher than those found in the present work. In addition, we did not found any alterations of the SNP frequencies in the MS samples.

Both polymorphisms studied in this work are localized to the –600 to –300-bp distal region and are therefore separated from the extensively studied proximal enhancer of the IL-2 promoter. This sequence is very conserved with respect to the mouse promoter. Dimethyl sulphate (DMS) and DNase I genomic footprinting studies within this distal region indicate that it may serve as a stable nucleation site for tissue-specific factors and as a potential initiator site for activation-dependent chromatin remodelling (Ward *et al*., 1998). For this reason, SNPs in this region are potential candidates for future functional promoter studies and for association studies in immune diseases.

In conclusion, as we have found a low frequency of both SNPs and no differences between MS patient and control groups, it appears that these polymorphisms have no significant influence in MS susceptibility.

Acknowledgements

This work was supported by grants from the Comisión Interministerial de Ciencia y Tecnología-Comisión Europea (1FD97/0596) and the Ministerio de Ciencia y Tecnología (PM 99/0101) Fondo de Investigación Sanitaria (01/3149). MF is a holder of an Agencia Española de Cooperación Internacional (AECI) fellowship.

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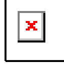

Multiple sclerosis association study with the TENR-IL2-IL21 region in a Spanish population

Journal:	*Tissue Antigens*
Manuscript ID:	TAN-Sep-2008-1090-BC.R2
Manuscript Type:	Brief Communication
Date Submitted by the Author:	16-Mar-2009

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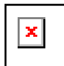
Keywords:	multiple sclerosis, 4q27, IL2, IL21, case-control association study, SNP



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Brief communication

Multiple sclerosis association study with the *TENR-IL2-IL21* region in a Spanish population

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Abstract

Polymorphisms from the *TENR-IL2-IL21* block in the 4q27 chromosome were recently associated with type I diabetes, celiac disease, rheumatoid arthritis and psoriasis. We undertook this study to investigate the potential role of polymorphisms rs3136534, rs6822844 and rs2069762 (-330 T/G IL2) in multiple sclerosis (MS)(805 patients of Spanish Caucasian origin and 952 health controls). We did not find evidence for association with any SNPs tested. Allele and genotype frequencies of the SNPs, which were studied, were similar in DRB1*15-positive or negative patients. After stratification of MS patients by clinical course, a weak association was observed with rs2069762 G allele and haplotype bearing this allele with secondary progressive MS, although these cases represent 22% of the MS cases. Our results did not show major influence of *TENR-IL2-IL21* locus on susceptibility or disease progression in MS. However, we could not exclude completely the effect in MS for this region. Additional studies, employing much larger sample sizes and analysis of additional polymorphisms in the gene and its flanking region will be required to ascertain their contributions to MS susceptibility.

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Key words: case-control association study; 4q27; multiple sclerosis; IL2; IL21; SNP.

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As a result of genome-wide association studies (GWAS), several polymorphisms in the chromosome 4q27 region have been recently associated with susceptibility to T1D and Graves´ disease (1), celiac disease and RA (2), psoriasis (3). The Wellcome Trust Case Control Consortium study reports that rs17388568 and rs3136534 from 4q27 *TENR-IL2-IL21* region are associated with T1D and Graves´ disease (1). van Heel and co-workers (2) indicate that rs6822844 is significantly associated with celiac disease in three ethnic cohorts. This SNP also mapped to the *TENR-IL2-IL21* high degree linkage disequilibrium (LD) block. The functional promoter polymorphism rs2069762 (-330 T/G IL2), which affects the gene expression, has been found associated with the secondary progressive course (SP) of multiple sclerosis (MS) in Caucasians by our group (4). The aim of this work was to assess the possible association of the rs3136534, rs6822844 and rs2069762 polymorphisms from *TENR-IL2-IL21* region with MS.

The pathogenesis of MS remains to be discovered although it is widely regarded as an autoimmune disease directed against myelin proteins and/or other oligodendrocyte or neuronal epitopes (5). The triggering antigen is unknown, but epidemiological evidence indicates that both genetic and environmental risk factors are involved, similar to other autoimmune diseases (6). The common autoimmune disorders collectively affect at least 5% of the population and it is considered that they share genetic risk factors. First of all, a strong association with the MHC chromosomal region, though it is supposed that only combinations of MHC class II alleles with other variant alleles that regulate the threshold of T-cell activation are critical in susceptibility to autoimmune disease (7). Several epidemiological studies have demonstrated the concurrence of MS and type I diabetes (8), MS and inflammatory bowel disease (9). The risk of MS is significantly increased in families with systemic lupus erythematosus than in the general population (10). However, other investigations on the rate of autoimmune disease in MS patients and their families did not identify an increased risk (11). Therefore, the results of studies on comorbid autoimmunity in multiple sclerosis are controversial (12).

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TENR-IL2-IL21 region includes three genes. *TENR* gene has testis-specific expression and unlikely could be implicated in MS etiology, while the interleukin-2 (IL-2) and interleukin-21(IL-21) genes are two strong positional and functional candidates both involved in immune function and regulation. The main non- redundant role of IL-2 *in vivo* is the maintenance of peripheral T cell tolerance; it plays a critical role in regulatory T cell (Treg) homeostasis, promoting the thymic development, peripheral homeostasis and suppressive function of these cells implicated in autoimmune mechanisms (12). IL-21 is known now to modulate both innate and adaptive immune responses (13). Recent data implicate IL-21/IL-21R system in autoimmunity in the animal model through functional modulation of Tregs (14, 15). Functional antagonism of IL-21 and Tregs is described in humans (16). *IL21* polymorphism is associated recently with genetic susceptibility to type 1 diabetes (17) and systemic lupus erythematosus (18). So far, the polymorphisms in these two genes can affect the susceptibility to MS.

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Our study sample consisted of 805 unrelated cases (68 % female and 32% male) with clinically defined MS according to Poser's criteria (19), and 952 regional controls (61 % female and 39% male), all of them Caucasians. The mean age in the sample collection of the cases was 36 years and mean age of controls was 38 years.

Patients´ blood samples were obtained from four public hospitals of Granada, Málaga and Seville. Patients were classified as either relapsing-remitting (RR) (78%) or SP (22%) MS cases (20), both groups had the same female/male relation (68% female, 32% male) and similar mean age at disease onset (RR-MS - 28.2+10.1 years, SP-MS - 30.0+12.0 years).

Controls were blood donors with no history of inflammatory disease attending the Centres for Blood Transfusion of Granada, Málaga and Seville. Studies were performed after obtaining written informed consent from all participants under protocols approved by the Institutional Review Boards of the Hospitals and Centres for Blood Transfusion.

The genotyping of the samples was performed by Taqman Pre-designed SNP Genotyping Assays (C__15859930_10, C__28983601_10, C__31136447_10) under the conditions

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recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA) and analyzed in 7900 HT Fast Real-Time PCR System (Applied Biosystems). Negative and positive controls were included in all analysis as a quality control measure. Samples were genotyped for the rs3135388 to identify the MS-associated DRB1*15 allele, as the presence of this allele and the rs3135388 A allele are highly correlated (21).

Statistical analysis was performed with standard statistical package (Epi Info v. 6.02; World Health Organization, Geneva, Switzerland). Hardy Weinberg equilibrium was tested using χ^2 goodness-of-fit test. Differences in minor allele frequencies between cases and controls were tested using Pearson´s χ^2 test on 2x2 contingency tables. Strength of association was given as odds ratio (OR) with a 95% confidence interval (C.I.). ORs and C.I. for comparisons between cases and controls were estimated by logistic regression. Haplotypes and linkage disequilibrium among the three markers (r^2) were estimated with Haploview (22). Power analysis was made using QUANTO v. 1.1 software (Gauderman J. and Morrison J.)

We had 97% power to reproduce OR 0.6-0.7 found in T1D and RA for rs6822844 (23), 80% power to reproduce OR 1.54 found in SP-MS in our previous study for rs2069762 (4) and 38% power to reproduce OR 1.1 found in T1D and RA for rs3136534 (1, 23).

The genotype and allele frequencies of studied polymorphisms in both MS patients and controls are summarised in Table 1. There were no significant Hardy-Weinberg equilibrium deviations in control and case samples for the three SNPs (P>0.05). All minor allele frequencies (MAF) observed in healthy controls were similar to those reported in UK, Dutch and Irish populations (2, 23, 3). In contrast to cited studies the allelic distribution was not significantly different between MS and control subjects for rs3136534 and rs6822844 in our study. The same negative result was obtained for rs2069762 in unstratified MS group.

Epidemiological evidence suggests that genetic factors may affect phenotypic expression of the disease. The genotype and allele frequencies were assessed after stratifying the case sample by RR-MS and SP-MS clinical courses. For rs3136534 and rs6822844 we did not reveal

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significant associations with disease course. We have found a weak association for *IL2* -330 T/G SNP in SP-MS group (uncorected allelic *P* = 0.03, OR 1.328 , 95% CI 1.03-1.72) but significance was lost after Bonferroni's correction (data not shown).

Overall haplotype analysis in MS cases did not show significant associations. Nevertheless we observed that patients with haplotype bearing rs2069762 G allele were at increased risk for a SP-MS clinical course (uncorrected *P* = 0.0176, after 10000 permutations performed *P*= 0.045) (Table 2), in contrast, no difference was viewed in the RR-MS (data not shown).

We also analyzed data on all studied polymorphisms after stratifying the cohort on the basis of multiple sclerosis associated HLA DRB1*1501 allele status. Evidence for association were not observed in either DRB1*15-positive or DRB1*15-negative group of patients.

Minor allele at rs6822844 is found as perfect proxy for T1D, CD and RA associated haplotypes with decreased frequencies in patients (23). However, this result was not replicated in the MS cases in our study, although we had 97% power to reproduce OR values 0.6-0.7 found in T1D and RA. Our findings do not support a link between allelic variation in rs6822844 and MS.

Genes that affect clinical outcome are thought to be more effective therapeutic targets than those which determine susceptibility (24). Previous study by our group showed an association between rs2069762 (*IL2* –330 T/G, named in that work as -384 counting from the translation start site) and SP-MS (4). In the present work we also obtained association of *IL2* -330 T/G SNP with SP clinical course of MS, but it did not support Bonferroni's correction for multiple comparisons. Generally the SP- MS group seems to be a more clinically homogeneous sample, as transition from RR to SP MS course occurs in more than 50 % of RR patients but it can take different periods of time and it is unpredictable (20, 25). Thus the power to detect genetic effect in SP-MS is expected to increase.

The functional -330 *IL2* gene promoter polymorphism affects the gene expression and the protein synthesis. The lymphocytes from healthy individuals carrying G/G genotype produce

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three times more IL-2 than their T/T and G/T counterparts (26) though gene expression analysis shows more complex pattern (27). Due to multiple functions of IL-2 the local availability of different IL-2 concentration in appropriate moments might lead to very different outcomes, from tolerance to autoimmunity. For naive T cells, enhanced IL-2 signaling can convert what is otherwise a tolerogenic response into one that includes the destruction of self-tissues (28). The novel polymorphisms in minimal *IL2* promoter in mice that affect the transcriptional activity (29) are suggested as candidates for a shared autoimmune disease susceptibility locus in autoimmune ovarian dysgenesis, insulin-dependent diabetes mellitus and experimental allergic encephalomyelitis. In vivo IL-2 not only expand Tregs but also strongly raise their inhibitory activity (30) and inhibits the development of inflammatory Th17 cells (31), both cell types strongly implicated in pathogenesis of MS (32).

Our results did not show major influence of *TENR-IL2-IL21* locus on susceptibility or disease progression in MS. However, we could not exclude completely the effect in MS for this region. Additional studies, employing much larger sample sizes and analysis of additional polymorphisms in the gene and its flanking region will be required to ascertain their contributions to MS susceptibility.

Acknowledgments

We thank the multiple sclerosis patients and volunteer acted as controls for making this study possible. We also thank Stewart A. Grace for his help with English version of the article. This work was supported by Grants PN-SAF2006-02023 and Junta de Andalucía P07-CVI-02551 to A.Alcina and Servicio Andaluz de Salud PI0168/2007 to F. Matesanz. M.Fedetz is a holder of a fellowship from Fundación IMABIS. D.Ndagire is a holder of AECI-Ministerio de Asuntos Exteriores fellowship.

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Table 1. Genotypic and allelic frequencies of rs3136534, rs6822844 and rs2069762 polymorphisms in patients with multiple sclerosis and healthy controls

	Healthy Controls n(%)	MS n(%)	*P** value	OR (95%CI)
rs3136534	n=889	n=781		
AA	401 (45)	383 (49)		1 reference
AC	400 (45)	326 (42)	0.12	0.853 (0.70-1.05)
CC	88 (10)	72 (9)	0.37	0.857 (0.61 -1.21)
A	1202 (68)	1092 (70)		
C	576 (32)	470 (30)	0.15	0.898 (0.78-1.04)
rs2069762	n=899	n=777		
TT	444 (49)	359 (46)		1
TG	378 (42)	345 (44)	0.24	1.129 (0.92-1.38)
GG	77 (9)	73 (10)	0.37	1.173 (0.83-1.66)
T	1266 (70)	1063 (68)		
G	532 (30)	491(32)	0.21	1.099 (0.95-1.27)
rs6822844	n=929	n=768		
CC	717 (77)	598 (78)		1
CA	191 (21)	160 (21)	0.97	1.004 (0.79-1.27)
AA	21 (2)	10 (1)	0.14	0.571 (0.27-1.22)
C	1625 (87)	1356 (88)		
A	233 (13)	180 (12)	0.47	0.926 (0.75-1.14)

* *P* values Pearson´s χ^2 test d.f.1

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Table 2. Haplotype frequencies (rs 3136534, rs2069762 and rs6822844) in SP-MS cases and healthy controls

Haplotype	Frequencies		Chi Square	P Value*
	SP-MS Cases	Controls		
CTC	0.290	0.317	0.837	0.3602
AGC	**0.356**	**0.289**	**5.64**	**0.0176****
ATC	0.260	0.264	0.015	0.9011
ATA	0.092	0.126	2.886	0.0893

Combined frequency of haplotypes presented is 99.8%
* Uncorrected
** P value after 10000 permutations performed is **0.045**

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Journal of Neuroimmunology 148 (2004) 212–217

Journal of Neuroimmunology

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Effects of the multiple sclerosis associated −330 promoter polymorphism in *IL2* allelic expression

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Received 11 August 2003; received in revised form 1 December 2003; accepted 1 December 2003

Abstract

The −330 *IL2* gene promoter polymorphism has been associated with multiple sclerosis (MS) [J. Neuroimmunol. 119 (2001) 101], but the basis underlying this association remains unknown to date. In the present work, we have found that *IL2* promoter-luciferase constructs, transfected in Jurkat cell line, showed twofold higher levels of gene expression in the −330 G allele. However, the transcriptional effect of this polymorphism in lymphocytes showed that the G allele was related to lower expression of *IL2*. This difference increased in the patient group. Divergence between in vivo and in vitro influence of the −330 *IL2* promoter polymorphic site suggests the existence of additional unknown polymorphisms affecting gene regulation. Our data show an increased *IL2* expression among GT and TT genotypes previously associated with susceptibility to MS.
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Keywords: Interleukin-2; Polymorphism; Multiple sclerosis; Expression quantification; Transfection; Real-time PCR

1. Introduction

Multiple sclerosis (MS) is characterized by chronic inflammation and demyelination in the central nervous system (CNS) most likely arising from an autoimmune process (Brosnan and Raine, 1996). The precise molecular and cellular mechanisms that provoke the myelin damage have still to be elucidated although both genetic and environmental contributions to the pathogenesis are inferred from epidemiologic studies (Compston, 2000; Willer and Ebers, 2000). Genes may influence susceptibility to the development of MS and the subsequent course of the disease (Kantarci et al., 2002). To date, a gene within or close to the MHC II class locus is the most likely candidate determinant of susceptibility, although the form of this association is not considered to be as straightforward as previously thought (Herrera and Ebers, 2003). MS is believed to be a complex trait in which interactions between small effects of the several susceptibility genes and environment increase the probability of inflammatory pathways deregulation and demyelination in CNS (Kalman et al., 2002).

A number of genetic studies looked for associations between MS and polymorphic alleles of candidate genes which were selected mainly on the basis of their involvement in the autoimmune pathogenesis and include immunorelevant molecules such as cytokines, cytokine receptors, immunoglobulins, T cell receptor subunits and myelin antigens (Oksenberg et al., 1996; Epplen et al., 1997; Weinshenker et al., 1997; Vandenbroeck et al., 1997; Roth et al., 1995). Importance of IL-2/IL-2R system for the T cell homeostasis at the levels of repertoire selection, the generation of suppressive regulatory T cells, T cell homing and clonal contraction via activation induced cell death (reviewed in Schimpl et al., 2002) indicates its relevance in the development of autoimmune disease.

Abbreviations: MS, multiple sclerosis; PBL, peripheral blood lymphocytes; RAE, relative allelic expression; RT-PCR, reverse transcriptase polymerase chain reaction; S.E.M., standard error of mean; SNP, single nucleotide polymorphism.

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doi:10.1016/j.jneuroim.2003.12.001

Studies in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, also give evidence for the implication of *IL2* locus in the susceptibility to this disease. The levels of IL-2 and *IL2* mRNA were found to be elevated in CNS during the induction and acute phase of EAE, and lowered during recovery (Kennedy et al., 1992). On the other hand, a genetic linkage analysis using a cross between susceptible and resistant mice revealed a locus affecting EAE severity which contains the *IL2* gene (Encinas et al., 1999). Vulnerability to EAE is markedly reduced in C57BL/6 mice lacking *IL2*, suggesting that this cytokine may play a critical role in autoimmune processes of the central nervous system (Petitto et al., 2000).

In a previous study we reported that the genotypes (G/T and T/T) at − 330 site (− 384 from the ATG) in the human *IL2* promoter are associated with the susceptibility to MS (Matesanz et al., 2001). This single nucleotide polymorphism is located in the upstream region of the *IL2* gene promoter–enhancer domain (John et al., 1998). Recently, Hoffmann et al. (2001) reported differences in protein production between the *IL2* promoter polymorphic variants in healthy controls. In the present study we evaluate the influence of the − 330 promoter polymorphism in the expression of the corresponding *IL2* alleles.

2. Materials and methods

2.1. Genotyping of healthy control subjects and patients with MS

Blood samples were obtained with the informed consent of unrelated Spanish Caucasoid patients with clinically definite multiple sclerosis who attended the Hospital Carlos Haya (Malaga, Spain) and healthy controls of similar age and genetic background. Patients had relapsing remitting or secondary progressive form of the disease (Lublin and Reingold, 1996). DNA was extracted by standard procedure (Ausubel et al., 1990). Genotyping of − 330 and + 114 SNPs was carried out by PCR-RFLP as previously described (Matesanz et al., 2001).

2.2. Cell cultures

Human Jurkat leukemia cell line was maintained in RPMI 1640 medium, supplemented with 10% fetal calf serum (Gibco), 2.0 mM glutamine, 50 μM 2-mercaptoethanol, and 50 mg/l gentamycin (Sigma)(complete medium).

2.3. Plasmid construction

Two variants of human *IL2* promoter–enhancer fragment from − 500 to + 1 containing G or T at − 330 site were prepared by PCR from − 330 *IL2* heterozygous individual genomic DNA as template and CGGGATCCCAGGAAACCAATAAC TTCC (forward) and GAAGATCTTGTGGCAGGAGTTGAGGTTA (reverse) oligonucleotides, creating a *Bam*HI and a *Bgl*II restriction sites. The amplification products were cloned into the pGEM-T vector (Promega). The insert sequences of the recombinants were confirmed by sequencing. Recombinants containing either the G or the T allele were prepared and the inserts were excised using restriction endonucleases *Bam*HI and *Bgl*II. Then the fragments were subcloned into luciferase vector pXP2 (Nordeen, 1988). The final plasmids pXP2-330IL-2/G-Luc and pXP2-330IL-2/T-Luc were sequenced to verify that no unwanted mutations were introduced in the process of cloning. Large-scale plasmid DNA was prepared using plasmid purification kit (Qiagen), following the recommended protocols. Experiments were performed with three independent plasmid preparations.

2.4. Transient transfection

Jurkat cells were transfected by electroporation. Briefly, plasmid DNA (40 μg) was mixed with exponentially growing Jurkat cells (20×10^6) in complete medium, and the cells were electroporated in an electrocell manipulator 600 (BTX, San Diego, CA) at 250 V and a capacitance of 975 μF. The transfected cells were cultured for 24 h, purified in a Histopaque-1077 gradient (Sigma), washed twice in complete medium and activated with phorbol-myristate acetate (PMA, 50 ng/ml) and ionomycin (1 μg/ml), both from Sigma. After various period of time (4, 14 and 20 h), activated cells from an independent flask and control transfected cells without activation were harvested ($300 \times g$, 5 min, 4 °C) and washed twice in phosphate-buffered saline (4 °C).

2.5. Luciferase activity analysis

Luciferase activity was evaluated using reagents from the Luciferase Assay System (Promega). Washed pellets of transfected cells were treated with 80 μl of 1 × Reporter Lysis Buffer for 5 min on ice. Cells were repipetted five times to ensure lysis of all the cells, lysates were spun down for 1 min and stored at − 80 °C.

For analysis, lysates were thawed on ice and the protein concentration was measured (Bradford method). Then 40 μg of protein (in a volume of less than 20 μl) from all supernatants were analyzed with Luciferase Assay Reagent (100 μl) and measured as quadriplicate for 30 s in luminometer F12 (Berthold Detection Systems). Control samples (transfected cells that had not been activated and activated cells that had been transfected with "empty" plasmid pXP2-Luc) contained equal minimal levels of luciferase activity (< 500 RLU). The level of luciferase activity is represented as RLU/40 μg of protein.

2.6. Relative allelic expression

Total RNA from + 114 G/T heterozygous individual PBL, stimulated for 4 h with PMA (50 ng/ml) and ion-

omycin (1 μg/ml), were extracted with Trizol and checked for accidental degradation. Total RNA (200 ng) were used for cDNA synthesis with MMLV reverse transcriptase (Invitrogen), RNAguard (Amersham Biosciencies) and oligonucleotide IP41 (GTTTCAGATCCCTTTAGTTC), a bridge between the 3rd and 4th exons.

Five microliters of cDNA was used to amplify a 372-bp fragment of the *IL2* (+1 to +372). PCR was performed in a 30-μl volume (forward primer ATGTACAGGATGCAACTCCT) under the following conditions: 95 °C for 5 min followed by 25 cycles of 20 s at 94 °C, 40 s at 54 °C, and 20 s at 72 °C and a final prolongation step of 10 min at 72 °C. During the last cycle, 1 pmol of ^{32}P oligonucleotide IP 41 labeled with [gamma-^{32}P] ATP plus 0.5 U of fresh Taq polymerase was added to the PCR tubes. Ten microliters of the final PCR product was used for restriction reaction with *Mwo*I (cuts the G allele) and electrophoresed on 10% PAGE. Bands were quantified by phosphoimager (Image Eraser, Amersham Bioscienses).

2.7. Real-time quantitative transcript analysis

Total RNA (200 ng) from 4 h activated PBL were converted into cDNA using MMLV RT enzyme and hexanucleotides. Three microliters was PCR amplified in a final volume of 25 μl with 75 mM Tris–HCl, pH 9, 4 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 1 mM dNTP, 0.1× SYBR Green I (Molecular Probes), 20 pmol of specific oligonucleotides and 0.6 U *Taq* polymerase (Biotools). The oligonucleotides used were loop incorporated primers to overcome the dimers and nonspecific products formation (Ailenberg and Silverman, 2003). The sequences were as follows: *IL2* forward (CAAGAAGCAGTTCTGTGGCCTTCTTG), reverse (GTAGAACGCACCTACTTCAAGTTCTAC); *UbcH5b*, forward (CAATTCCGAAGAGAATCCACAAGGAATTG), reverse (GTGTTCCAACAGGACCTGCTGAACAC). The real-time PCR consisted (for the *UbcH5b* as well as for the *IL2*) of one cycle 94 °C, 3 min, followed by 45 cycles of 94 °C 20s, 60 °C 20s, 72 °C 20s. The amplification and the detection of the product was performed with the iCycler iQ apparatus (BioRad). Every sample was analyzed in triplicate. No dimer formation was observed in any PCR product by the melting curve analysis. Reverse transcription real-time PCR efficiencies were calculated from the given slopes in the iCycler iQ software obtained from RT-PCR amplification of 6 twofold serial dilutions of RNA (200–6.25 ng). The mean normalized expression values were calculated from the obtained *ct* of the *IL2* and *UbcH5b*, and their respective standard curve slopes with the qgene software (Muller et al., 2002) http://www.wzw.tum.de/gene-quantification/).

2.8. Statistical analysis

Transfection data were analyzed by Student's *t*-test. Results are presented as means ± S.E.M. ($n=4$) for representative experiments. Significant differences in values were similar in all experiments, although actual values for experimental and control samples varied between experiments. Differences between allele ratios in groups and results of *IL2* RNA quantification were compared by Mann–Whitney test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. The −330 IL2 promoter polymorphism affects gene transcription

Our previous results showed that the −330 genotypes (G/T and T/T) were associated with susceptibility to MS. In order to examine the role of the −330 G/T polymorphism in transcriptional regulation of the human *IL2* gene, we performed transient transfection of Jurkat cells with reporter constructs pXP2-330IL-2/G-Luc and pXP2-330IL-2/T-Luc containing either nucleotide G or T at −330. Cells were stimulated with PMA–ionomycin after 24 h of transfection and the luciferase activity was quantified at different times after activation. Fig. 1 shows that reporter constructs containing the fragment of human *IL2* promoter with nucleotide G at −330 have twofold increased ($P<0.0001$) activity upon transfection compared with reporter constructs containing nucleotide T at −330. This difference was sustained at all studied time intervals after stimulation.

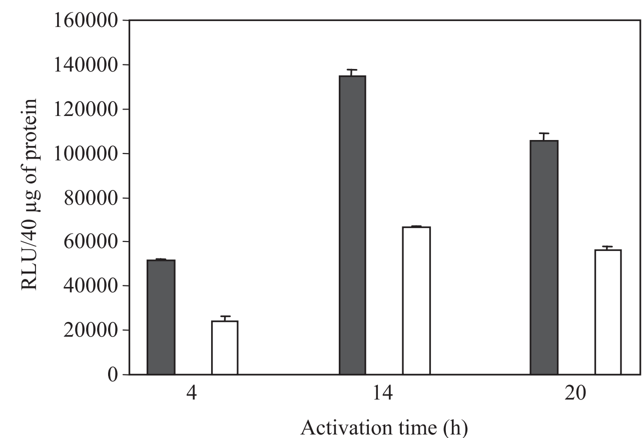

Fig. 1. Transcriptional activity of the −330 *IL2* G and T alleles in Jurkat cells. Cells were transiently transfected with a plasmid pXP2 containing a luciferase reporter gene controlled by the two −330 variants (G/T) of the *IL2* promoter–enhancer region (−501 to +1), and activated with PMA–ionomycin during 4, 14 and 20 h. Promoter activity was measured by conducting the luciferase assay as described in Materials and methods. Luciferase activity is expressed in relative light units/40 μg of protein. Results are represented as means ± S.E.M. ($n=4$) for representative experiments. White bars, the promoter activities of reporter constructs containing nucleotide T at −330; black bars, the promoter activities of reporter constructs containing nucleotide G at −330. RLU = relative light units.

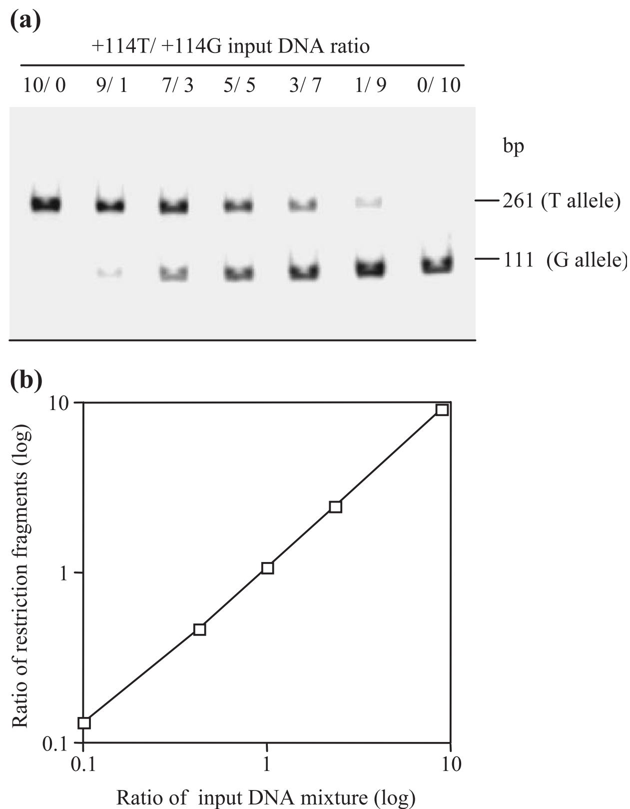

Fig. 2. Validation of relative allelic quantification procedure. (a) DNA mixtures containing different ratios of +114 T and +114 G DNA were used for PCR with final cycle radiolabelling then were digested with *Mwo*I and subjected to gel electrophoresis. (b) Results of gel phosphorimaging presented in graphic form demonstrate relationship between the input DNA template ratio and the ratio of restriction fragments.

3.2. The −330 promoter polymorphism associates with a differential IL2 allele expression in lymphocytes from multiple sclerosis patients

To determine the influence of the −330 G/T *IL2* promoter polymorphism in human lymphocytes, we measured the relative expression of G/T alleles by means of a +114 G/T SNP located in the first exon of the *IL2*. This SNP is silent, it does not change the IL-2 protein sequence and it can be used as a marker to quantify expression of maternal and paternal alleles in heterozygous cells by RT-PCR-RFLP method (Matesanz et al., 2000).

As we described (Matesanz et al., 2001), linkage disequilibrium exists between −330 and +114 *IL2* polymorphisms, there is no −330 G/G genotype in +114 G/T and +114 T/T individuals, therefore, the −330 G/+114 T haplotype does not exist. The +114 G allele is always found on the same haplotype as −330 G allele. In this way when we quantify the expression of the +114 G or T allele we can ascribe each one to a −330 G or T allele.

We performed PCR with a final cycle-labeling methodology (Kaijzel et al., 2001) using a ^{32}P-antisence primer that labeled the PCR product of one DNA chain. This method avoids the quantifications of heteroduplex which cannot be cut by the restriction enzyme. We validated the procedure of quantification by measuring the intensities of the fragments resulting from PCR-RFLP of DNA mixtures with a known distribution of +114 G and +114 T alleles. Fig. 2 shows that the ratios of band intensity in DNA mixtures after PCR-RFLP equals the ratios in the DNA input (R^2 = 0.9999).

Measurements of the +114 G/T allele ratio were performed in total RNA of activated PBL from 32 healthy and 23 MS patients with −330 GT or TT genotypes.In the healthy control group, expression of allele +114 G was significantly lower than expression of allele +114 T (ratio 0.88) for −330 G/T individuals in comparison with −330 T/T individuals (ratio 0.96, P = 0.0021). This difference increased in the MS patient group (ratio 0.69 for −330 G/T group, ratio 0.87 for −330 T/T group, P = 0.0009). Significant differences were observed comparing +114 G/T ratios for the −330 TT or GT genotypes between patients and healthy controls (Table 1).

3.3. The −330 G/G genotype promoter is linked to a lower level of IL2 RNA

We determined the *IL2* expression level in stimulated PBL of 23 healthy individuals bearing different genotypes for the −330 SNP by reverse transcription real-time PCR using SYBR Green I. Relative quantification method was applied to each sample using the *UbcH5B* housekeeping gene as reference (Hamalainen et al., 2001). The mean normalized expression (n = 3) between the *IL2* and *UbcH5B* was calculated according to Eq. (3) of Muller et al. (2002) by the *qgene96* software. The slopes of the standard curve graph performed to determine the PCR efficiency were −3.421 for the *IL2* and −3.693 for the *UbcH5b* housekeeping gene. Out of 23 RNA analyzed, 9 were of T/T, 9 were T/G and 5 were G/G genotype at −330 position. The mean normalized expression values for each genotype is represented in Table 2. The expression of the *IL2* is

Table 1
Relative allelic expression of *IL2*[a]

Genotype −330	+114 G/+114 T ratio
TT	−330 T+114 G/−330 T+114 T[b]
Healthy controls (n = 15)	0.96 ± 0.11[c]
MS patients (n = 14)	0.87 ± 0.08[d]
GT	−330 G+114 G/−330 T+114 T[b]
Healthy controls (n = 17)	0.88 ± 0.12[e]
MS patients (n = 9)	0.69 ± 0.12[f]

Mann–Whitney two-sided test.
[a] Mean ratio ± S.E.M.
[b] Haplotype frequencies: −330 T/114 T (MS = 0.294; Controls = 0.296); −330 G/114 G (MS = 0.313; Controls = 0.317); −330 T/114 G (MS = 0.391; Controls = 0.385).
[c] P(TT healthy controls/GT healthy controls) = 0.0021.
[d] P(TT healthy controls/TT MS patients) = 0.05.
[e] P(GT healthy controls/GT MS patients) = 0.0057.
[f] P(TT MS patients/GT MS patients) = 0.0009.

Table 2
Mean normalized expression (MNE) of *IL2* in activated PBL[a]

Genotype −330	MNE *IL2* ± S.E.M.
GG (n=5)	577 ± 458[b]
GT (n=9)	1142 ± 610[c]
TT (n=9)	936 ± 471[d]

[a] *P* analyzed by Mann–Whitney one-sided test.
[b] *P*(GG vs. GT) = 0.03.
[c] *P*(GT vs. TT) = 0.33.
[d] *P*(TT vs. GG) = 0.03.

significantly lower for samples with the G/G than with the T/T and T/G genotype.

4. Discussion

Our previous work showed that the *IL2* −330 G/T and T/T genotypes were associated with susceptibility to MS. In this study, we examined the influence of the −330 *IL2* promoter polymorphism on the gene expression in healthy and MS individuals. This was carried out by transfection of Jurkat cells with constructions containing two allelic variants upstream a reporter gene, measurement of the relative expression of the −330 G allele against the −330 T, and quantification of the total *IL2* expression in individuals carrying the three different −330 genotypes.

The transfection study in Jurkat cells demonstrated differential promoter activity between the G and the T alleles. The construction carrying G was twice more active than the one with T. Conversely, quantification of allelic expression in lymphocytes showed that the −330 T allele was associated with a higher level of transcription than the −330 G. These data are in concordance with our real-time quantification results, since a higher expression level of *IL2* mRNA was observed in the samples of individuals with −330 T/T and G/T genotypes compared with the ones bearing the −330 G/G genotype. These results are in disagreement with those of Hoffman et al. which described an enhancement in IL-2 production in individuals genotyped as −330 G/G.

The divergence between our results obtained in transfected Jurkat cells and the ones obtained in peripheral lymphocytes suggests that the −330 promoter polymorphism is influenced by factors linked to the −330 polymorphism. The transfection study in Jurkat cells showed the effect on the luciferase gene expression of an *IL2* promoter fragment isolated from other regulatory elements. They could be located at the far upstream promoter or in the gene where several RNA stability regulatory elements are defined (Ragheb et al., 1999). This regulation could be modified by polymorphisms or methylation pattern.

In the relative allelic quantification study of healthy controls, the ratio +114 G/T in −330 T/T genotype was close to 1 (0.96) as theoretically expected. However, in MS patients bearing the same promoter genotype, this value was significantly lower than 1 (0.87). Since we compared the −330 T/+114 G with the −330 T/+114 T haplotype, the difference cannot be attributed to sequence polymorphism at −330. The mechanism involved in this process is unknown but it seems to be associated with the +114 polymorphism.

In the data of +114 G/T relative allelic quantification for −330 G/T genotype, the ratio difference between healthy controls and MS patients is augmented (0.88 vs. 0.69, P=0.0057). This indicated that both haplotypes were differently affected by regulatory factors and this phenomenon ought to be distinct in cells from patients and healthy controls. The existence of a linkage disequilibrium with other unknown polymorphisms can influence an allelic transcription and this may be more evident for MS patients.

The position of this polymorphism is not at any known transcription factor consensus sequence. However, deletion studies of the human *IL2* promoter region, containing this polymorphism have shown that the elimination of the −361/−292 (from the transcriptional start site) sequence reduces the reporter gene activity in Jurkat cells to 40% (Williams et al., 1988). On the other hand, DNase I footprinting studies of the mouse distal −600/−300 bp *IL2* region revealed multiple tissue-specific hypersensitive sites (Ward et al., 1998). The *IL2* promoter sequences up to 600 bp are highly conserved between mouse and human (Novak et al., 1990). It has been postulated that the region outside the established *IL2* minimal enhancer may serve as a stable nucleation site for tissue-specific factors and as a potential initiation site for activation-dependent chromatin remodeling (Ward et al., 1998).

Our results reflect the complex regulatory mechanism of *IL2* gene expression and indicate that the influence of the −330 polymorphism is modulated by other elements. The higher expression of *IL2* −330 GT and TT genotypes, associated with susceptibility to MS, and the altered relative expression between alleles in MS patients supports the importance of *IL2* in the MS. On the other hand, the contribution of different *IL2* expression levels to the MS pathogenesis should be seen in the context of a multifactorial disease, considering interactions with other susceptibility genes.

Acknowledgements

This work was supported by grants PM 99/0101 from the *MCYT* and FIS PI020920 to A. Alcina, FIS 01/3149 to F. Matesanz, and FIS PI0210671 to L. Leyva. M. Fedetz is a holder of a fellowship from Hospital Carlos Haya Foundation.

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J Neurol (2007) 254:682–684
DOI 10.1007/s00415-006-0416-4

LETTER TO THE EDITORS

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IL2RA/CD25 polymorphisms contribute to multiple sclerosis susceptibility

Received: 18 May 2006
Received in revised form: 28 June 2006
Accepted: 12 September 2006
Published online: 10 April 2007

Sirs: Multiple sclerosis (MS) is a neurodegenerative disease of the central nervous system (CNS) whose primary mechanism of injury is by inflammatory/autoimmune demyelination and, to a variable degree, axonal damage. Epidemiological studies, genome screenings and case-control studies suggest that multiple genetic factors influence susceptibility to multiple sclerosis (MS) [1–5].

Genome-wide screens in multiplex families with MS [6], insulin-dependent diabetes mellitus (IDDM) [7] and adult-onset primary open angle glaucoma [8], have shown linkage to locus 10p15. A partial list of candidate genes located in this region includes *NET1, PRKCT, ITIH2, IL2RA, IL15RA, IT1H2, hGATA3*, the mRNA for open reading frame KIAA0019, and the gene for D123 protein. Amongst these, the interleukin 2 receptor alpha chain (*IL2RA/CD25)* gene has recently been associated with type 1 diabetes (T1D) in a study using a tag-SNP approach [9]. Furthermore, a polymorphism in the *IL2* promoter region (–330) not only affects its level of expression but is also associated with MS diseases progression (secondary progressive MS) [10]. The protein encoded by *IL2RA* plays a key role in the activity of the high affinity IL-2R/CD25 and in the differentiation and functioning of the CD4 + CD25+ regulatory T cells [11], involved in immunological tolerance. In fact, in one patient report [12], mutations affecting its expression resulted in autoimmunity, similar to what has been observed in IL-2 and IL-2R-deficient mice [11]. Therefore, considering all of these data, and the provisional evidence of the implication of IL-2 in MS, *IL2RA* is an interesting candidate MS susceptibility gene.

We used PCR-restriction fragment length polymorphism (PCR-RFLP) analysis to genotype 4 SNPs located in regulatory regions of the *IL2AR* gene (Figure 1) in a DNA collection of 346 MS patients, clinically defined according to Poser's criteria [13], and 413 ethnically matched (Caucasian) controls. Within the cases, 247 subjects had the relapsing-remitting form of MS (RR-MS) and 99 had the secondary progressive form (SP-MS). The study was carried out after obtaining written informed consent from all participants under protocols approved by the Institutional Review Board of the Hospital Carlos Haya, Málaga, and the Hospital Clínico and the Regional Centre of Blood Transfusions, both in Granada, Spain.

There was no evidence of deviation from Hardy–Weinberg equilibrium in controls or cases for any of the SNPs genotyped. The distribution of genotypes by affected status is shown in Table 1. Allele and genotype frequencies were compared using Pearsońs chi-square test. The data revealed a significant over-representation of the *IL2RA4* T allele among cases compared with controls, with frequencies of 0.54 and 0.48 respectively *(P* = 0.03), and more so for those with RR-MS (frequency = 0.55, *P* = 0.01). The estimated odds ratio (OR) for carriers of the T/T genotype of *IL2RA4* relative to homozygotes in the C allele was 1.51 (95% CI, 1.02–2.22; P = 0.04) and, again this was higher for RR-MS (OR = 1.69, P = 0.02). The estimated OR per T allele from logistic regression analysis was of 1.23 (95% CI, 1.01–1.49, P = 0.04), which again, was stronger for RR-MS (OR = 1.30, P = 0.02). Both of these associations were observed independently of age and sex, with ORs of 1.27 (P = 0.02) and 1.32 (P = 0.02) observed respectively after adjustment for sex and age,

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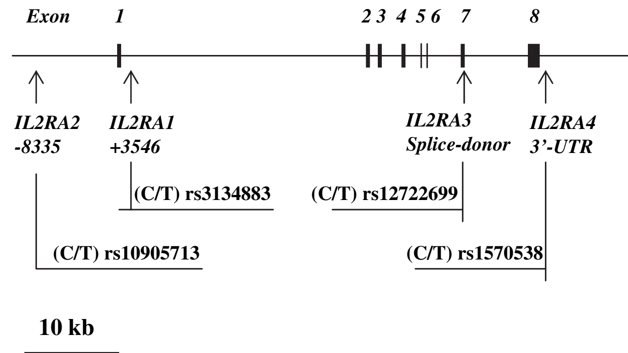

Fig. 1 Scheme of the *IL2RA* locus at semi quantitative scale, representing approximately 60 kb, indicating the position in the gene and the rs # of the four SNPs analysed according to ''NCBI SNP Human Genome''. Methodology details as primers and enzymes used are available from the authors. The (C/T) *IL2RA*1 polymorphism is located at the first intron, at +3546 bp from the transcription initiation site, at the Positive Regulatory Region IV (PRRIV) next to NF-AT-GAS motifs [16]. The (C/T) *IL2RA*2 is at −8335 bp of the transcription initiation start site, at the PRR CD28-responsive enhancer [17]. The (G/A) *ILR2RA*3 is located at splicing site donor consensus sequence of the exon 7. The *IL2RA4* polymorphism is located at the 3′ untranslated region +50704 bp from the initiation start site

Table 1 Distribution of genotypes of *IL2RA* polymorphisms in MS cases and controls*

		Controls *n* (%)	Cases *n* (%)	OR (95% CI)	P
IL2RA1	C/C	243 (58)	207 (60)	1.00	
	C/T	147 (35)	123 (36)	0.98 (0.73–1.33)	0.9
	T/T	28 (7)	16 (5)	0.67 (0.35–1.27)	0.2
			per T allele	0.90 (0.71–1.14)	0.4
IL2RA2	C/C	232 (56)	200 (58)	1.00	
	C/T	154 (37)	125 (36)	0.94 (0.70–1.27)	0.7
	T/T	32 (8)	21 (6)	0.76 (0.43–1.36)	0.4
			per T allele	0.90 (0.72–1.14)	0.4
IL2RA3	TT	418 (100)	346 (100)		
	TC				
	CC				
IL2RA4	C/C	119 (28)	79 (22)	1.00	
	C/T	193 (46)	161 (47)	1.26 (0.88–1.79)	0.2
	T/T	106 (25)	106 (31)	1.51 (1.02–2.23)	0.04
			per T allele	1.23 (1.01–1.49)	0.04

n, number of subjects; percentages shown in parenthesis.

* Statistical analysis. Comparisons of genotype and allele frequencies between healthy controls and MS patients were performed by contingency table 3 × 2 (genotypes) or 2 × 2 (alleles) chi-square (χ^2) test, using the package available from the web of the Institute of Human Genetics of the Technical University of Munich (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl). We performed haplotype frequency estimation testing for differences between cases and controls using the HaploView (MJ Daly and JC Barrett, Whitehead Institute, MA, USA) and FAMHAP12 (T. Becker and M. Knapp, University of Bonn, Germany) softwares. No association was found (data not shown).

the latter in 5-year categories. High linkage disequilibrium was observed between the *IL2RA2* and *IL2RA1* polymorphisms with an $r^2 = 0.8$. A comparison of haplotype frequencies between cases and controls using HaploView program found no evidence of an association.

Because the *IL2RA4* polymorphism is at the 3′- untranslated region (3′-UTR), it could affect the level of the IL-2 receptor alpha expression and consequently the amount of CD4 + CD25+ regulatory T cells (Fig. 1). This may be very relevant for controlling the autoimmune attack against myelin and neurons in MS and therefore influence disease progression or severity. Animal models of MS have shown that CD4 + CD25+ regulatory T cells are directly involved in the natural recovery from, and protection against, experimental autoimmune encephalomyelitis (EAE model) [14].

The apparently weak association of the *IL2RA4* polymorphism with MS may be explained by diseases complexity that makes the potential set of genes involved in the pathology in each patient heterogeneous and by the complex inheritance involving interactions between combinations of loci that may influence the immune response. On the basis of neurobiological and immunological markers, Luchinetti et al, [15] identified four fundamentally different patterns of demyelination in MS. Two of these patterns (I and II) were found to resemble T-cell-

mediated or T cell plus antibody-mediated autoimmune encephalomyelitis, while patterns III and IV were characteristic of primary oligodendrocyte dystrophy rather than autoimmunity. On the other hand, we cannot discard the possibility that the association observed at the *IL2RA4* polymorphism could be due this SNP being in linkage disequilibrium with other causal variants at the *IL2RA* region.

In conclusion, this is the first study that identifies a significant association between a *IL2RA* polymorphism and MS, employing a case-control approach with samples collected from Caucasians in Spain. Replication in independent samples is the next important step in the validation of this gene as genetic factor involved in MS.

Acknowledgements This work was supported by grants FIS PI 020920 and Federación Española de Esclerosis Múltiple (FEDEM) to A. Alcina, FIS PI041298 and SAS 304–04 to F. Matesanz and M. Fedetz is a holder of Fundación IMABIS fellowship.

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IL2RA/CD25 Gene Polymorphisms: Uneven Association with Multiple Sclerosis (MS) and Type 1 Diabetes (T1D)

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Abstract

Background: IL-2 receptor (IL2R) alpha is the specific component of the high affinity IL2R system involved in the immune response and in the control of autoimmunity.

Methods and Results: Here we perform a replication and fine mapping of the *IL2RA* gene region analyzing 3 SNPs previously associated with multiple sclerosis (MS) and 5 SNPs associated with type 1 diabetes (T1D) in a collection of 798 MS patients and 927 matched Caucasian controls from the south of Spain. We observed association with MS in 6 of 8 SNPs. The rs1570538, at the 3′- UTR extreme of the gene, previously reported to have a weak association with MS, is replicated here (P = 0.032). The most associated T1D SNP (rs41295061) was not associated with MS in the present study. However, the rs35285258, belonging to another independent group of SNPs associated with T1D, showed the maximal association in this study but different risk allele. We replicated the association of only one (rs2104286) of the two *IL2RA* SNPs identified in the recently performed genome-wide association study of MS.

Conclusions: These findings confirm and extend the association of this gene with MS and reveal a genetic heterogeneity of the associated polymorphisms and risk alleles between MS and T1D suggesting different immunopathological roles of IL2RA in these two diseases.

Citation: Alcina A, Fedetz M, Ndagire D, Fernández O, Leyva L, et al. (2009) IL2RA/CD25 Gene Polymorphisms: Uneven Association with Multiple Sclerosis (MS) and Type 1 Diabetes (T1D). PLoS ONE 4(1): e4137. doi:10.1371/journal.pone.0004137

Editor: Michael Nicholas Weedon, Peninsula Medical School, United Kingdom

Received October 13, 2008; **Accepted** November 21, 2008; **Published** January 6, 2009

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Funding: Financial support for the study was provided by the Ministerio de Educación y Ciencia (grants PN-SAF2006-02023 and TIN2007-67418-C03-03) and Junta de Andalucía (P07-CVI-02551) to A. Alcina and Servicio Andaluz de Salud de la Junta de Andalucía (grant PI0168/2007) to F. Matesanz. María Fedetz is a holder of a fellowship from Fundación IMABIS. Dorothy Ndagire is a holder of AECI-Ministerio de Asuntos Exteriores fellowship. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Multiple sclerosis (MS) is the most common central nervous system disease in young adults, and one of the leading causes of disability in this age group affecting over 2.5 million individuals world-wide [1] The prevalence and incidence rates in Spain are around 77/100 000 habitants and 5.3/100 000 habitants per year respectively similar to what has been found in Britain [2,3] The disorder, which is presumed to be autoimmune in nature, is characterized by inflammation and demyelination, with axonal and neuronal degeneration. Susceptibility to MS is thought to be conferred by the combination of many common gene variants (not aberrant gene products) and environmental factors, which are mostly unknown [1,4].

The most strongly associated region implicated in predisposition to MS is the major histocompatibility complex (MHC) on chromosome 6p21, specifically the HLA-DRB1* 1501 class II allele; but, this account for less than 50% of MS genetics [1,5]. Recently, other regions have been implicated in MS susceptibility and replicated in different independent populations as the interleukin 7 receptor alpha (*IL7RA*) [6–8], the interferon regulatory factor 5 (*IRF5*) gene [9] and the interleukin-2 receptor alpha (*IL2RA*) [8,10,11]. The *IL2RA* gene has also been associated with type 1 diabetes (T1D) [12–14] and localized the association region in two independent groups of SNPs, spanning overlapping regions of 14 and 40 Kb encompassing *IL2RA* intron 1 and the 5′ regions of *IL2RA* and the RNA binding motif protein 17 (*RBM17*) genes.

Diverse autoimmune diseases may coexist in the same individual and in families, suggesting they might share common susceptibility gene variants implying a common etiology [15,16]. For example, in families with systemic lupus erythematosus (SLE), other autoimmune mediated diseases, such as MS and rheumatoid arthritis (RA) [17], or families with T1D and MS in Sardinian population [18], occur more frequently than in the general population. Such observations and others suggest the existence of shared genes or involvement of common biochemical pathways in these diseases. This hypothesis is supported by numbers of reports

on genes that are associated with more than one autoimmune disease, for example the Protein Tyrosine Phosphatase Nonreceptor 22 (PTPN22) has been associated with T1D [19], RA [20], and SLE [21], the IRF5 with SLE [22], inflammatory bowel diseases [23], RA [24] and MS [9], the FCRL3 with RA, autoimmune thyroid disease, SLE [25] and MS [26], among several other examples. However, population-based studies of >30 000 MS first-degree relatives found no increase in autoimmune disease [27].

As the associated SNPs analyzed in a genome wide association scan (GWAS) [8], candidate-gene association study in MS [10] and T1D [13] were not the same [28] we considered interesting to test if the TID-associated SNPs in the *IL2RA* region [13] were also associated with multiple sclerosis (MS) in our cohort of MS patients. In addition, we confirmed one of the two polymorphisms that have been associated with MS in the GWAS [8] and the one associated with MS in our previous candidate-gene association study of *IL2RA* gene [10].

Methods

Study subjects

Case samples comprised 798 patients with clinically defined MS according to Poser's criteria [29] They were obtained from four public hospitals: the Hospital Clínico in Granada (n = 130), the Hospital Virgen de las Nieves de Granada (n = 153), the Hospital Carlos Haya in Málaga (n = 357) and the Hospital Virgen de la Macarena in Seville (n = 158) all three cities within a 200 km radius in the South of Spain. The mean age at the sample collection of the cases was 36 years and mean age of controls at interview was 38 years. The percentage of females was 68% for cases and 59% for controls. All of them were classified as RR (relapsing-remitting) or SP (secondary progressive) MS cases. Controls were 927 blood donors with no history of inflammatory disease attending the blood banks of Granada (n = 619), Seville (n = 138) and Málaga (n = 170). The study was approved by the Ethics Committees of each of the hospitals participating in the study and written informed consent was obtained from all participants.

Genotyping

The SNPs were selected for being associated with MS (rs1570538, rs2104286, rs12722489) [8,10] or with T1D (rs10795791, rs4147359, rs7090530, rs41295061, rs35285258) [13] in previous studies.

High-molecular-weight DNA was isolated from whole blood using the Flexigene Kit (Qiagen, Hildren, Gemany) according to the manufacturer's protocol. We studied 8 SNPs located in the IL2RA locus as has been indicated in Fig. 1. rs1570538 was genotyped by restriction fragment length polymorphism method Primer sequences used were as follows: forward, TCATGTGA-CATCTGGAGGGTTA and reverse, AAAATGAATTTCGT-CAATTCGAG, restriction enzyme MwoI. The rest of the SNPs were genotyped by TaqMan technology under conditions recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). A total of 10 negative controls and 8 duplicates were included for each SNP as a quality control measure.

Statistical Analysis

The statistical studies to compare allelic and genotypic distribution between patients and controls were performed by Pearson χ^2 test or Fisher's exact test on 2×2 and 2×3 contingency tables, respectively, using the package available from the web of the Institute of Human Genetics of the Technical University of Munich (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) and the SPSS 15.0 statistical package. Hardy Weinberg equilibrium was tested using χ^2 goodness-of-fit test and no significant deviation was observed for any of the SNPs in this study (all $P>0.05$). Logistic regression analysis was used to calculate odds ratios (ORs) and 95% Confidence Intervals (C.I.s) for genotypes. To evaluate whether the best model is statistically significant, a permutation test was applied to obtain a *p*-value. The Haploview v.3.3 software [30] was used to generate haplotypes, perform haplotype association analysis, and to determine linkage disequilibrium (LD) measures (r^2) between the polymorphisms. P-values reported are unadjusted except for the most associated SNPs in whose case they were corrected for multiple testing assuming 8 independent SNP markers.

Assuming a minor allele frequency between 6.8–48.4% and a 0.05% prevalence of MS in the Spanish population, we estimated that at the 5% significance level, this study had between 44–92% power to detect genotypic relative risks reported in the first association study. The power that we had to detect effects was actually lower than in the original MS and T1D studies where larger cohorts were used (Table 1).

Results

Eight polymorphisms in the *IL2RA* gene were genotyped in 798 MS patient samples and 927 sex-matched Caucasian controls from the south of Spain. The linkage disequilibrium (LD) and their precise localization in the chromosome are indicated in the Fig. 1. The rs1570538 is located at the 3′-untranslated region (3′- UTR) of *IL2RA* [10], rs2104286 and rs12722489 are located at the 5′-proximal intron 1 region of the *IL2RA* [8], and the SNPs rs10795791, rs4147359, rs7090530, rs41295061 and rs35285258 are located at the 5′-upstream region of the *IL2RA*, in the intergenic region between *IL2RA* and *RBM17* genes [13].

We found the additive effect of allele dosage as the most plausible genetic model for rs2104286, rs7090530 and rs35285258. The recessive model seemed to fit better for rs1570538 (Table S1). The best model for all the other SNPs was also the additive one among the four tried: additive effect of allele dosage (allelic tests), recessive action, dominant action, additive+dominant (genotypic tests). Thus, we used the additive dosage model although in those SNPs there was not statistically significant support for the chosen model to be the best.

Six out of eight polymorphisms exhibited allelic association with MS, with nominal P-values ranging from 0.0016 to 0.033 (Table 1). All of them also showed significant association when genotypes instead of alleles and the Cochran-Armitage trend test where used (P values from 0.0016 to 0.033, Table S2). The Cochran-Armitage trend test has better power for near-additive risks models [31] than the Fisher exact test.

rs1570538 has been shown to be weakly associated with multiple sclerosis in our previous study of *IL2RA*/MS in a cohort of 346 cases and 413 controls (allelic P-value = 0.04) [10] and showed here increased statistical significance with our actual extended cohort (allelic association P = 0.033; OR for minor allele = 0.86, 95% CI, 0.75–0.99; genotype association (Cochran-Armitage trend test P = 0.033). We did not find any statistically significant evidence for MS association with either rs41295061 nor for rs12722489 by using the Cochran-Armitage trend test (P = 0.279 and P = 0.133 for rs12722489 and rs41295061 respectively) or the Fisher exact test in genotypes (P = 0.501 and P = 0.243 for rs12722489 and rs41295061 respectively) and alleles (P = 0.278 and P = 0.135 for SNPs rs12722489 and rs41295061 respectively). The strongest association signals in this study were

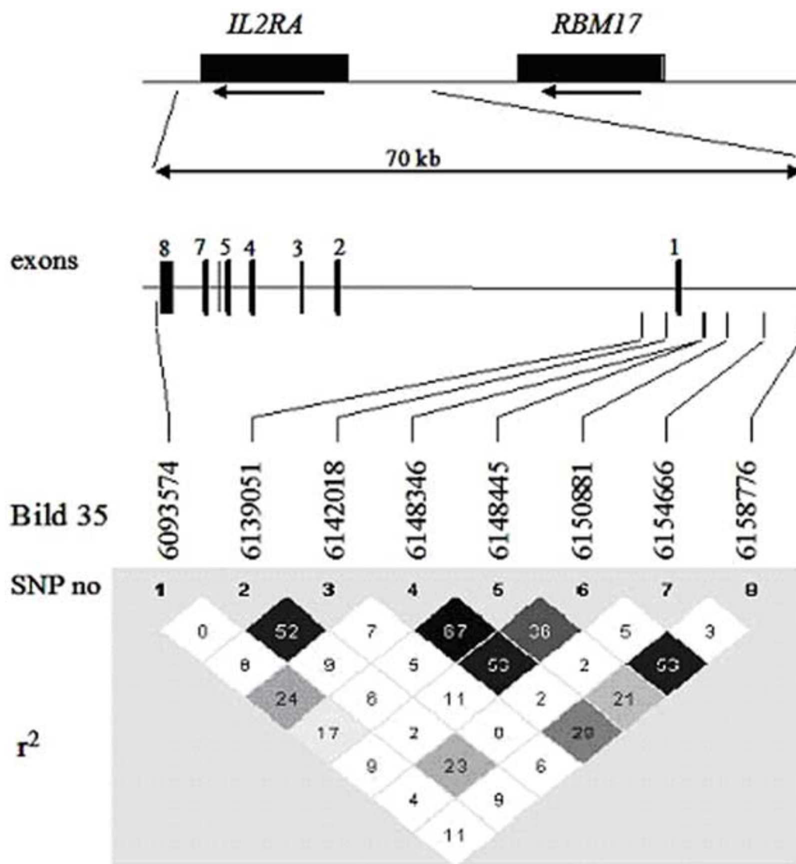

Figure 1. Schematic illustration of *IL2RA* locus showing the positions of the polymorphisms based on the NCBI Bild 35 and the linkage disequilibrium (LD) structure of the *IL2RA* gene as pairwise r^2-values. The rs41295061 and the rs35285258 in this study correspond to ss52580101 and ss52580135, respectively in the T1D study. [13] rs35285258 is in complete LD with rs11594656 ($r^2 = 1$) from the T1D study. [13] Both ss (submitted snp) numbers can be found as rs (reference snp) numbers in the SNPdb of NCBI.
doi:10.1371/journal.pone.0004137.g001

observed for rs7090530 and rs35285258 which, both allelic and genotype association, survived after correction for multiple tests by Bonferroni (Table S2). For the rs7090530, the data revealed a significant over-representation of the C allele among cases compared with controls (OR = 1.23, 95% C.I. = 1.07–1.41, P = 0.003). Very similar data were obtained for the T allele of rs35285258 (OR = 1.26, 95% C.I. = 1.09–1.46, P = 0.0016).

As observed in Fig. 1, rs7090530 and rs35285258 are in high LD with a pairwise $r^2 = 0{,}53$. rs1570538, rs2104286, rs10795791 and rs4147359, are in partial LD ranging from 0.29 to 0.09 with rs35285258. rs12722489 and rs41295061, negative for association with MS in this study, were in very low LD with rs35285258 ($r^2 = 0.06$ and 0.033, respectively). Conditioned to rs35285258, rs7090530 did not add any significant association, neither did the other SNPs (P-values 0.2377, 0.1718, 0.5885, 0.3112, 0.4517, 0.2342 and 0.3116 for the seven SNPs respectively). Thus, the rs7090530 association with MS could be explained by its strong LD with rs35285258.

Discussion

The aim of this study was to determine whether there is a common origin of the association of certain polymorphisms in the region of the *IL2RA* gene in MS and T1D. The fine mapping of the *IL2RA* region in T1D has located two ancestrally distinct causal alleles that are marked by two independent groups of SNPs at the first intron and the 5′ region of the *IL2RA* gene [13]. The genome wide association study of MS showed association with two SNPs (rs2104286 and rs12722489) [8] located at the 5′ region of *IL2RA* [10], that were not in linkage disequilibrium with those most associated with T1D [13]. The 3′ UTR polymorphism, rs1570538, which has been shown to be weakly associated with MS in our previous study [13] is replicated here in a larger cohort showing increased statistical significance. This could be due to the LD that keeps with rs35285258 (r^2 0.11) and shows a haplotype-specific effect.

The analysis of polymorphisms in this study reflects the existence of a heterogeneous association between T1D and MS that suggest different immunopathological mechanisms. It is notable that rs41295061, the most associated with T1D [13], was not associated with MS in this study.

rs35285258, belonging to the other independent group of SNPs associated with T1D [13] showed the maximal association in this study. However, the rs35285258 risk allele is C in the T1D study [13] while it was T in our analysis of MS. This is also the case for the rs4147359 and rs7090530 whose risk alleles were the contrary to those observed for T1D [13]. This type of observation has also been described for the *FCRL-3* gene, encoding a member of the Fc receptor-like family, specifically the C allele of FCRL3_3 variant has been associated with susceptibility to several autoimmune diseases [26] but showed to be protective for MS [27,28], and Addison's disease [32].

We replicated the association of only one (rs2104286) of the two SNPs identified in the GWA study of MS [8]. Moreover SNPs

Table 1. Allele distribution and frequency of *IL2RA* SNPs in patients with multiple sclerosis and healthy controls.

SNP	Alleles, n (%)			OR minor allele [95% CI]	P-value		
		Cases	Controls		This work[a]	GWAS/MS[b]	T1D[c]
rs1570538[d]	C	734 (47.8)	851 (51.6)	0.86 [0.75–0.99]	0.033/0.04[d]		
SNP1	T	802 (52.2)	799 (48.4)				
rs2104286	T	1237 (82.3)	1426 (79.1)	0.81 [0.68–0.96]	0.017	2.16×10^{-7}	
SNP2	C	265 (17.7)	378 (20.9)				
rs12722489	G	1216 (88.6)	1186(87.2)	0.87 [0.69–1.10]	0.278	2.96×10^{-8}	0.52
SNP3	A	156 (11.4)	1742(12.7)				
rs10795791	A	933 (64.4)	1094 (60.6)	0.85 [0.74–0.98]	0.027		1.4×10^{-6}
SNP4	G	515 (35.6)	710 (39.4)				
rs4147359	G	1105 (72.6)	1228 (69.0)	0.84 [0.72–0.98]	0.023		1.7×10^{-5}
SNP5	A	417 (27.4)	552 (31.0)				
rs7090530[e]	A	741 (48.9)	996 (54.2)	1.23 [1.07–1.41]	0.003		3.6×10^{-10}
SNP6	C	767 (51.0)	840 (45.8)				
rs41295061[f]	C	1436 (94.5)	1605 (93.2)	0.80 [0.60–1.07]	0.135		2.8×10^{-12}
SNP7	A	84 (5.5)	117 (6.8)				
rs35285258[e, g]	C	921 (62.4)	1193 (67.7)	1.26 [1.09–1.46]	0.0016		5.3×10^{-4}
SNP8	T	555 (37.6)	569 (32.3)				

[a]P-values = Pearson's goodness-of-fit chi-square (df = 1).
[b]GWAS/MS combined analysis including 1540 family trios, 2322 case subjects, and 5418 control subjects.[8]
[c]T1D study from 2965 cases and 2548 controls [13].
[d]Data from our previous work [10]. IL2RA/MS association study from 346 cases and 413 controls included in the present study.
[e]rs7090530 and rs35285258 survived Bonferroni correction.
[f]rs41295061 named ss52580101 in T1D study [13].
[g]rs35285258 named ss52580135 in T1D study [13].
doi:10.1371/journal.pone.0004137.t001

rs1570538, rs2104286, rs12722489, previously associated with MS, have lower levels of statistical significance than SNPs rs10795791, rs4147359, rs7090530, and rs35285258 from the T1D study when typed in MS. This may be due to that the MS associated SNPs come from a non exhaustive study of the locus and they capture the causal SNP with low LD.

It is unknown whether there is any causal polymorphism in the SNPs analyzed here and whether it can affect the level of expression of the *IL2RA* product because none of these polymorphisms are located in known regulatory regions. rs4147359 G allele and the T allele of rs10795791 create a putative recognition site for GATA-1 and GATA-2 transcription factors, respectively. Although these two SNPs did not survive the Bonferroni correction and had no effect conditioned on rs35285258, they showed a significant haplotype-specific effect (data not shown). The A allele of rs7090530 disrupted a putative CpG dinucleotide, as it does the SNP rs11597367 described in the T1D study [13], located at the 5′ region of the IL2RA at position 6147540 and in total LD ($r^2 = 1$) with rs35285258. Methylation of the CpG dinucleotides could be important for gene transcription regulation as it has been demonstrated for the IL2 gene [33]

The T1D-risk alleles have been associated with reduced soluble IL2R alpha concentrations. Thus, as the MS susceptibility alleles correspond with the contrary ones to those associated in the T1D study, we assume that the MS risk alleles might be associated with the high concentration phenotype of soluble IL2RA protein. Several aspects of the immune response could also be affected in an opposite fashion to those in the T1D study, for instance, the activity and functioning of activated Th1 and T regulatory cells (CD4+CD25+) but at the moment, the relevance and the role of this potential phenotypic data in the pathogenesis of MS is unknown.

In summary, our results replicate and extend the association found in the *IL2RA* gene region with MS and reveal differences in the polymorphisms and risk alleles associated with T1D which may reflect distinct roles that such gene variants may have in these two pathologies.

Supporting Information

Table S1 Test to evaluate whether the best model is statistically significant.
Found at: doi:10.1371/journal.pone.0004137.s001 (0.03 MB DOC)

Table S2 Genotype distribution for 8 IL2RA SNPs in MS cases and healthy controls and P values for Fisher exact test and Cochran-Armitage trend test performed in genotypes.
Found at: doi:10.1371/journal.pone.0004137.s002 (0.03 MB DOC)

Acknowledgments

We thank the patients with multiple sclerosis and persons acted as controls for making this study possible.

Author Contributions

Conceived and designed the experiments: AA FM. Performed the experiments: AA MF DN FM. Analyzed the data: AA MMAG FM. Contributed reagents/materials/analysis tools: AA OF LL MG CA CD ML GI FM. Wrote the paper: AA FM.

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Journal of the Neurological Sciences 190 (2001) 69–72

Journal of the Neurological Sciences

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The −174/−597 promoter polymorphisms in the interleukin-6 gene are not associated with susceptibility to multiple sclerosis

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Received 20 February 2001; received in revised form 8 June 2001; accepted 24 July 2001

Abstract

Interleukin-6 (IL-6) has been implicated in the etiology of experimental autoimmune encephalomyelitis (EAE) in transgenic animals and contributes to neuropathology in humans. A single nucleotide polymorphism (SNP) at position −174 in the IL-6 gene promoter (IL-6pr) appears to influence IL-6 expression. Complete linkage disequilibrium was observed between the −174 and the −597 alleles. The aim of this study was to investigate the possible influence of −174/−597 IL-6pr polymorphisms on susceptibility to multiple sclerosis (MS). Genotyping of the −597 variant was performed by an RFLP method in 131 MS patients [88 relapsing–remitting (RR-MS), 43 secondary progressive (SP-MS)] and 157 healthy subjects. No differences were found between MS patients and controls with respect to the distribution of −597 IL-6pr genotypes. Neither was found when genotypes were analyzed according to the clinical course of the disease (RR-MS or SP-MS). Future studies focusing on complex transcriptional interactions between the IL-6pr and 3′ flanking region polymorphic sites will be necessary to determine the IL-6 haplotype influence on susceptibility to MS. © 2001 Published by Elsevier Science B.V.

Keywords: Multiple sclerosis; Cytokine; Interleukin-6; Gene polymorphism; SNP; Immunopathology

1. Introduction

A complex genetic etiology is thought to underlie susceptibility to multiple sclerosis (MS). Interleukin-6 (IL-6), an important mediator of inflammatory and immune responses, is also produced within the CNS and plays a significant role in pathophysiology [1]. Elevated levels of IL-6 expression in this area may contribute to several disorders, e.g., Alzheimer's disease, Parkinson's disease, AIDS dementia complex, CNS trauma and bacterial and viral meningitis [2]. IL-6 has also been detected in MS brain [3] and in up-regulated transcription seen in mononuclear cells from the peripheral blood and cerebrospinal fluid of MS patients [4]. People with the disorder have higher levels of IFN-gamma, TNF-alpha and IL-6-secreting dendritic cells than healthy subjects [5]. Global spontaneous IL-6 production has been reported to be markedly higher in relapse MS patient groups than in remission or control groups [6].

Important evidence of IL-6 involvement in the MS pathological process has been collected in the MS animal model. Transgenic mice that constitutively overexpress IL-6 in the CNS exhibit a type of neuropathology which includes perivascular cell infiltration and blood–brain barrier disruption [7]. IL-6 knockout mice show resistance to myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis (EAE), while administration of IL-6 causes typical EAE [8].

Polymorphisms in the promoter regions of cytokine genes can modulate cytokine expression and modify the immune response [9–12]. Characterization of the IL-6 promoter region (IL-6pr) has revealed the presence of several genetic polymorphisms: −597 [13], −174 [14,15], −393 AnTn run [15] and −572 [16]. A single nucleotide

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0022-510X/01/$ - see front matter © 2001 Published by Elsevier Science B.V.
PII: S0022-510X(01)00595-0

change from G to C at position −174, located immediately adjacent to the multiresponsive element, has been found to suppress IL-6 transcription [15]. Complete linkage disequilibrium has been observed between the −597 and the −174 alleles. All the samples homozygous for allele G at −597 were homozygous for allele G at −174. All the samples homozygous for A at −597 were homozygous for C at −174. Heterozygous samples at position −597 were invariably heterozygous at −174 [17].

In this investigation, the distribution of −597 IL-6pr polymorphism in a group of MS patients and control subjects of the same geographic and ethnic origin was analyzed.

2. Materials and methods

2.1. Study subjects

Peripheral blood samples were obtained with informed consent from 131 unrelated Caucasian MS patients attending the Malaga and Granada neurological clinics (Southern Spain), and from 157 healthy blood donors of the same ethnic origin and geographic area. All patients had clinically defined MS (Poser criteria, 1983): 88 were assigned to a ‘Relapsing–Remitting’ group and 43 to a ‘Secondary Progressive’ group. The mean age at disease onset was 27.6 years. The patient sample was composed of 83 (63.4%) women and 48 (36.6%) men.

2.2. PCR-RFLP analysis

RFLP was performed with the −597 IL-6pr instead of −174 itself since both sites are at complete linkage disequilibrium, and the former yields a clear pattern of bands after digestion with the *Fok-I* restriction enzyme [17]. It is therefore possible to determine −597 and −174 IL-6 genotypes by typing with only one of these polymorphic sites. DNA was extracted by standard procedures [18]. For the determination of −174/−597 IL-6pr polymorphism, about 100 ng of DNA was PCR-amplified with the following oligonucleotides (both shown 5′–3′): GGAGTCACACAC TCCACCT (forward) and CTGATTGGAAACCTTATTAAG (reverse). PCR conditions were one cycle at 94 °C for 5 min; 35 cycles at: 94 °C for 30 s, 54 °C for 40 s and 72 °C for 50 s; and one cycle at 72 °C for 10 min. Amplification yielded a band of 525 bp. An aliquot of the PCR product was digested at 37 °C for 2 h with *Fok-I* (New England Biolabs). The band of 525 bp and digested fragments of 459 and 66 bp were separated by 12% polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide, and the products were visualized with ultraviolet light.

2.3. Statistical analysis

Comparisons of genotype frequencies between healthy controls and total MS patients, RR-MS and SP-MS subjects were calculated by the Chi-squared test for independent variables using the Stalcalc program (EpiInfo, version 6.0; Stone Mountain, GA). Mean age at disease onset was compared by the *t*-test. The Hardy–Weinberg equilibrium was tested by the Goodness of Fit test using the gene frequencies of healthy individuals.

3. Results

The distribution of the −597 IL-6pr genotypes in multiple sclerosis patients and healthy controls is shown in Table 1. The RFLP detection system is schematized in Fig. 1. Frequencies of −597 IL-6 G/G, −597 G/A and −597 A/A were 46.5%, 42.0% and 11.5% in controls and 43.5%, 48.1% and 8.4% in the total patients group. The gene frequencies of the IL-6pr genotypes were in Hardy–Weinberg equilibrium. No statistically significant differences were found between total MS patients and healthy controls with respect to the −597 genotype or allele distribution.

In order to establish whether there was an association with the clinical course of the disease (RR-MS and SP-MS) rather than with susceptibility, the −597 IL-6pr variants in the different groups were examined. Age at disease onset was also examined. No statistically significant differences in the distribution of IL-6pr genotypes were found between the RR-MS and SP-MS groups. Similarly, no association between IL-6pr genotypes and age at MS onset was observed (data not shown).

Table 1
Genotype distribution of −597 IL-6 promoter polymorphism in MS patients and in healthy controls[a]

−597 IL-6pr genotype	All MS patients, $n=131$	RR-MS group, $n=88$	SP-MS group, $n=43$	Controls, $n=157$
GG	57 (43.5)	40 (45.5)	17 (39.5)	73 (46.5)
GA	63 (48.1)	41 (46.6)	22 (51.2)	66 (42.0)
AA	11 (8.4)	7 (7.9)	4 (9.3)	18 (11.5)

RR: relapsing–remitting course.
SP: secondary progressive course.
[a] Number of individuals (%) is represented.

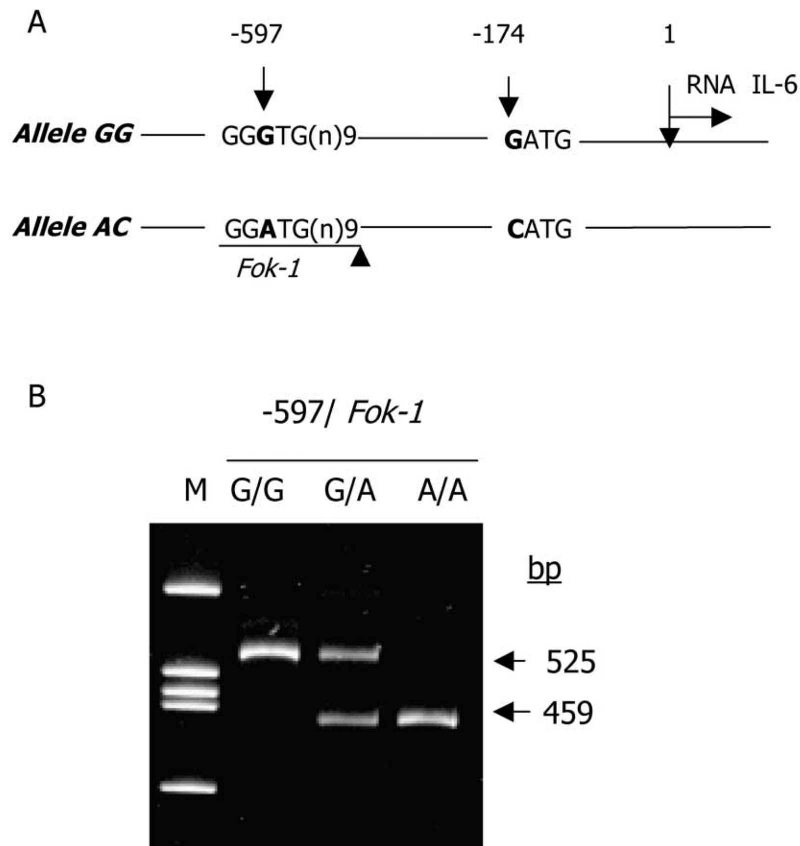

Fig. 1. Scheme representing the polymorphic sites in the IL-6 promoter region. (A) Complete linkage disequilibrium between the −597 and −174 nucleotides (bold letter) results in the existence of two alleles [17]. Restriction site sequence for the *Fok-I* enzyme used is shown. SNP positions are numbered in reference to the starting of gene transcription [15]. (B) PCR-RFLP analysis of the −597 polymorphism in polyacrylamide gel electrophoresis. The size of the digested PCR products and the different −597 genotypes are shown. M stands for molecular size markers.

Our data indicate that the −597/−174 IL-6pr genotypes have no major role in susceptibility to MS and in determining the clinical course of the disease or age of onset.

4. Discussion

IL-6 transcription is regulated by different factors binding to the promoter region. This may cause variations in expression levels and may influence susceptibility to different autoimmune and inflammatory diseases. −174 IL-6pr G/C polymorphism has recently been positively correlated with some disease states [15,19,20]. The present paper is the first report addressing the associations between −174/−597 IL-6pr polymorphism and susceptibility to MS.

Other authors have recently investigated polymorphisms at the 3′ flanking region in respect to MS. Minisatellite polymorphism located in the 3′ flanking region may predispose to alterations in the onset of MS [21]. Variable numbers of polymorphic tandem repeats in the 3′ flanking region (IL-6vntr-3′), whose C allele is associated with the altered activity of IL-6 in vivo, have been analyzed in MS patients. No difference in allelic distribution between patients and healthy controls has been found [22].

Other studies have demonstrated an association between the C allele of the IL-6vntr-3′ and delayed onset and reduced risk of Alzheimer's disease (AD) [23]. There were no associations with −174 IL-6pr polymorphism [24]. However, in haplotype analysis, a strong linkage imbalance between IL-6vntr-3′ and −174 IL-6pr has been observed. The interaction between these polymorphisms influences the risk of AD [24].

In the present investigation, no evidence was obtained to suggest that −174/−597 IL-6pr polymorphisms are susceptibility factors for the development of MS. Recent investigations [25] involving transfection experiments have suggested that base differences at distinct polymorphic sites (−572, −597, −392/−373 An/Tn and −174) do not act independently of one another. Thus, IL-6 transcription is affected not by a simple additive mechanism but by cooperative interactions determined by the haplotype. This might explain the negative results obtained in the present study. Further studies using new data on complex transcriptional interactions between IL-6 polymorphic sites are necessary to determine IL-6 haplotype influence on susceptibility to MS.

Acknowledgements

This work was supported by Grant No. 1FD97/0596 from the Comisión Interministerial de Ciencia y Tecnología-Comisión Europea and PM 99/0101 from the Ministerio de Ciencia y Tecnología to A.A. M. Fedetz is a holder of an Agencia Española de Cooperación Internacional (AECI) fellowship.

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Journal of Neuroimmunology 195 (2008) 146 – 150

Journal of Neuroimmunology

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The high producer variant of the Fc-receptor like-3 (*FCRL3*) gene is involved in protection against multiple sclerosis

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Received 25 August 2007; received in revised form 14 January 2008; accepted 16 January 2008

Abstract

Some polymorphisms in the *FCRL3* gene, a member of the Fc-receptor like family, have been associated with several autoimmune diseases and recently with multiple sclerosis (MS). We performed a case–control study of three SNPs in *FCRL3* gene in 645 MS patients and 786 controls, all Caucasians from the South of Spain. Genotype and allele frequencies of two SNPs (rs7528684/*FCRL3_3* and rs7522061/*N28D*), which were in high linkage disequilibrium ($r^2=0.87$), differed between MS cases and controls. The C allele of *FCRL3_3* was found to be protective for MS (per allele OR = 0.81, 95% C.I. = 0.70–0.94; *P*-value = 0.007) as was the G variant of *N28D*, but no association was found for rs11264799/*FCRL3_4*. Haplotype analysis confirmed these associations with highly consistent effect sizes for haplotypes carrying the C allele of *FCRL3_3*.
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Keywords: Multiple sclerosis; *FCRL3*; SNP; Polymorphisms; Genetic factor

1. Introduction

Multiple sclerosis (MS) is a prototypic idiopathic neurodegenerative disease of the central nervous system (CNS) whose primary mechanism of injury is by inflammatory/autoimmune demyelination and, to variable degree, axonal damage. The clinical manifestations, prognosis and pathological features vary, both amongst entities within the broad spectrum of demyelinating disease and amongst subtypes of MS. There is strong evidence supporting that both genetic and epidemiological factors play important roles (Kantarci and Weinshenker, 2006; Dyment et al., 2004; Fedetz et al., 2006; Leyva et al., 2005; Matesanz et al., 2004). How these genetic and environmental factors exert their biological effects so as to account for the clinicopathological heterogeneity in MS is not well defined (Kantarci and Weinshenker, 2006).

Recently, a functional variant, *FCRL3_3* (rs7528684), in the Fc-receptor like-3 (*FCRL3*) gene promoter, which alters the binding affinity of nuclear factor-kappaB and regulates *FCRL3* expression, has been reported to be associated with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), autoimmune thyroid disease (AITD) (Kochi et al., 2005) and multiple sclerosis, the latter finding from a Spanish case–control study (Martínez et al., 2007). The strongest association with RA has been observed for homozygous carriers of the C allele of the

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doi:10.1016/j.jneuroim.2008.01.004

FCRL3_3 (rs7528684) polymorphism, located at −169 of the *FCRL3* promoter. This allele was found to produce higher promoter activity in a reporter gene assay and the binding affinity for the NF-kB transcription factor was altered (Kochi et al., 2005). The *FCRL3* gene is located at the 1q21 locus and the 1q21–23 region has been implicated in several autoimmune diseases such as psoriasis (Capon et al., 2001) and MS (Dai et al., 2001). Several *FCRL* and *FCG* receptor genes (*FCRL5, FCRL4, FCR*, *and FCRL2*) are located in this region.

FCRL3 encodes a glycoprotein that is a member of the immunoglobulin receptor superfamily, and although its precise function remains unknown, it contains immunoreceptor tyrosine-based activation and inhibition motifs in its cytoplasmic domain, suggesting that it plays a role in immune cell regulation (Ravetch and Bolland, 2001).

To determine whether *FCRL3* is associated with susceptibility to MS, we genotyped three SNPs, two in the promoter region and one in the 3rd exon producing an amino acid change, in 645 MS patients and 786 controls in a Caucasian population from the South of Spain and compared with the results of Martínez et al. (2007) recently obtained in a multiple sclerosis-association study with an independent Spanish cohort.

2. Methods

2.1. Study subjects

Both cases and controls were recruited in Granada, Málaga and Seville, all three cities within a 200 km radius in the South of Spain. Case samples comprised 645 patients with clinically defined MS according to Poser's criteria (Poser et al., 1983). They were obtained from three public hospitals: the Hospital Clínico in Granada ($n=130$), the Hospital Carlos Haya in Málaga ($n=357$) and the Hospital Virgen de la Macarena in Seville ($n=158$). The mean age at the sample collection of the cases was 36 years and mean age of controls at interview was 38 years. The percentage of females was 68% for cases and 52% for controls. All of these patients were classified as either RR or SP-MS cases. Controls were 786 blood donors with no history of inflammatory disease attending the blood banks of Granada ($n=411$), Sevilla ($n=186$) and Málaga ($n=189$). The study was approved by the Ethics Committees of each of the hospitals participating in the study and written informed consent was obtained from all participants.

2.2. Genomic DNA isolation

High-molecular-weight DNA was isolated from whole blood using the Flexigene Kit (Qiagen, Hildren, Germany) according to the manufacturer's protocol.

2.3. SNP selection

All subjects were genotyped for 3 SNPs in the *FCRL3* gene, one C/T SNP (rs7528684) at the −169 position in the 5′ promoter region of the gene, another 5′ promoter G/A polymorphism (rs11264799) at position −110 of the gene, and a 3rd exonic polymorphism (rs7522061) at the 374G/A, which leads to the amino acid substitution, *N28D.* These three SNPs were designated as *FCRL3_3*, *FCRL3_4,* and *N28D* respectively consistent with previous studies (Kochi et al., 2005; Owen et al., 2007).

2.4. Genotyping

The three *FCRL3* polymorphisms were genotyped at the Madrid node (CNIO) of the Spanish National Genotyping Centre using Taqman. Assays on Demand C_1741825_10, C_17411826_10 and C_1741833_20 were supplied by Applied Biosystems. A total of 10 negative controls and 8 duplicates were included for each SNP as a quality control measure.

2.5. Statistical analysis

Hardy–Weinberg equilibrium was tested using χ^2 goodness-of-fit test. Differences in minor allele frequencies between cases and controls were tested for using Pearson's χ^2 test on 2×2 contingency tables. Odds ratios (ORs) and 95% Confidence Intervals (CIs) for comparisons between cases and controls were estimated by logistic regression using Stata SE v8. Haplotype frequencies were estimated and linkage disequilibrium (LD) measures (r^2) calculated using the Haploview software (Barrett et al., 2005) and compared between cases and controls using FamHap (v.12) software (Becker and Knapp, 2004). Assuming a minor allele frequency of 35% and a 0.05% prevalence of MS in the Spanish population (Fernández et al., 1994), we estimated that at the 5% significance level, this study had 99% power to detect genotypic odds ratios of the order reported in the first association study of RA in the Japanese population (OR=2.15, 95% C.I.=1.58–2.93). *P*-values reported are unadjusted for multiple testing unless stated otherwise. *P*-values were corrected for multiple testing by applying a permutation test based on 10,000 permutations, in each of which case–control status was randomly assigned conserving the observed proportions, χ^2 statistics calculated for each SNP tested and the maximum recorded for each permutation. The proportion of permutations for which the maximum χ^2 statistic was greater than that observed in the actual data for a given SNP was used as an estimate of the associated *P*-value, corrected for multiple testing.

3. Results

3.1. Polymorphisms and study groups

There was no evidence of departure from Hardy–Weinberg equilibrium among controls or cases for any of the three SNPs genotyped. We found that *FCRL3_3* and *N28D* were in high LD ($r^2=0.87$), with lower LD observed for each of these with *FCRL3_4* ($r^2=0.53$ and 0.49 respectively). The frequency of the C allele of *FCRL3_3* appeared to be higher in our control group (45.1%) than in the Japanese controls (37%) (Kochi et al., 2005), but similar to that among White North American controls (44.8%) (Hu et al., 2006) and to

Table 1
Genotype distributions for the three *FCRL3* SNPs in cases and controls

SNP/genotype	Cases *n* (%)	Controls *n* (%)	OR (95%CI)	*P*-value
N28D				
AA	209 (32.4)	211 (26.8)	1.00	
AG	315 (48.8)	406 (51.7)	0.78 (0.62–1.00)	0.047
GG	121 (18.8)	169 (21.5)	0.72 (0.53–0.98)	0.035
FCRL3_4				
GG	341 (53.1)	402 (52.2)	1.00	
AG	251 (39.1)	294 (38.2)	1.01 (0.81–1.26)	0.955
AA	50 (7.8)	74 (9.6)	0.80 (0.54–1.17)	0.249
FCRL3_3				
TT	227 (35.2)	226 (29.1)	1.00	
CT	318 (49.3)	400 (51.6)	0.79 (0.63–1.00)	0.052
CC	100 (15.5)	150 (19.3)	0.66 (0.49–0.91)	0.010

OR: odds ratio; CI: confidence interval.

that observed among controls in a recently published Spanish study of MS (44.9%) (Martínez et al., 2007). Genotype frequency distribution for the 3 SNPs between males and females in cases and controls were not different (data not shown).

3.2. Association of the FCRL3 variants with MS

Table 1 summarises the estimated genotype-associated odds ratios (ORs). There was no evidence that *FCRL3_4* was associated with MS (minor A allele frequencies of 27.2% and 28.1%, $P=0.6$). The variant alleles of both *N28D* and *FCRL3_3* were less common in cases than in controls (frequency=43.2% vs 47.3%, $P=0.027$ and frequency=40.2% vs 45.1%, $P=0.007$ respectively). After adjustment for multiple testing, association with *N28D* was only marginally significant (adjusted-$P=0.051$) and it was estimated that carriers of at least one G allele (dominant model) were at reduced risk of MS (OR=0.77, 95%CI=0.61–0.96). On the other hand, the association with *FCRL3_3* remained clearly statistically significant after correction for multiple testing (adjusted-$P=0.015$). Risk appeared to decrease with the number of C alleles carried in *FCRL3_3* and the estimated OR per C allele (codominant additive model) was 0.81 (95% CI=0.70–0.94, $P=0.007$).

3.3. FCRL3_3 haplotype association with MS

We examined whether haplotypes of *FCRL3* determined by the three SNPs genotyped were associated with the disease.

Table 2
Haplotype distribution between MS patients and healthy controls

Haplotype[a]		MS (*n*=642)	Controls (*n*=770)	OR (95% C.I.)	*P*-value
hap 1	AGT	0.564	0.525	1.445 (1.246–1.676)	0.037
hap 2	GAC	0.273	0.285	0.927 (0.786–1.094)	0.469
hap 3	GGC	0.127	0.159	0.771 (0.624–0.952)	0.016[b]
hap 4	GGT	0.034	0.027	1.263 (0.817–1.953)	0.296

[a] Allele order: *N28D*/*FCRL3_4*/*FCRL3_3*.
[b] Bonferroni corrected *P*-value<0.05.

Table 3
Combined data analysis of the Martínez at al. data and this work respect to the *FCRL3_3* polymorphism association with multiple sclerosis

	T vs C	(TT+TC) vs CC (Risk allele T)	TT vs (CT+CC) (Risk allele C)
OR (T allele)[a], 95% CI	1.24 (1.10–1.4)	1.4 (1.12–1.74)	0.77 (0.65–0.91)
P-value	0.0003	0.0025	0.0031

[a] OR per C allele 0.80 (IC95%)=0.71–0.90), *P*-value=0.0003.

There was evidence of a difference in haplotype distribution between cases and controls (Global $\chi^2=10.36$, *P*-value=0.016, based on 3 degrees of freedom). Further haplotype analysis performed with the HaploView software (Barrett et al., 2005) is summarised in Table 2. We found that the GGC haplotype (hap 3 in Table 2) composed of the G allele at *N28D*, the G allele at *FCRL3_4* and the C allele at *FCRL3_3* was associated with protection from MS (OR=0.77, 95%CI=0.62–0.95, *P*-value=0.016). Hap1 (AGT) carrying the risk-associated allele T of *FCRL3_3*, was associated with increased risk (OR 1.45, 95%CI=1.25–1.68, $P=0.037$).

4. Discussion

This study investigates the association of three SNPs in the *FCRL3* gene with multiple sclerosis. Polymorphisms in this gene have been associated with different autoimmune disorders such as RA, Graves disease, Addison's disease and MS in Caucasian populations and with Hahimoto's thyroiditis in a Japanese cohort. The association of the *FCRL3_3* C allele with RA has been replicated in Japanese and Canadian populations, however with diminished effect sizes compared to the initial association studies (Ikari et al., 2006; Newman et al., 2006; Thabet et al., 2007). In contrast to these findings, well powered association studies of the *FCRL3_3* C allele in Caucasian cohorts from North America, UK and Spain have failed to replicate the association of *FCRL3_3* with RA and type 1 diabetes (T1D) (Martínez et al., 2006; Hu et al., 2006; Turunen et al., 2006; Smyth et al., 2006). However, this polymorphism has been associated with other autoimmune diseases such as Addison's disease and Graves' disease in Caucasian populations (Simmonds et al., 2006; Owen et al., 2007).

Our data are in accordance with the results recently obtained in a MS association study by Martínez et al. (2007), based on a Spanish series of 400 cases and 508 controls. In both studies, genotype frequencies at the SNP *FCRL3_3* differed similarly and significantly between MS patients and controls, and differential distribution between cases and controls at the *FCRL3_4* was not significant for both studies. A combined analysis of both cohorts (Table 3) increased the significance of association and revealed that the C allele of *FCRL3_3* is protective for MS (OR=0.80, $P=0.0003$). Similar results have been reported for Addison's disease in Caucasian patients for which the *FCRL3_3* C allele appears to be protective (Owen et al., 2007).

The C allele of *FCRL3_3* has been shown to alter the binding affinity of NF-kB, which correlates with higher levels of

FCRL3 expression in B cells both in vivo and in vitro (Kochi et al., 2005), suggesting that the association with different autoimmune pathologies is due to a "higher producer phenotype" (Vandenbroeck and Goris, 2003). This "higher producer phenotype" may be a genetic determinant implicated in conferring protection against MS and Addison's disease. Therefore, the same *FCRL3* allele seems to be associated with opposing effects for two inflammatory diseases, MS and RA, suggesting different pathogenic mechanisms. The experimental treatment of a MS group with a TNF alpha blocker, Lenercept, was found to produce an exacerbation rather than a decrease of inflammation process (Kantarci and Weinshenker, 2006). The similar products, Etanercept and Infliximab, have been tried in RA and Crohn's disease and some patients receiving these treatments experienced inflammatory-demyelination in the CNS (Kantarci and Weinshenker, 2006). It is also possible that this allele plays a role in susceptibility only in the presence of certain triggers or in interaction with other genetic variants specific to each disease or unevenly distributed among populations. Indeed, an epistatic interaction between the *FCRL3* and NFkappaB1 genes for RA susceptibility has been described in a Spanish study. Furthermore, a restriction of the association of *FCRL3_3* with RA to *PTPN22* 1858T-homozygous individuals has been reported in a Canadian population (Martínez et al., 2006; Newman et al., 2006).

The apparently moderate association of the *FCRL3_3* and *N28D* polymorphisms with protection against MS might be explained by disease complexity that makes the potential set of genes involved in the pathology in each patient heterogeneous and by the complex inheritance response and disease heterogeneity (Dyment et al., 2004; Comi et al., 2000; Steinman et al., 2002; Lucchinetti et al., 2004; Pittock et al., 2007). On the other hand, we cannot rule out that the association observed between the *FCRL3_3* polymorphism and MS may be due to a causal SNP located elsewhere within the block of linkage disequilibrium (LD) that contains the 5′ region of the *FCRL3* gene, or with other causal variants at the near chromosomal region, such as the *FCRL2* gene which could have more relevance in the MS pathology. Although the SNPs analyzed in this work were chosen on the basis of maximal association and LD in the FCRL gene cluster (*FCRL5, FCRL4, FCRL3, FCRL2, FCRL1* and *CD5L*) in different populations and autoimmune diseases in previous works (Kochi et al., 2005; Owen et al., 2007).

Given the heterogeneity in associations of *FCRL3* with different pathologies in different populations, future large-scale association studies of the extended haplotype block containing both *FCRL3* and *FCRL2* across different ethnic groups will be useful to illustrate the mechanisms by which different genetic and environmental factors contribute to complex diseases in subjects with distinct ethnic backgrounds. In summary, all these data suggest that this locus may affect susceptibility to different autoimmune diseases across different populations. The fact the initial association (Martínez et al., 2007) of *FCRL3_3* with MS has been replicated and extended in this work in an independent case–control study with almost double the sample size, indicates that the evidence for the involvement of *FCRL3* in multiple sclerosis susceptibility is solid.

Acknowledgements

We thank the MS patients for making this study possible. Studies were performed after obtaining written informed consent from all patients under protocols approved by the Institutional Review Board of the Hospital Carlos Haya and Blood bank of Málaga, Spain. This work was supported by Grants Plan Nacional SAF2006-02023 from MEC to A. Alcina and PI041298 to F. Matesanz, CP03/0042 to L. Leyva and PAI CTS-507 to O. Fernández. M. Fedetz is a holder of a fellowship from Fundación IMABIS.

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Tissue Antigens ISSN 0001-2815

BRIEF COMMUNICATION

The T244I variant of the interleukin-7 receptor-alpha gene and multiple sclerosis

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Key words
association; interleukin-7 receptor-alpha gene; multiple sclerosis; polymorphism; single nucleotide polymorphism; Taqman

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Received 15 February 2008; revised 26 March 2008, 5 May 2008; accepted 12 May 2008

doi: 10.1111/j.1399-0039.2008.01075.x

Abstract

Several but not all studies have provided evidence for the association between multiple sclerosis (MS) and the T244I variant of the interleukin-7 receptor-alpha gene (*IL7RA*), rs6897932. We performed a new replication case–control study in 599 MS patients and 594 healthy controls, all Caucasians from the south of Spain. The genotype and allele frequencies differed between MS cases and controls. The *IL7RA* rs6897932 C allele and the CC genotype were found to be factors for disease susceptibility [per allele odds ratio (OR) 1.32, 95% CI 1.1–1.6, $P = 0.0031$; per CC genotype *vs* TT + TC genotypes, OR 1.5, 95% CI 1.18–1.87, $P = 0.0007$]. The combined data analysis included 3324 cases and 5032 controls of Europeans and Americans of European origin resulting in stronger association with similar OR ($P = 1.9 \times 10E{-}9$). These findings in our sample support previous reported association studies between *IL7RA* rs6897932 and MS.

Several studies have provided evidence for the association between multiple sclerosis (MS) and the T244I variant of the interleukin-7 receptor-alpha gene (*IL7RA*), rs6897932, but some others have been negative. As consistent replication of an effect for *IL7RA* in various populations would provide stronger evidence of causality (1, 2), we investigated in this study whether the *IL7RA* rs6897932 is associated with MS in an independent set of Caucasian population from the south of Spain.

MS is chronic inflammatory and demyelinating disease of the central nervous system estimated to affect over 2 million individuals worldwide. The disease manifests itself by immune-mediated demyelination and damage to axons resulting in progressive neurological disability in the absence of apparent ongoing infection (3). Genes influence susceptibility to MS; however, results of linkage and association studies are inconsistent, aside from the identification of human leukocyte antigen (HLA) class II haplotypes where the HLA-DRB1*1501 allele is a well-established genetic risk factor for MS (4–8). This locus, however, does not account for the whole genetic component of MS, although former and recently performed large linkage and association studies suggest that genes other than those in the HLA locus have small effects (4). Numerous candidate gene studies have also been performed in MS, but findings from one population have been difficult to replicate in other populations (9). The protein kinase C alpha (*PRKCA1*) gene (10, 11), the interleukin-2 receptor-alpha

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gene (*IL2RA*) (12, 13) and the *IRF5* gene (14) are some examples of genes reported to be associated with MS in more than one population.

Recently, the *IL7RA* has been found to be associated with MS in several independent family-based or case–control data sets (13, 15–18), suggesting a role for this pathway in the pathophysiology of this disease. The likely causal single nucleotide polymorphism (SNP), rs6897932, located within the alternatively spliced exon 6 of *IL7RA*, has a functional effect on gene expression. The SNP influences the amount of soluble and membrane-bound isoforms of the protein by putatively disrupting an exonic splicing silencer (16). However, the complex role of IL7RA in T-cell development (19), combined with the hitherto undefined molecular mechanisms of MS, suggests several possible means by which aberrant or suboptimal functioning of IL7RA may be implicated in the disease.

In our study, control and patient sample genotype frequencies were distributed according to Hardy–Weinberg equilibrium (controls, $P = 0.08$; MS, $P = 0.55$). As observed in Table 1, a significant difference in the overall distribution of genotype frequencies was found between patients and controls for this SNP ($P = 0.003$). The data showed a significant overrepresentation of the C allele among cases compared with controls, with frequencies of 0.778 and 0.725, respectively ($P = 0.003$). These results show that the *IL7RA* rs6897932 C allele was associated with increased predisposition to MS. The estimated odds ratio (OR) per C allele from logistic regression analysis was 1.32 (95% CI 1.1–1.597, $P = 0.0031$), while the *IL7RA* rs6897932 T allele was associated with protection (OR 0.755, 95% CI 0.63–0.91, $P = 0.0031$). This protective effect has also been detected by Lundmark et al. (15) in one of the haplotypes formed by four SNPs, including the T allele of rs6897932 with a maximal significance ($P = 0.0002$). One interesting observation was that the distribution of genotypes formed by these two alleles reflects different mode of heritability. According to the number of copies needed in order to modify the risk, the C allele requires two 'C' copies (recessive model) to increase the risk of MS (CC *vs* CT + TT, OR 1.5, 95% CI 1.18–1.87, $P = 0.0007$). The magnitude of this finding is in very close agreement with the study by O'Doherty et al. (20) who reported (CC *vs* CT + TT) an OR of 1.6 (95% CI 1.1–2.3, $P = 0.0049$) in an Olmsted County (USA) cohort of 208 patients with MS and 413 controls. Carriers of T allele need only one 'T' copy for providing a reduced risk of MS (CT + TT *vs* CC, OR 0.672, 95% CI 0.53–0.85, $P = 0.0007$) because two copies of 'T' produced similar reduction risk than one 'T' copy (dominant model).

The minor allele frequency (MAF) for this polymorphism in healthy controls of our Spanish population (MAF = 0.275 for the T allele) was similar to those previously described in the North Americans from European origin (MAF = 0.265) (16), Nordic and Swedish group (MAF = 0.3) (15), an European group (MAF = 0.28) (16), an Olmsted County cohort (MAF = 0.30) (20) and a group from Belfast (MAF = 0.264) (20), showing no evidence of stratification, thus permitting a combined analysis as is shown in the Table 2. The combined data analysis included 3995 cases and 5977 controls, resulting in stronger association ($P = 1.9 \times 10E{-9}$) and showed that the T carrier of *IL7RA* rs6897932 is protective for MS (OR 0.76, 95% CI 0.7–0.83), which confirmed significant association with MS in this nonoverlapping case–control group. Recently, the association of this polymorphism in two independent European populations (French and German) has been reported (21). In that study, the association of the *IL7RA* polymorphism seems to be negative for the German population ($P = 0.17$) as it was for the Belfast cohort, indicated in Table 2 ($P = 0.638$) (20). Therefore, the consistency of the association of the *IL7RA* rs6897932 in all European populations is not complete.

Table 1 Genotype and allele frequencies distribution analysis of the *IL7RA* rs6897932 polymorphism in MS cases and healthy controls

	Controls[a] (n = 594)	MS cases[a] (n = 599)
Genotypes[b]		
C/C	304 (51.3)	365 (61)
C/T	254 (42.7)	202 (33.7)
T/T	36 (6)	32 (5.3)
P (global)		0.003
Allotypes		
C	862 (72.5)	932 (77.8)
T	326 (27.5)	266 (22.2)
Allelic_χ^2 *P* value		0.003
OR per C allele (95% CI)		1.325 (1.1–1.6)
Genotypic association[c]		
CC *vs* CT + TT (1.0, reference)		
OR		1.5
95% CI		1.18–1.87
χ^2		11.53
P		0.0007

CI, confidence interval; MS, multiple sclerosis; OR, odds ratios.
[a] MS cases were clinically defined according to Poser's criteria (23) and controls were ethnically, sex- and age-matched, unrelated Caucasian from the south of Spain.
[b] The genotyping of the samples was performed by Taqman (Applied Biosystems, Foster City, CA) as previously described (12).
[c] Comparison of allelic and genotypic distributions between patients and controls was performed by Pearson chi-squared test or Fisher exact test on 3 × 2 and 2 × 2 contingency tables, respectively, using the package available from the web of the Institute of Human Genetics of the Technical University of Munich (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) and the SPSS 15.0 statistical package. Hardy–Weinberg equilibrium was tested using chi-squared goodness-of-fit test. Logistic regression analysis was used to calculate OR and 95% CI. Given the size of this study, we had a power of 80% to detect an OR of 1.4.

Table 2 Allele and carriership frequencies difference and combined analysis between data sets from Sweden, Americans (European origin), EUR, Olmsted County, Belfast and ours (Spanish)

	Allele frequencies difference		Allele carriership (TT + CT *vs* CC)	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Set origin (*n*, MS cases/CTL)				
USA (438/479)	0.78 (0.63–0.97)	0.025	0.77 (0.59–0.99)	0.048
EUR (1077/2725)	0.8 (0.71–0.89)	0.00011	0.79 (0.68–0.91)	0.001
Sweden (1210/1234)	0.8 (0.70–0.90)	0.0005	0.76 (0.65–0.89)	0.00095
Olmsted County, USA (208/413)	0.7 (0.5–0.9)	0.005	n.p.[a]	
Belfast (463/532)	0.95 (0.8–1.16)	0.638	n.p.[a]	
Spanish (599/594)	0.75 (0.63–0.91)	0.0031	0.67 (0.53–0.84)	0.0007
Combined (*n*, MS cases/CTL)	0.79 (0.74–0.84) (3995/5977)	$1.2 \times 10E-11$	0.76 (0.7–0.83) (3324/5032)	$1.9 \times 10E-9$

CI, confidence interval; CTL, controls; EUR, European group; MS, multiple sclerosis; n.p., not provided; OR, odds ratios.
[a] Not provided in the O'Doherty paper (20).

We have also assessed the association of *IL7RA* polymorphism with interferon (IFN)-beta therapy response in a group of patients who were classified as responders and nonresponders with IFN-beta treatment, as previously described (22). The estimated frequencies were not different between IFN-beta responders ($n = 209$) and nonresponders ($n = 107$) ($P = 0.64$). Stratification by age at onset and genotypes showed absence of statistical significance ($P = 0.8$).

The effect that this polymorphism may have in the *IL7RA* product and its role in MS is not completely clear (15, 16). Lundmark et al. (15) have reported altered expression of the genes encoding IL7RA and its ligand, IL7, in the cerebrospinal fluid (CSF) compartment of individuals with MS but not in the peripheral blood mononuclear cells (PBMCs) and more important the analysis of *IL7RA* mRNA expression in CSF PBMCs in relation to the genotype of the rs6897932, did not uncover any correlation ($P = 0.81$). In *in vitro* experiments, the study by Gregory et al. (16) has shown that the rs6897932 affects alternative splicing of exon 6. Transcripts that include exon 6 encode a membrane-bound IL7RA, whereas transcripts that skip exon 6 produce a variation T244I located in an important transmembrane domain of IL7RA encoding a predicted soluble form of the protein. The MS-associated 'C' allele resulted in twofold increase in transcript that skipped exon 6 compared with transcripts containing the 'T' allele. Therefore, these data suggest that carriers of the 'C' allele at rs6897932 produce less membrane-bound IL7RA protein compared with carriers of the 'T' allele, leading to a further increase of the soluble form of IL7RA. Thus, the low and intermediate levels of IL7RA soluble form are more beneficial than higher levels in the suppression of the disease.

In summary, our study replicates the association of *IL7R* SNP rs6897932 with MS in an independent population providing a stronger evidence of causality for this SNP. In spite of this solid implication of SNP in MS, different level of attributable risk to disease susceptibility have been reported, varying from 0.2% (together with *IL2RA*) in the study of Hafler et al. (13) to 12% in the study of Lundmark et al. (15). This outcome indicates that other types of genome variants should be required for the development and progression of MS, which may vary among populations and is consistent with a complex disease model in which multiple genes and environmental factors contribute to the phenotype.

Acknowledgments

We thank the patients with multiple sclerosis and persons acted as controls for making this study possible. Studies were performed after obtaining written informed consent from all participants under protocols approved by the Institutional Review Board of the Hospital Carlos Haya, Hospital Virgen de las Nieves, Hospital Clínico San Cecilio and Blood Bank of Málaga and Granada, Spain. This work was supported by Grants from the PN-SAF2006-02023 and Junta de Andalucía P07-CVI-02551 to AA and SAS PI0168/2007 to FM, CP03/0042M to LL and PAI CTS-507 to OF. MF is a holder of a fellowship from Fundación IMABIS (Instituto Mediterraneo para el Avance de la Biomedicina y la Investigación Biosanitaria). DN is a holder of AECI (Agencia Española de Colaboración Internacional)-Ministerio de Asuntos Exteriores fellowship.

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BRIEF COMMUNICATION

OAS1 gene haplotype confers susceptibility to multiple sclerosis

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Key words
2′,5′-oligoadenylate synthetase 1; gene polymorphism; haplotype; multiple sclerosis; restriction fragment length polymorphism

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Received 12 May 2006; revised 12 August 2006; re-revised 23 August 2006; Accepted 11 September 2006

doi: 10.1111/j.1399-0039.2006.00694.x

Abstract

Multiple sclerosis (MS) is associated with genetic susceptibility and unknown environmental triggers, possible viral infections, but the specific etiological mechanism that subsequently develops into an inflammatory/autoimmune cascade of events is poorly understood. Recently, genetic variants of 2′,5′- oligoadenylate synthetase 1 (*OAS1*) gene, a critical enzyme involved in innate antivirus response, have been associated with differential enzyme activity and type 1 diabetes in both case–control and family studies. We hypothesized that polymorphisms in the *OAS1* gene could influence the susceptibility to MS. To test this hypothesis, we conducted a case–control study of 333 patients with MS and 424 healthy controls and genotyped two *OAS1* single nucleotide polymorphisms (SNPs) by restriction fragment length polymorphism method: rs 10774671, A/G SNP altering the splicing site at the seventh exon, and rs 3741981, a nonsynonymous (Ser162Gly) A/G SNP in the third exon. Haplotype but not single-marker analysis revealed an association of the haplotype created by the G allele at rs 10774671 and the A allele at rs 3741981 with the susceptibility to MS (P value $= 8.8 \times 10^{-5}$). Subjects carrying this haplotype had an increased risk of MS comparing with those not carrying it (odds ratio = 4.7, 95% confidence interval 2.1–10.9). Our findings indicate that the *OAS1* gene polymorphisms may confer susceptibility to MS or serve as markers of functional variants and suggest that OAS1 activity is involved in the etiology of the disease. Future studies in a larger sample and association analysis with functional variants will clarify the role of the *OAS1* gene in the susceptibility to MS.

Multiple sclerosis (MS) is a chronic neurodegenerative disease of the central nervous system that is characterized by inflammatory demyelination and, to a variable degree, axonal damage. Epidemiological and genetic findings suggest that MS is an acquired autoimmune disease triggered by unknown environmental factor in genetically susceptible individuals (1). Several cases of virus-induced demyelinating encephalomyelitis in human beings and in experimental models as well as the presence of immunoglobulin G oligoclonal bands in the cerebrospinal fluid indicate that the causative factor may be viral (2, 3). The virus may remain dormant in the central nervous system but then becomes activated in adulthood and provoke direct lysis of oligodendrocytes or could initiate immunopathology (4). The possible causative agents were studied, but conclusions are not definite. However, the absence of identifiable infectious agent, especially viral, does not rule out its presence at a certain time-point and the concomitant long-term triggering of an autoimmune cascade of events thereafter (1).

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The central nervous system mounts an organized innate immune response during systemic bacterial or viral infection (5). Two different classes of pattern-recognition receptors (PRRs), including multiple Toll-like receptors and several RNA helicases, are responsible for the recognition of various viral conserved molecular motifs in different cellular compartments and induce antiviral responses through different signaling pathways. However, signaling through these PRRs converges on type I interferon (IFN) induction and leads to the elimination of viruses (6).

Among the critical proteins with antiviral activity induced by type 1 IFNs, there is a family of 2′,5′adenylate synthetase enzymes (oligoadenylate synthetase, OAS) that catalyzes the synthesis of oligodadenylates of general structure ppp $(A2'p9)_n$ A (2-5A). OAS enzymes require double-stranded RNA structures, such as viral genomes or single-stranded transcripts that possess significant double-stranded character, to become activated (7). The functional 2-5A oligomers bind to and activate latent endoribonuclease, RNA degrading enzyme, which was also shown to produce a strong stimulation of transcription for genes that suppress virus replication and promote cellular apoptosis (8, 9). Studies on inbred strains of laboratory mice demonstrate that mutation in exon 4 of their *Oas1b* gene plays an important role in susceptibility to West Nile Virus (10, 11), meanwhile wild-type mice are resistant to this virus (12).

In humans, the activity of the OAS1 enzyme is found to be controlled by a single nucleotide polymorphism (SNP) at the splice-acceptor site of exon 7 in the *OAS1* gene (13). The G allele at acceptor site is associated with high enzyme activity. It conserves the splice site and generates a p46 enzyme isoform, while A allele ablates the splice site that results in the generation of p48 and p52 isoforms. This SNP (rs 10774671) is reported to influence susceptibility to type 1 diabetes in a case–control study (14). The association of the *OAS1* gene with type I diabetes (T1D) in a family-based study (15) is attributed to rs 3741981 (*OAS1* third exon) as a more likely functional candidate than rs 10774671. SNP in the seventh exon of *OAS1* gene (rs 2660), which is in almost complete linkage disequilibrium with SNP at the splice-acceptor site of the exon 7 (13), is associated with the outcome of hepatitis C viral infection (16).

The aim of this study was to assess the influence of *OAS1* gene polymorphisms in susceptibility to MS. For this purpose, we chose two SNPs: one, A/G polymorphism at the splice-acceptor site of exon 7 (rs 10774671) and another, non-synonymous A/G SNP in the third exon of gene (rs 3741981), which leads to amino acid substitution (Ser162Gly).

Our study sample consisted of 333 unrelated cases with clinically defined MS according to Poser's criteria (17) and 424 ethnically matched unrelated Caucasian controls of similar age range (donors of the regional blood bank). The study was approved by the Ethics Committee of Hospital, and written informed consent was obtained from all the participants. The restriction fragment length polymorphism method was used for genotyping of these SNPs. Primer sequences used were as follows: rs 10774671 – forward, TCCAGATGGCATGTCACAGT and reverse, TAGAAGGCCAGGAGTCAGGA, restriction enzyme *Alu*I; and rs 3741981– forward, GGATCAGGAATGGACCTCAA and reverse, 3719 GGAGAACTCGCCCTCTTTCT, restriction enzyme *Ssi*I. Amplification conditions included initial denaturation at 94°C for 2 min, followed by 28 cycles of 94°C for 20 s, 62°C for 40 s and 72°C for 30 s, with a final extension for 7 min at 72°C.

In both panels, the genotype frequencies of both SNP were distributed according to Hardy–Weinberg equilibrium. Single-marker results were analyzed by χ^2 statistics. No significant difference was observed between patient and control genotype or allelic frequencies (Table 1). Stratification of patient group by clinical course [relapsing–remitting (RR) or secondary progressive (SP)], age at the onset of disease and gender did not reveal any association with OAS1 genotypes. Since linkage disequilibrium with a disease locus may be stronger for haplotypes than for single alleles (18), we examined whether haplotypes of *OAS1*, determined by studied SNPs, could reveal an association with the disease. Haplotype analysis was performed using Haploview software (19). Linkage disequilibrium characteristics between two markers in our sample set were as follows: $D' = 0.898$ and $r^2 = 0.588$. We found that the haplotype GA created by the G allele at rs 10774671 and by the A allele at rs 3741981 (hap 4) was 4.6-fold more frequent in unstratified patient group with MS (P value $= 8.8 \times 10^{-5}$) as shown in Table 2. Global $\chi^2 = 23.53$, d.f. was 3 and P value

Table 1 Genotype and allelic frequencies of *OAS1* polymorphisms in patients with multiple sclerosis and healthy controls[a,b]

	Patients ($n = 333$)	Controls ($n = 424$)	χ^2	d.f.	P
rs 10774671					
Genotype					
AA	120 (36.0)	156 (36.8)			
AG	164 (49.3)	212 (50.0)	0.36	2	0.84
GG	49 (14.7)	56 (13.2)			
Allele					
A	404 (60.7)	524 (61.8)	0.20	1	0.65
rs 3741981					
Genotype					
AA	87 (26.1)	119 (28.0)			
AG	180 (54.1)	219 (51.7)	0.48	2	0.79
GG	66 (19.8)	86 (20.3)			
Allele					
A	354 (53.2)	457 (53.9)	0.08	1	0.78

n, number of the subjects genotyped.

[a] The values given in parenthesis are in percentages.

[b] Statistical analysis of genotype and allelic frequencies between the groups was carried out using the contingency table 3 × 2 (genotypes) and 2 × 2 (alleles) χ^2 statistics (http://faculty.vassar.edu/lowry/VassarStats.html).

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Table 2 The haplotype frequency estimation for the patients with multiple sclerosis and control group[a]

	Haplotype (rs 10774671–rs 3741981)	Patients	Control	χ^2	*P*
hap1	AA	0.493	0.534	2.54	0.1113
hap2	GG	0.356	0.372	0.44	0.5061
hap3	AG	0.114	0.085	3.43	0.0640
hap4	GA	0.037	0.008	15.38	8.8105E-5

[a] Analyzed by HAPLOVIEW software package (19); global $\chi^2 = 23.53$, d.f. = 3, $P < 0.0001$ in the analysis by FAMHAP software (20).

was less than 0.0001 in analysis by FAMHAP (version 12) software (20). Odds ratio (OR) was calculated for hap 4 *vs* three other haplotypes using two-way contingency table analysis. Subjects carrying this haplotype had a 4.7-fold increased risk of MS [95% confidence interval (CI) 2.1–10.9]. Haplotype frequencies were also estimated by THESIAS (21) software, and similar results were obtained. Our results demonstrated that hap 4 was associated with RR [*P* value = 6.04E-5, OR 5.1 (95% CI 2.2–12.2)] and SP [*P* = 0.003, OR 4.8 (95% CI 1.6-14-4)], the clinical courses of MS.

We have assessed the association of GA haplotype with IFN-beta therapy response in a group of patients that were classified as responders and non-responders with IFN-beta treatment, as described by Leyva et al. (22). The estimated haplotype frequencies were not different between IFN-beta responders ($n = 139$) and non-responders ($n = 56$).

Report by Field et al. (14) presents data suggesting that polymorphism G/A at the splice site of exon 7 in *OAS1* gene could influence susceptibility to T1D, the frequencies of genotypes GG and GA are found to be significantly increased in patient with T1D. These investigators also show that the minor G allele at this SNP has a higher frequency in individuals with high enzyme activity than in those with low enzyme activity (13).

In the study of Tessier et al. (15), significant increased risk of T1D is related only with homozygosity for the minor allele of each of the *OAS1* SNPs (rs 10774671, rs 3741981 and rs 3177979), a recessive effect of these polymorphisms is shown by FBAT analysis. Results of transmission disequilibrium test performed by these authors suggest that rs 3741981 may be the sole functional variant accounting for the genetic effect in T1D in contrast to the hypothesis of Field et al. OAS locus is characterized by a long range linkage disequilibrium between polymorphisms (15). Due to this fact, we are in agreement with the opinion of Tessier et al. (15) that the studied SNPs could be merely markers for a haplotype containing a functional variant that determines the susceptibility to disease.

In conclusion, we found an association of a haplotype consisting of the SNPs in exons 3 and 7 (splice-acceptor site) of the *OAS1* gene, which is involved in innate host antiviral response, with MS. Because these two markers did not show any association with MS in single-marker analysis, the causative variant may be located in an adjacent region. Future studies in a large case–control or family-based sample will clarify the role of *OAS1* gene in susceptibility to MS.

Acknowledgments

This work was supported by grants FIS PI 020920 and Federación Española de Esclerosis Múltiple (FEDEM) to A. A. and by grants FIS PI041298 and SAS 304-04 to F. M. M. F. is a holder of Fundación IMABIS fellowship.

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RESULTS AND DISCUSSION

VI. RESULTS AND DISCUSSION

VI.1. The role of polymorphisms in *IL2* gene in susceptibility to MS

Articles I-IV

The aim of the **study I** was to see if the polymorphisms in *IL2* gene were associated with MS. Two polymorphisms, one in the promoter, at position *-330* (*-384*, from start codon) and another – synonimous SNP in the first exon at position *+114*, were genotyped in a cohort of 173 patients and 153 controls. In the control group, the allele frequencies showed little or no difference from the previously reported distribution in healthy UK Caucasians (Reynard et al., 2000). We found that genotypes at *-330 G/T* polymorphisms were associated with the susceptibility SPMS. The G allele was over represented in these patients (30% comparing to 22% in control group), although the difference did not reach statistical significance, probably due to small size of SPMS group. Japan colleagues did not reproduce this association in their cohort; the frequency of *-330G* allele was very similar in Japanese patients and controls (Kikuchi et al., 2002). This result can be explained by small sample group or ethnic differences.

Study II was designed to check if two more SNP in the *IL2* promoter, *-475* and *-631*, which could influence gene expression, are polymorphic in our population and associated with MS.

Both polymorphisms are localized to the -600 to -300-bp distal region and are therefore separated from the extensively studied proximal enhancer of the *IL2* promoter. This sequence is very much conserved with respect to the mouse promoter. Genomic footprinting studies within this distal region in mice indicate that it may serve as a stable nucleation site for tissue-specific factors and as a potential initiator site for activation-dependent chromatin remodelling (Ward et al*.,* 1998). For this reason, SNPs in this region are

potential candidates for functional promoter studies and for association studies in immune diseases.

Both, patient and control groups showed a very low frequency of allele *T* at *-475* and allele *A* at *-631*. The frequencies of the *-631 A* allele (0.01) and the *-475 T* allele (0.03) given in the SNP database are even higher than those found in the present work. In addition, we did not found any alterations of the SNP frequencies in the MS samples. Our results suggest that these polymorphisms do not contribute to MS susceptibility.

In **study III** we established as one of the objectives to replicate the *-330 IL2* association in larger cohort, consisted of 805 unrelated cases and 952 regional controls. In this case group, where SPMS patients represented 22% of cohort, we showed the genotype, allelic and haplotype associations although the statistical significance of genotype and allelic associations did not support the Bonferroni corrections for multiple comparisons.

This study was also dedicated to analysis of the role in susceptibility to MS of SNPs from the gene region 4q27 associated with several autoimmune and inflammatory diseases by recent GWAS studies where *IL2* gene maps (Todd et al., 2007; van Heel et al., 2007; Liu et al., 2008) as the common autoimmune disorders are considered to share genetic risk factors (Gupta et al., 2005; Corporaal et al., 2002; Marrie et al., 2007). We selected two SNPs from 4q27 *TENR-IL2-IL21* region: rs3136534 associated with T1D and Graves´ disease (Todd et al., 2007) and rs6822844, significantly associated with celiac disease in three ethnic cohorts (van Heel et al., 2007; Zhernakova et al., 2007). There was not evidence for association with any SNPs tested as in whole MS group as after stratification. Allele and genotype frequencies of the SNPs, which were studied, were similar in *DRB1*15*-positive or negative patients and in patients with RRMS and SPMS clinical course.

Although minor allele at rs6822844 is found as perfect proxy for T1D, CD and RA associated haplotypes with decreased frequencies in patients (Zhernakova et al., 2007), this result was not replicated in the MS cases in our study although we had 97% power to reproduce OR values 0.6-0.7 found

in T1D and RA. Our study had 80% power to discard the associations with OR more then 1.15. We neither obtained higher OR or had enough power to reproduce OR 1.1 found in T1D and RA for rs3136534 (Todd et al., 2007; Zhernakova et al., 2007).

However, in haplotype analysis, the patients with haplotype bearing rs2069762 *G* allele (*-330G IL2*) were at significant increased risk for a SPMS clinical course (*P*=0.045 after 10000 permutations performed) (Table 1). In contrast, no difference was viewed in the haplotype frequencies of RRMS when compared with healthy controls.

Haplotype	Frequencies		Chi Square	P Value*
	SP-MS Cases	Controls		
CTC	0.290	0.317	0.837	0.3602
AGC	**0.356**	**0.289**	**5.64**	**0.0176****
ATC	0.260	0.264	0.015	0.9011
ATA	0.092	0.126	2.886	0.0893

Combined frequency of haplotypes presented is 99.8%
* Uncorrected
** P value after 10000 permutations performed is **0.045**

Table 1. Haplotype frequencies (rs 3136534, rs2069762 and rs6822844) in SP-MS cases and healthy controls.

The power to detect genetic effect in SPMS is expected to increase due to that SP MS group seems to be more clinically homogeneous sample. The RRMS and SPMS are considered as the two stage of the same disease, however the transition from RRMS to SPMS course occurs in approximately 50 % of RR patients, it can take different periods of time and is unpredictable (Lublin & Reingold, 1996; Tremlett et al., 2008). We did not exlude the influence of *IL2* gene locus on MS clinical course.

Thus, our results discarded the major influence of *TENR-IL2-IL21* on susceptibility, but they did not exclude the role of this locus in MS. It will be

necessary to perform the studies with significantly bigger cohort and a higher density of markers to confirm or discard the implication of *IL2* locus in disease.

In the **study IV** we analyzed the functional effects of the associated with MS in our previous study *-330 G/T* promoter polymorphism in *IL2* allelic expression. This SNP is located in the upstream region of the *IL2* gene promoter – enhancer domain (John et al., 1998). The impact of region encompassing 370 bp 5' of the transcription start site on *IL2* gene expression has been studied using series of 5'-deletion constructs transfected into the Jurkat T lymphoid line (Williams et al., 1988). It was demonstrated that this region is sufficient to promote inducible transcription. It contains two discrete regions from -42 to -169 and -289 to -361 bp relative to the transcription start site which are critical for inducible expression of the IL-2 gene. *IL2* gene polymorphism is shown to influence cytokine production levels in peripheral blood lymphocytes after anti-CD3/CD28 stimulation (Hoffmann et al., 2001).

We used three different methods to study functional effect: the transfection of Jurkat cells with two constructions containing *-330* allelic variants (*G/T*) of the *IL2* promoter – enhancer region (-501 to + 1) upstream a reporter gene; measurement of the relative expression of the -*330 G* allele against the -*330 T* in samples of MS patients and controls; quantification of the total *IL2* expression in PBL of healthy individuals carrying the three different -*330* genotypes.

The transfection study in Jurkat cells demonstrated differential promoter activity between the *G* and the *T* alleles (Figure 27). The construction carrying G was twice more active than the one with *T*. Conversely, quantification of allelic expression in lymphocytes showed that the *-330 T*allele was associated with a higher level of transcription than the *-330 G.* These data were in concordance with our real-time quantification results, since a higher expression level of *IL2* mRNA was observed in the samples of individuals with *-330 T/T* and *G/T* genotypes compared with the ones bearing the -*330 G/G* genotype (Table 2).

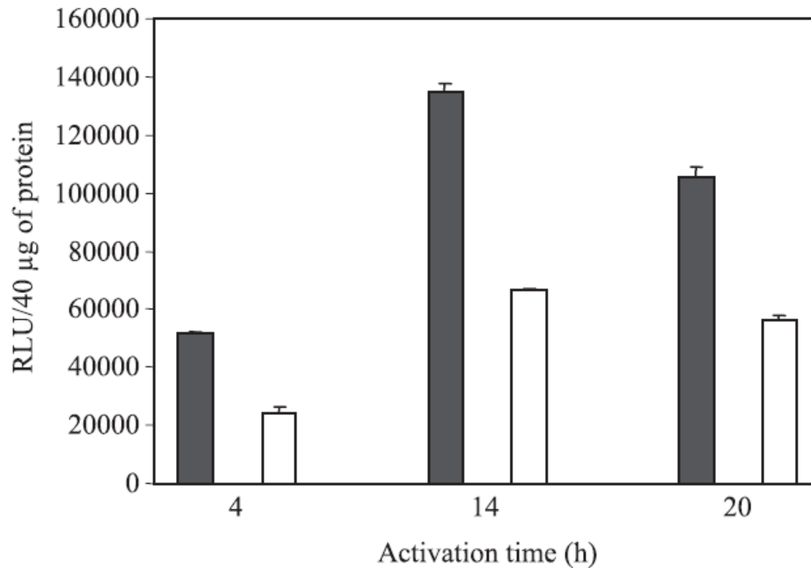


Figure 27. Transcriptional activity of the *-330 IL2 G* and *T* alleles in Jurkat cells.

These results are in disagreement with those of Hoffman and coworkers (2001) which described an enhancement in IL2 production in individuals bearing *-330 G/G.* The divergence between our results obtained in transfected Jurkat cells and the ones obtained in peripheral lymphocytes suggests that the *-330* promoter polymorphism is influenced “in vivo” by factors linked to the *-330* polymorphism. The transfection study in Jurkat cells showed the effect on the luciferase gene expression of an *IL2* promoter fragment isolated from other regulatory elements. They could be located at the far upstream promoter or in the gene where several RNA stability regulatory elements are defined (Ragheb et al., 1999). This regulation could be modified by polymorphisms or methylation pattern.

In the relative allelic quantification study of healthy controls, the ratio + *114 G/T* in *-330 T/T* genotype was close to 1 (0.96) as theoretically expected. However, in MS patients bearing the same promoter genotype, this value was significantly lower than 1 (0.87). Since we compared the *-330 T/ + 114 G* with the *-330 T/ + 114 T* haplotype, the difference cannot be attributed

to sequence polymorphism at *-330*. The mechanism involved in this process is unknown but it seems to be associated with the + *114* polymorphism.

Genotype − 330	MNE *IL2* ± S.E.M.
GG ($n=5$)	577 ± 458[b]
GT ($n=9$)	1142 ± 610[c]
TT ($n=9$)	936 ± 471[d]

[a] *P* analyzed by Mann–Whitney one-sided test.
[b] *P*(GG vs. GT) = 0.03.
[c] *P*(GT vs. TT) = 0.33.
[d] *P*(TT vs. GG) = 0.03.

Table 2. Mean normalized expression (MNE) of IL2 in activated PBL[a].

In the data of + *114 G/T* relative allelic quantification for *-330 G/T* genotype, the ratio + *114 G/T* in MS patients is augmented if compare with healthy controls (0.88 vs. 0.69, *P*= 0.0057). This indicated that both haplotypes were differently affected by regulatory factors and this phenomenon ought to be distinct in cells from patients and healthy controls.

The position of this polymorphism is not at any known transcription factor consensus sequence. However, deletion studies (Williams et al., 1988) have shown that the elimination of the -361/-292 sequence reduces the reporter gene activity in Jurkat cells to 40%.

The results of this study reflect the complex regulatory mechanism of *IL2* gene expression and indicate that the influence of the *-330* polymorphism is modulated by other elements and is altered in MS that supports the importance of *IL2* in the MS. On the other hand, the contribution of different *IL2* expression levels to the MS pathogenesis should be seen in the context of a multifactorial disease, considering interactions with other susceptibility genes.

VI.2. Analysis of *IL2Ra* /CD25 locus polymorphisms in MS

Articles V and VI

Genome-wide linkage analysis in multiplex families with MS has shown potential linkage on chr 10p15 (Akesson et al, 2003), which contains the *IL2Ra/CD25* gene. The linkage and association with *IL2Ra* gene has also been described for T1D using genome-wide screens (Concannon et al., 2005) and a tag- SNP approach (Vella et al., 2005). We considered interesting to study *IL2Ra* as candidate MS susceptibility gene.

In **study V** we tested four SNPs in IL2R*a* gene regulatory regions. *The C/T IL2RA1* polymorphism is located at the first intron, at +3546 bp from the transcription initiation site, at the Positive Regulatory Region IV (PRRIV) next to NF-AT-GAS motifs (Kim et al., 2001). The *C/T IL2RA*2 is at −8335 bp of the transcription initiation start site, at the PRR CD28-responsive enhancer (Yeh et al., 2001). The *G/A ILR2RA*3 is located at splicing site donor consensus sequence of the exon 7. The *IL2RA4* SNP is located at the 3′ untranslated region +50704 bp from the initiation start site (Figure 28).

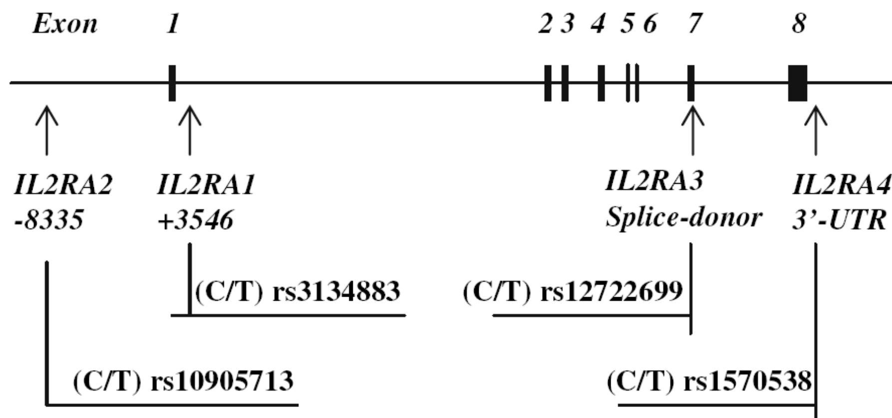


Figure 28. Scheme of the IL2RA locus with location of studied SNPs.

We found a statistically significant association for *IL2RA4* polymorphism (P=0.04 for whole group, P=0.02 for RR-MS group). It is at the 3′-untranslated region (3′-UTR), which could affect the level of the *IL2Ra* expression. This study was the first implicating *IL2Ra* gene in the susceptibility to MS.

In study VI we performed a replication and fine mapping of the *IL2RA* gene region analyzing 3 SNPs previously associated with MS and 5 SNPs associated with T1D. The aim of this study was to determine whether there is a common origin of association in the *IL2RA* gene with MS and other autoimmune diseases. A fine mapping of the *IL2RA* region in T1D has located two ancestrally distinct causal alleles that are marked by two independent groups of SNPs spanning overlapping regions of 14 and 40 kb, encompassing *IL2Ra* intron 1 and the 5' regions of *IL2Ra* and *RBM17* (Lowe et al., 2007) (SNPs 4-8). Furthermore, these authors have associated *IL2Ra* T1D susceptibility genotypes with lower circulating levels of soluble IL-2Ra ($P = 6.28 \times 10^{-28}$), biomarker of peripheral inflammation.

In **study VI** eight polymorphisms in the *IL2RA* gene were genotyped in 798 MS patient samples and 927 sex-matched Caucasian controls from the south of Spain.

Six out of eight polymorphisms exhibited allelic association with MS, with nominal P-values ranging from 0.0016 to 0.033 (Table 3). rs1570538 has been shown to be weakly associated with multiple sclerosis in our previous study (**study V**) in a cohort of 346 cases and 413 controls (allelic P-value = 0.04) and showed here increased statistical significance with our actual extended cohort (allelic association P = 0.033; OR for minor allele = 0.86, 95% CI, 0.75–0.99; genotype association (Cochran-Armitage trend test P = 0.033).

We did not find any statistically significant evidence for test (P = 0.279 and P = 0.133 for rs12722489 and rs41295061 respectively) or the Fisher exact test in genotypes (P = 0.501 and P = 0.243 for rs12722489 and rs41295061 respectively) and alleles (P = 0.278 and P = 0.135 for SNPs

SNP	Alleles, n (%)			OR minor allele [95% CI]	P-value		
		Cases	Controls		This work[a]	GWAS/MS[b]	T1D[c]
rs1570538[d]	C	734 (47.8)	851 (51.6)	0.86 [0.75–0.99]	0.033/0.04[d]		
SNP1	T	802 (52.2)	799 (48.4)				
rs2104286	T	1237 (82.3)	1426 (79.1)	0.81 [0.68–0.96]	0.017	2.16×10^{-7}	
SNP2	C	265 (17.7)	378 (20.9)				
rs12722489	G	1216 (88.6)	1186(87.2)	0.87 [0.69–1.10]	0.278	2.96×10^{-8}	0.52
SNP3	A	156 (11.4)	1742(12.7)				
rs10795791	A	933 (64.4)	1094 (60.6)	0.85 [0.74–0.98]	0.027		1.4×10^{-6}
SNP4	G	515 (35.6)	710 (39.4)				
rs4147359	G	1105 (72.6)	1228 (69.0)	0.84 [0.72–0.98]	0.023		1.7×10^{-5}
SNP5	A	417 (27.4)	552 (31.0)				
rs7090530[e]	A	741 (48.9)	996 (54.2)	1.23 [1.07–1.41]	0.003		3.6×10^{-10}
SNP6	C	767 (51.0)	840 (45.8)				
rs41295061[f]	C	1436 (94.5)	1605 (93.2)	0.80 [0.60–1.07]	0.135		2.8×10^{-12}
SNP7	A	84 (5.5)	117 (6.8)				
rs35285258[e, g]	C	921 (62.4)	1193 (67.7)	1.26 [1.09–1.46]	0.0016		5.3×10^{-4}
SNP8	T	555 (37.6)	569 (32.3)				

Table 3 Allele distribution and frequency of IL2RA SNPs in patients with MS and healthy controls

a- P-values = Pearson's goodness-of-fit chi-square (df = 1); b- GWAS/MS (IMSGC, 2007); c- T1D study (Lowe, 2007)
d- (Matesanz, 2007); e- rs7090530 and rs35285258 survived Bonferroni correction;
f- rs41295061 named ss52580101 in T1D study (Lowe, 2007); g- rs35285258 named ss52580135 in T1D study (Lowe, 2007).

rs12722489 and rs41295061 respectively). The strongest association signals in this study were observed for rs7090530 and rs35285258 which are in high LD with a pairwise $r^2 = 0.53$. rs1570538, rs2104286, rs10795791 and rs4147359, are in partial LD ranging from 0.29 to 0.09 with rs35285258. Rs12722489 and rs41295061, negative for association with MS in this study, were in very low LD with rs35285258 ($r^2 = 0.06$ and 0.033, respectively). Conditioned to rs35285258, rs7090530 did not add any significant association, neither did the other SNPs (P-values 0.2377, 0.1718, 0.5885, 0.3112, 0.4517, 0.2342 and 0.3116 for the seven SNPs respectively). Thus, the rs7090530 association with MS could be explained by its strong LD with rs35285258.

The analysis of polymorphisms in this study reflects the existence of a heterogeneous association between T1D and MS that suggest different immunopathological mechanisms. It is notable that rs41295061, the most associated with T1D, was not associated with MS in this study. However, the rs35285258, belonging to another independent group of SNPs associated with T1D, showed the maximal association in this study but different risk allele. Another example of such allelic heterogeneity has been observed at the shared AI locus *PTPN22* and *FcRL3* (Criswell et al., 2005; Smyth et al., 2008; Barrett et al., 2008). While T1D, RA and CD all show disease associations that map to the same R620W variant (rs2476601), it is the 620W variant that associates with risk to T1D and RA, but protection from CD. This allele was not associated with MS as in study from our lab as in others (Matesanz et al., 2005; Begovich et al., 2005; de Jager et al., 2006).

It is unknown whether there is any causal polymorphism in the SNPs analyzed here and whether it can affect the level of expression of the *IL2RA* product because none of these polymorphisms are located in known regulatory regions. Rs4147359 G allele and the T allele of rs10795791 create a putative recognition site for GATA-1 and GATA-2 transcription factors, respectively. Although these two SNPs did not survive the Bonferroni correction and had no effect conditioned on rs35285258, they showed a significant haplotype-specific effect. The A allele of rs7090530 disrupted a

putative CpG dinucleotide, as it does the SNP rs11597367 described in the T1D study, located at the 5′ region of the IL2RA at position 6147540 and in total LD ($r^2 = 1$) with rs35285258. Methylation of the CpG dinucleotides could be important for gene transcription regulation as it has been demonstrated for the IL2 gene (Murayama et al., 2006).

Maier and coworkers (2009) have replicated partially our study VI (Figure 29), confirming that polymorphism rs41295061 is not associated with MS and and SNP rs11594656 (pefect proxy (r^2=1) of rs35285258) is associated with MS. These authors have also shown that multiple variants independently correlated with susceptibility to MS and T1D. Unexpectedly, the minor allele at rs2104286, related with protection from both MS and T1D, was associated with decreased sIL-2R*α* levels, while, rs11594656 risk allele for MS and protective for T1D is associated with higher levels of sIL-2R*α*. Soluble IL-2Rα concentrations were found to correlate with rs11594656 genotype in quiescent SLE (Carr et al., 2009).

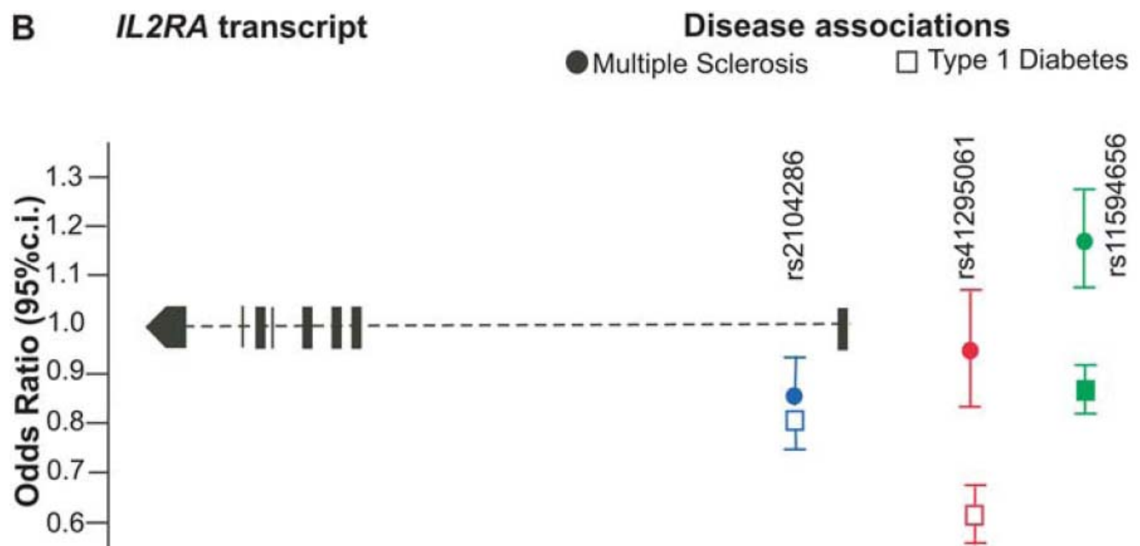


Figure 29. Association of IL2RA SNPs with MS and T1D (Maier et al., 2009).

GWA studies for both RA (Barton et al., 2008) and SLE (Harley et al., 2008) and candidate gene association studies in SLE (Carr et al., 2009) and juvenile idiopathic arthritis (Hinks et al., 2009) have also observed heterogeneity in associations at *IL2Ra* (Figure 30). Differential association of autoimmune diseases and SNPs within the *IL2RA* locus suggests that the *IL2RA* pathway may prove to play different, as yet undefined, roles in each disease.

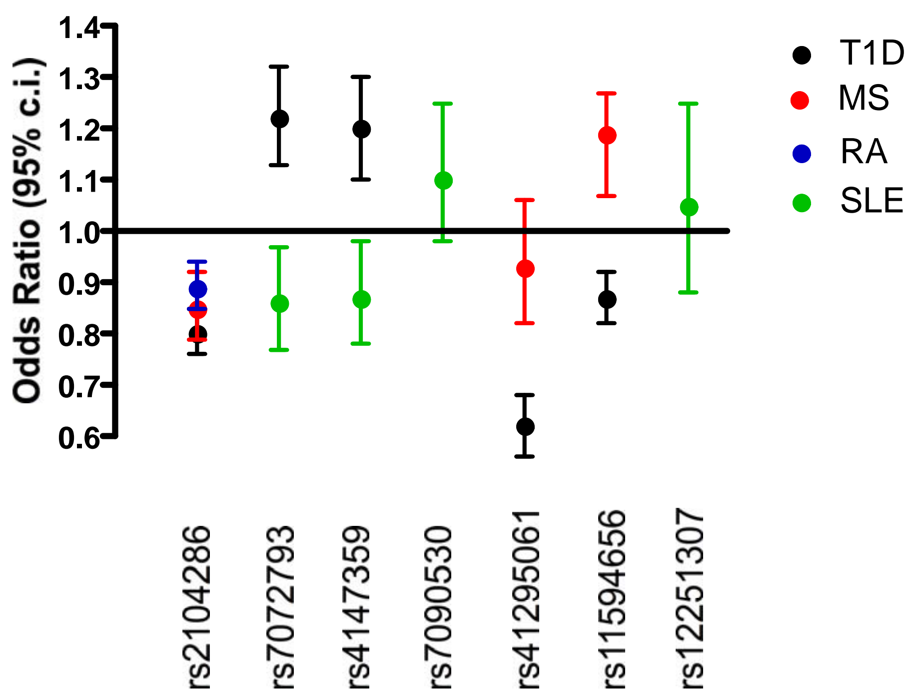


Figure 30. Comparison of *IL2RA* variants genotyped in T1D, MS, RA and SLE. Minor allele associations with disease are shown (Maier et al., 2009).

It is unknown whether there is any causal polymorphism in the SNPs analyzed and whether it can affect the level of expression of the IL2R*a* product because none of these polymorphisms are located in known regulatory regions. The analysis of polymorphisms in this study reflected the heterogeneity in T1D and MS risk alleles that suggest different immunopathological mechanisms.

VI.3. IL6 gene polymorphisms in MS

Article VII

Following the concept of shared genetic determinants between autoimmune diseases, we established as objective of our **study VII** to investigate the role of *IL6* gene in susceptibility to MS associated previously with rheumathoid arthritis (Fishman et al., 1998).

Our search for the possible effect of this polymorphism on susceptibility to MS did not show differences in the distribution of *-174 IL6* genotypes, when compared healthy controls with MS group, or stratifying according to clinical course (RRMS or SPMS), age at disease onset or gender.

Nevertheless, according to several reports, this common polymorphism at position -174 in the promoter region of the *IL-6* gene seems to have a role in a variety of diseases and conditions. After coronary artery bypass surgery, patients with the *−174 C/C* allele show higher plasma IL-6 levels than patients with the *−174G/G* allele (Brull et al., 2001). A lower frequency of the *−174C/C* genotype is observed in JCA patients than in the normal population (Fishman et al., 1998). However, the relation between *–174G/C* and circulating IL-6 is not completely consistent in the literature. Whereas several studies indicate that *–174G/C* is associated with plasma levels of IL-6, particularly in inflammatory situations (Jones, 2001; Brull, 2001), no association between *-174G/C* and circulating IL-6 was found within healthy women (Qi et al., 2006).

The *C/G* variation at position −174 is also associated with susceptibility to T1D (Jahromi et al., 2000) but no association is observed with SLE in Caucasian German patients (Linker-Israeli et al., 1999) or in IBD patients (Klein et al., 2001). *IL6* gene allelic variations and common haplotypes were related to cartilage degrading conditions (Noponen-Hietala et al., 2005; Pola et al., 2005). The polymorphism rs2069827, which resides in the 5' promoter and is in strong LD with *–174G/C*, was consistently associated with adiposity (Qi et al., 2007).

Another point of view is that IL-6 molecules seem to contribute to the development of the pain sensation and this effect can be modified by genomic variations, especially in the promoter area of the gene. The homozygous –*174G/G IL6* genotype was significantly correlated with pain scores (Oen et al., 2005), that could be of importance also in MS patology.

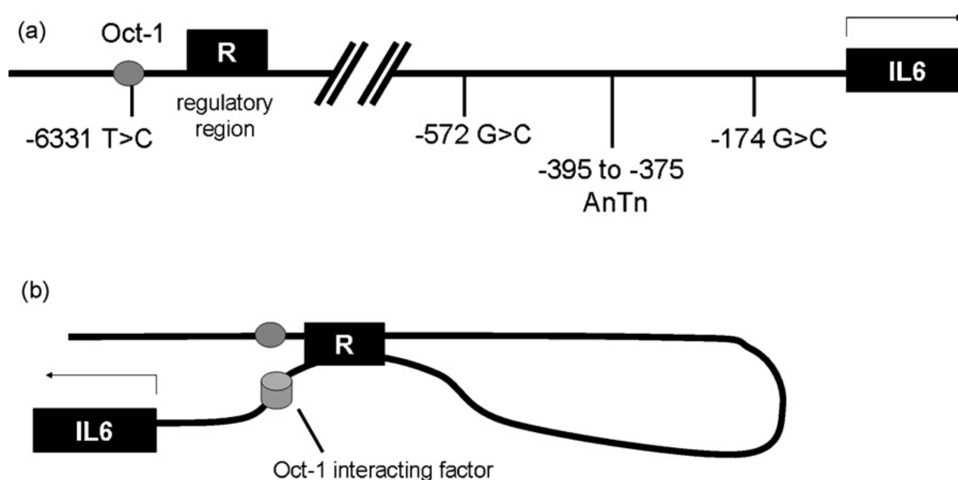


Figure 31. Map of the regulatory region of IL6.
a. IL6 −6331 T allele creates an Oct- binding site; b. Possible mechanism by which the distal promoter SNP, −6331T may regulate gene expression by binding to a proximal Oct-1-interacting factor, bringing the regulatory region in close proximity to the transcriptional machinery (Smith&Humphries, 2008)

It has been suggested that more than one polymorphic site in the *IL6* gene may be functional and influence gene transcription probably through complex interactions determined by the haplotype (Terry et al., 2000). The recent work discovered that IL-6 regulation is controlled not only by the proximal promoter, but also strongly by an element in the distal promoter -5307 to -5202 (Figure 31) (Samuel et al., 2008).

Thus, although in **study VII** we did not obtain evidence to suggest that proximal *IL6* promoter polymorphism is susceptibility factor for the development of MS; in the light of new data it would be interesting to reinvestigate the role of *IL6* gene promoter polymorphisms in MS.

VI.5. FcRL3 in MS susceptibility

Article VIII

Polymorphisms in *FCRL3* gene have been associated with several autoimmune diseases in a Japanese cohort (Kochi et al., 2005) and with different autoimmune disorders such as RA, Graves disease, Addison's disease (Owen et al., 2007) and MS Martinez et al., 2007) in Caucasian populations. The association of the *FCRL3_3* C allele with RA (Kochi et al. , 2005) has been replicated in Japanese and Canadian populations, however with diminished effect sizes compared to the initial association studies (Ikari et al., 2006;Newman et al., 2006; Thabet et al., 2007). In contrast to these findings, well powered association studies of the *FCRL3_3* C allele in Caucasian cohorts from North America, UK and Spain have failed to replicate the association of *FCRL3_3* with RA and T1D (Martínez et al., 2006, Hu et al., 2006, Turunen et al., 2006; Smyth et al., 2006).

These contradictive results evidence a complex pattern of disease association and could point to environmental impact on the susceptibility in autoimmune diseases.

In our **study VIII** we attempt to determine whether *FCRL3* is associated with susceptibility to MS. We genotyped three SNPs in 645 MS patients and 786 controls in a Caucasian population from the South of Spain and compared with the results of Martínez and coworkers (2007) recently obtained in a MS-association study in an independent Spanish cohort. The SNPs investigated in this work were chosen on the basis of maximal association oin other autoimmune diseases in previous studies. They were: a functional variant in the gene promoter at the −*169* position (FCRL3_3 (rs7528684), which alters the binding affinity of nuclear factor-kappa B and regulates *FCRL3* expression, another 5′ SNP (rs11264799) at position −*110* and a non-synonymous SNP (rs7522061) in 3rd exon.

The association of *-169 C/T FCRL3* with MS has been confirmed in this work. A combined analysis of two Spanish cohorts, using the data of Martinez and coworkers (2007) (Table 4) increased the significance of association and

revealed that the C allele of *FCRL3_3* providing "higher producer phenotype" is protective for MS, in difference to other autoimmune disease, where it is susceptible. Similary, recent study showed an association of *-169 C* allele with protection in Addison disease (Owen et al., 2007). This heterogeneity suggests different pathogenic mechanisms underlying distinct autoimmune diseases.

FcRL3_3	T vs C	(TT+TC) vs CC (Risk allele T)	TT vs (CT+CC) (Risk allele C)
OR (T allele)[a], 95% CI	1.24 (1.10–1.4)	1.4 (1.12–1.74)	0.77 (0.65–0.91)
P-value	0.0003	0.0025	0.0031

[a] OR per C allele 0.80 (IC95%)=0.71–0.90), *P*-value=0.0003.

Table 4. Combined analysis of two Spanish cohorts.

Haplotype analysis of combined data also confirmed this association with highly consistent effect sizes for haplotypes carrying the *C* allele of *FCRL3_3*. On the other hand, we cannot rule out that the susceptibility allele at *FCRL3* may lie elsewhere within the block of LD that contains the 5´ region of the *FCRL3* gene or within the adjacent *FCRL2* transcript, which could have more relevance in the MS pathology.

The asociation of *FCRL3_3* with MS has been replicated and extended in this study indicating to solid evidence for the involvement of *FCRL3* in MS susceptibility.

VI.5. IL7Ra gene in susceptibility to MS

Article IX

Validation of genetic association studies requires replication using independent data sets in order to search for functional variants relevant to

disease ethiology (Ioannidis et al., 2001). **Study IX** was designed to evaluate the *IL7Rα* gene role in susceptibility to MS in our sample collection.

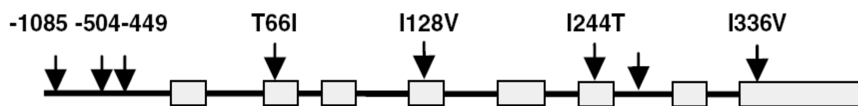
CD127 (*IL7Rα*) gene is polymorphic and its haplotypes has been recently associated with MS. Several studies, including GWAS, have provided evidence for the association of MS and the *T244I* variant of the IL7Rα, rs6897932 (IMSGC, 2007; Gregory, 2007; Lundmark et al., 2007). It is located in exon 6 increasing the skipping of this exon during splicing as was showed in transfection assays. The presence of the MS associated *C* allele resulted in a twofold increase in the skipping of exon 6 compared with transcripts containing the *T* allele. This skipping is predicted to increase production of soluble vs. membrane-bound IL-7Rα for individuals carrying the risk allele at rs6897932, which is likely to affect the IL7 signaling that again may influence the formation of Treg in the thymus. Therefore, suggesting a role for this pathway in the pathophysiology of the disease, we decided to evaluate in our MS collection the significance of *T244I* polymorphism in disease susceptibility.

Our data showed a significant overrepresentation of the rs6897932 *C* allele among cases compared with controls, thus this allele was associated with increased predisposition to MS. The combined data analysis of 3324 cases and 5032 controls of Europeans and Americans of European origin resulted in very strong association ($P = 1.9 \times 10^{-29}$). In recent GWAS by Baranzini and coworkers (2008) *IL7Rα T244I* also showed the strongest association, further supporting a functional role of rs6897932 in disease susceptibility.

This association was seen the same in both *HLA-DRB1*1501/1503* carriers and non-carriers indicating an HLA-independent effect of the *IL7R* marker (Gregory et al., 2007; Lundmark et al., 2007). In IMSGC subset, the population-attributable risk percent was 40.1% for *HLA-DRB1*1501/1503* and 16.4% for the *C/C* genotype of rs6897932, while the combined effect for these two markers was 49.6%. Similary, no interaction between *HLA-DRB1*1501* and rs6897932 could be detected in the Lundmark and coworkers (2007) investigation. At the functional level, the latter investigators reported increased expression of mRNA for *IL7Rα* and its ligand, *IL7*, in the

cerebrospinal fluid of MS patients compared with individuals with other neurological diseases. However, this was not related to the rs6897932 genotype.

***IL7Rα* gene polymorphisms**



Haplotypes

1.	G	TG	T	G	C	A	A
2.	G	TA	C	A	T	T	A
3.	T	TA	C	A	C	A	A
4.	G	CA	C	A	C	T	G

Figure 32. SNP composition of the *IL7Ra* haplotypes (McKay et al., 2008)

McKay and coworkers (2008) have demonstrated that two *IL7Ra* haplotypes, which represented SNPs in the promoter and non-synonimous polymorphisms, including *T244I*, are highly associated with the proportion of the mRNA encoding the soluble isoform of *IL7Ra*. The haplotype 1 containing associated with MS susceptibility allele *C* (Figure 32) produced more of this isoform that could reduce IL7 or TSLP signaling, favoring less T cell proliferation and survival, particularly in T cell subsets with limiting expression of CD127, while haplotype 2 produced less soluble isoform.

Our findings in Spanish cohort confirm previous reports on the association between *IL7Ra* gene and MS.

VI.6. OAS1 gene as possible candidate in MS susceptibility

Article X

The OAS1 gene encodes a protein of the 2′,5′-oligoadenylate synthase family, a group of enzymes that play an important role in innate antiviral defense. Induced by interferon-α, they catalyze the synthesis of 2′,5′-oligomers of adenosine, which consequently activates latent RNAase resulting in the degradation of viral and cellular RNA, thus inducing apoptosis of infected cells.

The minor allele (*G*) of an *OAS1* SNP (rs10774671) that alters splicing and increased enzymatic activity, was found to be associated, in a case-sibling control study, with T1D (Field et al., 2005). The *G* allale conserves the splice site and generates a p46 enzyme isoform while *A* allele ablates the splice site resulting in the generation of p48 and p52 isoforms. The association of the *OAS1* gene with T1D in a family-based study (Tessier et al., 2006) was attributed to rs3741981 (*OAS1* 3rd exon) as a more likely functional candidate than rs10774671. SNP in the 7th exon of *OAS1* gene (rs 2660), which is in almost complete LD with SNP at the exon 7th splice acceptor site (Bonnevie-Nielsen et al., 2005), was associated with the outcome of hepatitis C virus infection (Knapp et al., 2003).

In our **study X** we assessed the influence of *OAS1* gene polymorphisms in susceptibility to MS. For this purpose we chose 2 SNPs, one – A/G polymorphism at the exon 7 splice-acceptor site (rs 10774671) and another – nonsynonymous A/G SNP in 3rd exon of gene (rs 3741981), which leads to amino acid substitution (Ser162Gly).

Haplotype but not single-marker analysis revealed an association with the susceptibility to MS. We found that the haplotype *GA* created by the *G* allele at rs 10774671 and by the *A* allele at rs 3741981 was 4.6-fold more frequent in unstratified patient group with MS.

Our data are in agreement with the conclusions of Tessier and coworkers (2006) considering the studied SNPs in *OAS1* gene as merely markers for a haplotype containing a variant, or cluster of variants, that affects OAS

levels/activity and, consequently, disease susceptibility. In large scale study they confirm the results of Field and coworkers (2005) although suggesting that the splicing site SNP rs10774671 may well be part of the functional effect, but it cannot account for this effect exclusively. Because the association is in a long range LD region (Figure 33), there may be an additive effect of several functional variants. This effect appears to be modest, pending ascertainment of the maximally associated SNP in this LD block.

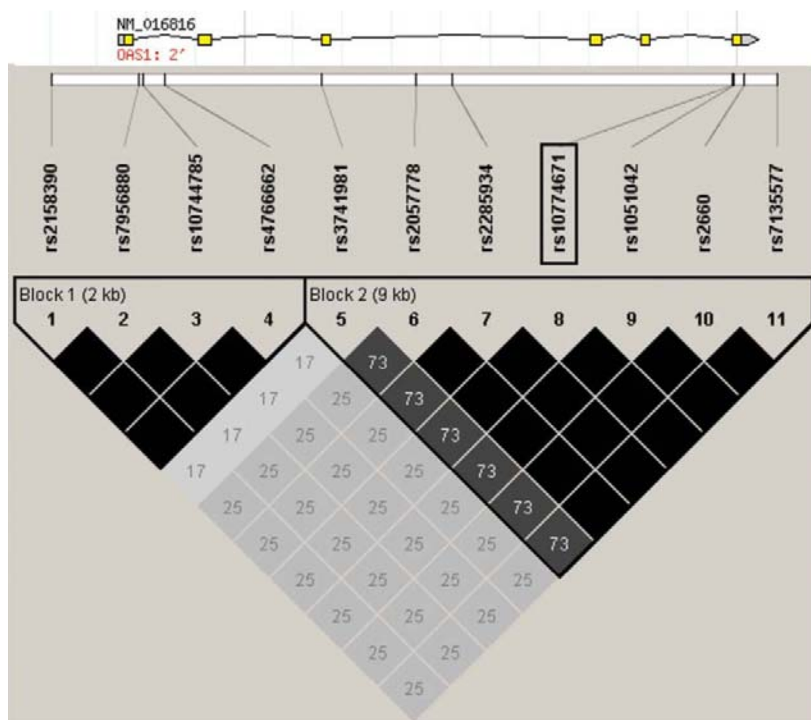


Figure 33. Haplotype block structure of the human OAS1 gene in the CEPH cohort. Pairwise r^2 values (r^2=1 in black colour) (Lim et al., 2009)

No evidence for association of *OAS1* with T1D in unaffected siblings or T1D cases was found by Smyth and coworkers (2006), although these authors have seen a possibility of an effect at or near *OAS1* in T1D and supposed very small and/or susceptible to population-specific effects,

requiring replication in extremely large population-specific studies. Nevertheless, first analysis of a cross-GWAS results database does reveal *OAS1* locus association with T1D and Crohn's disease (Johnson et al., 2009) demonstrating that previous candidate gene centered associations can be replicated via *in silico* analysis of GWAS results.

Lim and coworkers (2009) recently found that the highest virus accumulation occurred in donors with two copies of the hypofunctional rs10774671 *OAS1* variant. Their data suggest that OAS1 activity may influence the probability of initial infection, but not the severity or symptomatic quality of infection in man that is interesting from the point of view of MS ethiology/pathogenesis.

Our findings indicate that the *OAS1* gene polymorphisms may confer susceptibility to MS and suggest that OAS1 activity is involved in the etiology of the disease.

CONCLUSIONS

VII. CONCLUSIONS

1. The -330 promoter polymorphism of *IL2* gene is associated with the susceptibility to SPMS and affects the allelic expression. The regulatory effect of this polymorphism is modulated by other elements "in vivo" and the expression is altered in MS that supports the importance of *IL2* in MS.

2. The - 475 and -631 *IL2* promoter polymorphisms do not contribute to MS susceptibility.

3. Our results discarded the major influence on susceptibility to MS of the *TENR-IL2-IL21* locus SNPs associated with T1D, CD and RA but they did not exclude the role of this locus in MS progression.

4. For the first time the MS association with the *IL2RA* locus and the heterogeneity of T1D and MS risk alleles from this locus have been described, that suggests different immunopathological mechanisms of these diseases.

5. It was not obtained evidence that *IL6* promoter polymorphism is a susceptibility factor for the development of MS.

6. The functional variant *-169C* of the *FCRL3* gene promoter described as risk factor for several autoimmune diseases is a protective allele for MS.

7. *IL7Ra* rs6897932 C allele is confirmed as risk factor for MS in Spanish population.

8. Haplotype but not single-marker analysis revealed an association with MS of the *OAS1* gene involved in innate antivirus response. These data

support the implication of the IFN type I pathway in MS and suggest that *OAS1* association with MS could be related with its antiviral activity.

FUTURE PERSPECTIVES

VIII. FUTURE PERSPECTIVE OF MS GENETIC RESEARCH

With the completion of the Human Genome Project in 2003 and the International HapMap Project in 2005, researchers now have a set of research tools that make it possible to find the genetic contributions to common diseases. Three GWAS has been performed in MS. The results of GWAS in MS genome and transcriptome hold great promise for the identification of susceptibility genes. Although almost all variants seem to have modest effect sizes and, even when combined, still only very little of the genetic risk for disease can be explained (Peltonen, 2007). The GWAS are still underpowered to find small main effects. In future studies cohorts in the tens of thousands will be needed to have sufficient power to detect the majority of real but modest genetic effects. Several approaches are now taking into account to improve the capability of the GWAS to determine the genetic factor of multifactorial diseases.

The entire genome has not yet been studied by GWA approaches. Other types of polymorphisms, which include structural polymorphisms (in copy number of elements - deletion, duplication, insertion, translocation, inversion), mRNA precursors also contribute to genomic variation. Furthermore, copy number variation (CNV) in genomic regions harbouring dosage-sensitive genes may cause or predispose to a variety of human genetic diseases (Hurles et al. , 2008) (Figure 34). Several recent studies have reported an association between CNV and autoimmunity in humans such as SLE, psoriasis, Crohn's disease, RA and T1D. As an example - the recent discovery of MS association with a 5 bp insertion-deletion polymorphism in the promoter region of *IRF5* affecting the expression of this transcription factor involved both in the type I interferon and the toll-like receptor signalling pathways (Kristjansdottir et al., 2008).

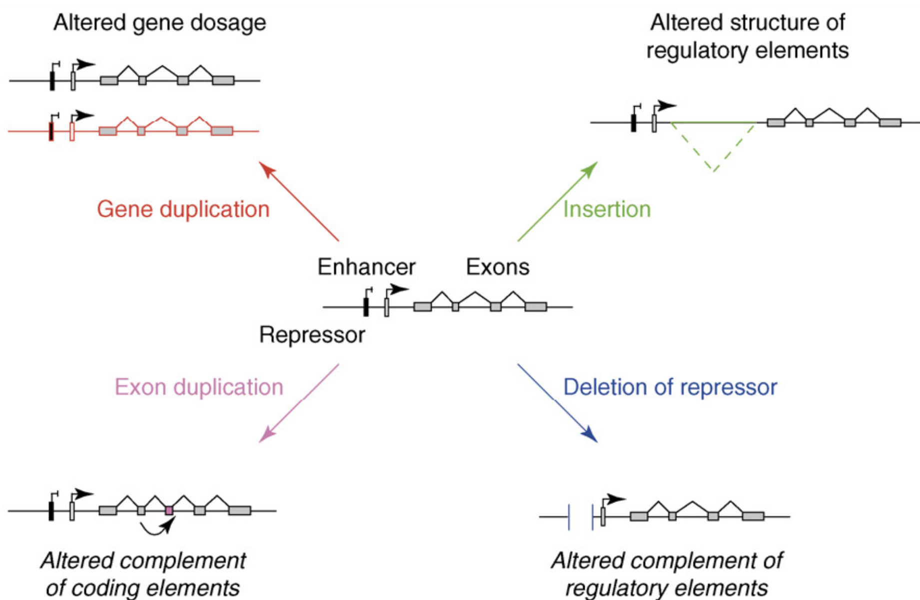


Figure 34. Influence of structural variation on gene regulation. (Hurles et al., 2008).

Recently considerations of candidate genetic loci that contribute to disease pathogenesis was extended to include the SNP variants residing within boundaries of genes encoding microRNAs and SNPs within microRNA-target sites in mRNAs. Most recent analysis of relationships between structural features and gene expression patterns of disease-linked SNPs, microRNAs and mRNAs of protein-coding genes in association to phenotypes of 15 major human disorders supports the idea that variations in DNA sequences associated with multiple human diseases may affect phenotypes in trans via non-protein-coding RNA intermediaries interfering with functions of microRNAs (Glinsky, 2008).

Many of the causal variants for the genes that have been discovered have not yet been identified. The associated variants identified in the GWAS might only be imperfectly correlated with the causal variants and, therefore, their genetic effect on disease susceptibility might be underestimated. Even some genes, for which a causal variant seems to be already identified, probably

contain additional as-yet unidentified causal variants. A complex challenge in nearest future will be to select the most promising genetic results and to design effective functional studies that will connect genetic variation with disease physiology (Lettre & Rioux, 2008). The identification of causal variants will require re-sequencing efforts of large sample sizes.

Genome-wide pharmacogenomics studies will be very important to address the question of genetic heterogeneity in MS by analysis of the correlation between different genotypes and the clinical response to therapy given the significant variation in response to currently available treatments (Oksenberg et al., 2008).

The methods for analysis of associations need to be improved with emphasis on systematic evaluations of genes in disease susceptibility. It is possible that single locus methods do not reflect the correct underlying model of association and the data might currently not be analyzed to its full potential. As one example, recently developed multilocus association testing method (localized haplotype clustering) had better success detecting disease associated variants than a single-marker analysis of imputed HapMap SNPs (Browning & Browning, 2008). A pathway analysis approach characterizing the polygenic basis has been recently proposed to identify major contributors to disease susceptibility, multiple rare or low-penetrance variations and interacting environmental factors (Torkamani et al., 2008). Such analysis and interpretation provide a new perspective on how large genomic data set information can be processed to implicate the role of biologic pathways that would otherwise be left unrecognized.

The analysis of GWAS results has shown that complete explanations for disease heritability unachieved without the consideration of all factors contributing to disease risk as genetic, epistasis, gene–environment, epigenetic, epigenetic–environment. There is growing evidence that such interactions contribute to complex diseases rather than single genes.

Epistasis, or interactions between genes and, in more wide understanding, also between genes and environment, has long been recognized as fundamentally important to understanding the structure and

function of genetic pathways. The expectation is that modest success of GWAS studies in identifying MS-causing genes and phenotypic variation, will be improved at least in part, as a consequence of searching for epistatic interaction. Several models for epistasis have been developed (Marchini et al., 2005) and new methods for discovering such interections appear continuously, which employ different approaches.

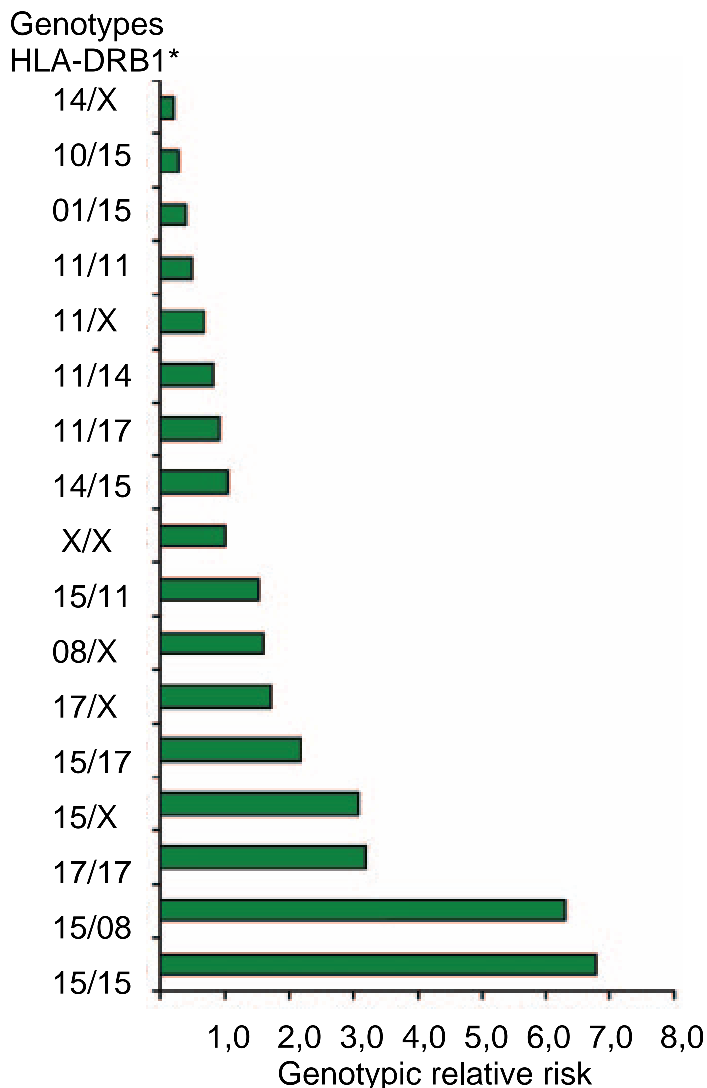


Figure 35. Genotypic relative risks for MS for combinations of alleles at the HLA-DRB1 locus (Ramagopalan & Ebers, 2009).

It has been proposed an evaluation of the joint effects of regulatory and proteincoding variants to genome-wide expression phenotypes that could be useful for global survey of epistatic effects in humans; it involves prioritisation of variants to be tested in order to highlight an underappreciated angle of functional variation (Dimas et al., 2008).

MS well illustrates the gene–gene interaction in complex disease. As one example, several epistatic interactions have been documented for the main association in the major histocompatibility complex where *HLADRB1*15* haplotypes exert the single strongest effect on MS risk. A genotype rather than allele- or haplotype-dependent hierarchy described (Figure 35). At an individual level this has been grouped into three types - dominant negative, synergistic and epistasis that influence clinical phenotype (Ramagopalan & Ebers, 2009). Understanding this epistatic interaction could be critical for treatment or prevention.

The relatively small results of GWAS efforts also may suggest that other mechanisms than disparities in the DNA sequence may be involved in MS pathophysiology. In the last decade, there have been several findings implying the significance of epigenetic modifications as causal factors for multifactorial diseases like MS. Genome-wide epigenetic modification plays a pivotal role in regulating gene expression through chromatin structure and stability, tissue-specific and embryonic developmental specific gene regulation, and genomic imprinting. DNA methylation and histone modifications, two of the major chromatin remodeling processes, also serve to integrate environmental signals for the cells to modulate the functional output of their genome. A failure to maintain epigenetic homeostasis in the immune response leads to aberrant gene expression, contributing to immune dysfunction and in some cases the development of AI in genetically predisposed individuals. Epigenetic factors might be affecting MS induction and clinical severity by modulating such diverse biological processes as X chromosome inactivation, viral infections, myelin protein production and Th1 type immune response differentiation (Kürtüncü & Tüsün, 2008).

Epigenetic studies of MS and other related disorders are still in its early stage, nevertheless the emerging epigenetic tools, complementing classical genetic research, will be the ultimate method of choice to explore the molecular causes underling the development of complex diseases. Understanding how small molecules interact with the epigenome will allow designing in future a new generation of epigenetic drugs for curing complex diseases (Liu L et al., 2008).

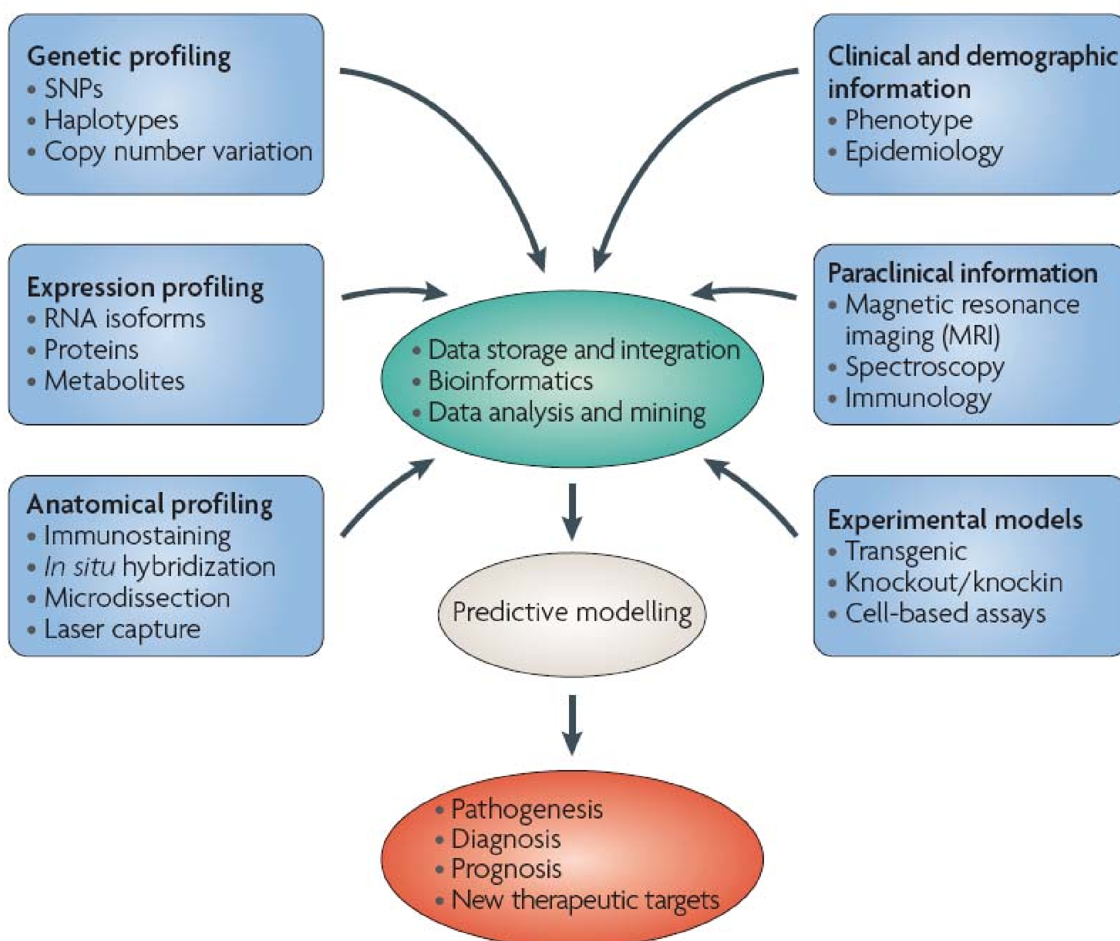


Figure 36. Systems-level analysis of MS (Oksenberg et al., 2008)

The combination of approaches and the integration of knowledge from genomic, transcriptional, proteomic and clinical sources will be necessary to make a real progress in understanding of MS genetics. Due to accumulated knowledges on the redundancy and gene–gene interactions of cellular and molecular pathways, the future genetic studies of MS ought to change the single gene strategy to an integrative approach (Figure 36).

The analysis of the gene or protein networks themselves rather that their individual elements will provide the most important functional and quantitative information for the development of increasingly accurate predictions of differential diagnosis, treatment outcome, drug-associated toxicity and disease prognosis.

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IX. REFERENCES

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ADDITIONAL PUBLICATIONS

X. ADDITIONAL PUBLICATIONS

Fedetz M, Matesanz F, Cáliz R, Ferrer MA, Collado MD, Alcina A, Martín J. Lack of association between –384 and 114 *il-2* gene polymorphisms and rheumatoid arthritis. J Rheumatol 2003; 30: 435–7.

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Lack of Association Between –384 and 114 *IL-2* Gene Polymorphisms and Rheumatoid Arthritis

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ABSTRACT. ***Objective***. To investigate the association of 2 single nucleotide polymorphisms (SNP) at positions –384 and 114 in the human interleukin 2 (*IL-2*) gene with susceptibility to and severity of rheumatoid arthritis (RA).

***Methods*.** Genotyping for these *IL-2* variants was performed by a polymerase chain reaction restriction fragment length polymorphism method in 174 RA patients and 153 control individuals.

Results. No statistically significant differences were observed when the –384 and 114 *IL-2* genotype distributions between RA patients and healthy controls were compared. In addition, no association was found between the *IL-2* genotypes with any demographic and clinical variables tested.

Conclusion. Our results provide no evidence for genetic association conferred by the –384 and 114 *IL-2* SNP with respect to susceptibility and severity of RA. (J Rheumatol 2003;30:435–7)

Key Indexing Terms:
INTERLEUKIN 2 GENE PROMOTER SUSCEPTIBILITY
SINGLE NUCLEOTIDE POLYMORPHISM RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology characterized by chronic synovial inflammation in multiple joints with progressive loss of articular cartilage and bone destruction. Twin and family studies provide evidence to support the involvement of both genetic and environmental factors in the etiopathogenesis of RA[1]. So far, HLA class II genes on chromosome 6 are the only loci that have been confidently proved to be associated with RA susceptibility. Despite all these associations, HLA genes account for only about one-third of the genetic predisposition to the disease, indicating that genes outside the HLA region also contribute to the disease[2].

Attractive candidates for additional susceptibility or progression factors are cytokine genes. Cytokines are important mediators of the immune and inflammatory response and play an important role in the pathophysiology of joint inflammation and destruction in RA[3].

Interleukin 2 (IL-2) is produced by activated T cells and has a powerful immunoregulatory effect on a variety of immune cells[4]. Dysregulation of the IL-2/IL-2 receptor system could lead to functional or pathological alterations in the immune system including autoimmunity[5]. Although great discrepancies in IL-2 mRNA and protein measurements have been observed between different studies[6,7], it is generally accepted that spontaneous IL-2 mRNA expression is decreased in peripheral blood and synovial tissue and in fluid of RA patients compared with healthy controls[8,9]. One possible explanation for the differences observed in IL-2 concentrations between RA patients and controls is variation in the 5' promoter region of the *IL-2* gene.

The occurrence of common features of autoimmune diseases and the co-association of multiple autoimmune diseases in the same individual or family suggests the existence of common genetic factors that predispose to autoimmunity[10]. Moreover, analyzing the results of 23 autoimmune or immune mediated disease genome-wide scans revealed non-random clustering of susceptibility loci between different human autoimmune diseases[11]. Further, studies in the IDDM and EAE NOD mouse model showed evidence of linkage to *IL-2*; *Idd3* diabetes resistant gene was co-localized with the EAE-resistant gene in a genetic interval less than 0.15 cM containing *IL-2* gene[12].

These considerations suggest that *IL-2* is a strong candidate in autoimmune diseases. We investigated the contribution of the 2 recently described variations in the *IL-2* locus, one of them located in the promoter region of the gene, to the susceptibility and/or severity of RA.

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Supported by grants SAF00-213 from CICYT, FIS01/3149 and PM 99/0101 from MCYT. Dr. Fedetz is the recipient of an AECI Fellowship.

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Submitted March 28, 2002; revision accepted August 30, 2002.

MATERIALS AND METHODS

Study participants. The subjects enrolled in this study included 174 Spanish patients with RA and 153 healthy volunteer blood donors from the Granada area (southern Spain). The patients were from the Rheumatology Department of the Virgen de las Nieves Hospital in Granada, Spain. All patients were

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diagnosed according to the revised criteria of the American College of Rheumatology[13]. Information on their clinical and demographic variables and on HLA typing has been published[14].

Detection of IL-2 gene polymorphisms. The *IL-2* promoter –384 mutation and the 114 polymorphism were analyzed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) as described[15]. Briefly, for the –384 polymorphism determination, a 131 bp fragment was amplified using the following primers: forward GTGATAGCTCTAATTCATGC and reverse ATTCACATGTTCAGTGTAGTTCT; the amplified product was digested with the enzyme *Bfa* I that recognizes the G allele. For detection of the 114 polymorphism a 262 bp fragment was obtained using the primers: forward ATGTACAGGATGCAACTCCT and reverse TGGTGAGTTTGGGATTCTTG; digestion of the PCR product with the restriction enzyme *Mwo* I produces 2 fragments of 111 and 151 bp from the 114-G allele.

Statistical analysis. For association studies, *p* values were calculated by chi-square method or Fisher's exact test when appropriate. SPSS Version 10.0 software was used to analyze the data. For nonparametric data analysis, Mann-Whitney U test was used for ordinal variables and Fisher's exact test for dichotomous variables.

RESULTS

The distribution of the –384 and 114 *IL-2* genotypes and alleles in RA patients and controls is shown in Table 1. The study population was found to be in Hardy-Weinberg equilibrium. As reported[15], the –384 and 114 *IL-2* variants were in linkage disequilibrium, since we found 6 different genotype combinations of the 9 theoretically possible. No statistically significant differences were observed when the –384 and 114 *IL-2* genotype distributions between RA patients and controls were compared, suggesting that these *IL-2* polymorphisms do not influence susceptibility to RA. Next, to investigate a possible association of the *IL-2* polymorphisms with disease severity, we analyzed demographic and clinical characteristics of RA patients according to their *IL-2* genotypes, and no associations were found with any of the variables tested (data not shown).

DISCUSSION

Many studies have examined the relationship between cytokine gene polymorphism and susceptibility to and clinical severity of diseases[16,17]. This is the first investigation of an association of *IL-2* polymorphisms at position –384 and 114 with RA, and our results provide no evidence for genetic association conferred by these single nucleotide polymorphisms with respect to susceptibility and severity of RA.

Significant differences in allele frequencies have been reported between ethnically diverse populations regarding cytokine gene polymorphism[18,19], suggesting that polymorphisms within cytokine genes may be responsible for the ethnic-based differences in the susceptibility to a variety of diseases, although this theory has not been confirmed. Similarly, the –384 *IL-2* genotype frequencies in our control population show few or no differences from the reported distribution among Caucasians and were different from African-American subjects[19,20]. These differences in the *IL-2* genotype frequencies observed in different populations may exist as a result of the selective characteristics of different infectious diseases.

In addition to the *IL-2* polymorphism analyzed in this study, several other *IL-2* polymorphisms have been reported that are located in different regions of the *IL-2* locus[21,22]. The dinucleotide repeat polymorphism in the 3' untranslated portion of the *IL-2* gene has been reported to be associated with a subset of RA patients and with ulcerative colitis, but not with juvenile rheumatoid arthritis[23,24]. It is worth noting that a recent genome-wide screen in multiplex RA revealed that the microsatellite marker D4S1647 had the most significant linkage with RA outside the HLA region[25]. Of interest, the microsatellite D4S1647 is situated in the chromosome 4q25, whereas *IL-2* gene is located in the region 4q26–27. Therefore, it would be of interest to investigate the possible existence of other polymorphisms in both the promoter and the coding region of the *IL-2* gene and to test them for potential involvement in the genetic predisposition to RA. On the other hand, the biology of IL-2 is also modulated by the IL-2 receptor, and it is possible that *IL-2* receptor polymorphism, individually or in combination with *IL-2* gene polymorphism, may also be important in the pathogenesis of RA.

Our results appear to rule out the relevance of –384 and 114 *IL-2* polymorphisms in the susceptibility to or severity of RA, although the participation of IL-2 protein in the immunopathogenesis of RA is not brought into question by these data.

Table 1. Distribution of the IL-2 –384 and 114 genotypes and alleles in RA patients and healthy controls.

IL-2	RA Patients, n = 174		Controls, n = 153	
	n	%	n	%
–384 Genotypes				
G/G	19	10.9	14	9.1
G/T	65	37.4	50	32.7
T/T	90	51.7	89	58.2
–384 Alleles				
G	103	29.6	78	25.5
T	245	70.4	228	74.5
114 Genotypes				
G/G	74	42.5	71	46.4
G/T	83	47.7	63	41.2
T/T	17	9.8	19	12.4
114 Alleles				
G	231	66.4	205	67
T	117	33.6	101	33

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Journal of Neuroimmunology 163 (2005) 165–171

Journal of Neuroimmunology

www.elsevier.com/locate/jneuroim

IFNAR1 and *IFNAR2* polymorphisms confer susceptibility to multiple sclerosis but not to interferon-beta treatment response

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Received 23 November 2004; accepted 18 February 2005

Abstract

We investigated the role of three polymorphisms in the *IFNAR1* (SNPs 18417 and -408) and *IFNAR2* (SNP 11876) genes in multiple sclerosis (MS) susceptibility and in the IFNβ treatment response in a group of 147 patients and 210 controls undergoing interferon therapy during the last 2 years. Only the 18417 and the 11876 SNPs showed an association with disease susceptibility ($p = 0.001$ and 0.035, respectively) although no differential genotype distribution were observed between interferon responders and non-responder MS patients. No alteration of the expression level of IFNAR-1 was observed with respect to the −408 genotypes or to interferon treatment response. These data suggest a role for the IFNAR pathway in susceptibility to MS.
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Keywords: Type I interferon receptor; Polymorphism; Multiple sclerosis; Association; Treatment

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory–demyelinating disease characterized by multifocal damage of the central nervous system (CNS) and caused presumably by an autoimmune process that induces demyelination, destruction of oligodendrocytes, and axonal injury (Trapp et al., 1998).

IFNAR1 and *IFNAR2* encode two subunits of the heterodimeric type I interferon receptor (IFNAR) and are important because interferon-beta (IFNβ) is known to exert its biological activities by the interaction with these subunits. In MS controlled clinical trials, IFNβ therapy has been shown to decrease clinical relapses, reduce brain MRI activity and possibly slow progression of disability (McCormack and Scott, 2004). However, a long-term shift in the natural history of the disease has not been demonstrated (IFNβ Multiple Sclerosis Study Group, 1993; PRISMS study group, 1998; European Study Group on Interferon β-1b in secondary progressive MS, 1998). A significant number of patients are refractory to interferon therapy. MS lymphocytes tend not to be responsive to type I IFN actions and, in some circumstances, circulating IFN may not transduce a signal via IFNAR. Abnormalities ranging from the transmembrane receptor genes *IFNAR1* and *IFNAR2*, interactions between both chains of the receptor, cytoplasmic

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doi:10.1016/j.jneuroim.2005.02.010

proteins associated with this heterodimer, or the ultimate complex that interacts with IFN sensitive response elements in the nucleus, may be responsible for IFN resistance (Brod, 1998). The mechanisms by which IFNβ exerts its disease-modifying effect are not completely understood, but among other activities, IFNβ is known to reduce T cell activation, inhibit IFNγ effects and blood–brain barrier leakage, and induces an immune deviation, either by inhibiting Th-1 or by promoting Th-2 cytokine production (Dhib-Jalbut, 2002). The biological responses of IFNβ are initiated by the interaction of this cytokine with its cell surface heterodimer receptor (IFNAR), shared by all type 1 interferons which include about 15 cytokines (13 isotypes of IFNα one IFNβ, one IFNω), (Novick et al., 1994). This binding brings together two receptor chains, IFNAR1 and IFNAR2 (Croze et al., 1996), whose interaction induces a cascade of signalling pathways resulting in the secretion or production of a number of proteins called IFN-stimulated gene products with antiviral, antiproliferative and immunomodulatory activities (Dhib-Jalbut, 2002; Yang et al., 2000). These subunits are encoded by two different genes located on chromosome 21q (Lutfalla et al., 1992), where several polymorphisms have been described (McInnis et al., 1991; Muldoon et al., 2001; Sriram et al., 2003). Two of these polymorphisms induce amino acid substitutions in the mature proteins (*IFNAR1* 18417 [G→C] V168L and *IFNAR2* 11876 [T→G] F10V) and the third is located at the *IFNAR1* promoter region. The object of this study was to assess the role of these cytokine receptor subunits *IFNAR1* and *IFNAR2* polymorphisms in the genetic control of MS and address the pharmacogenomic impact on IFNβ treated patients.

2. Materials and methods

2.1. Study subjects

The study included 147 patients with clinically defined MS according to Poser's criteria (Poser et al., 1983) (100 relapsing–remitting and 47 secondary progressive), on IFNβ therapy: 42 received IFNβ1b subcutaneously (Betaferon®, Schering) at 250 μg/48 h, 47 IFNβ1a intramuscularly (Avonex®, Biogen) at 30 μg/week, and 58 IFNß1a subcutaneously three times weekly (Rebif®, Serono), 53 with 22 μg and 5 with 44 μg. Criteria to classify patients as non-responders to IFNβ were: an increment of one or more relapses with respect to the previous year or an increase in EDSS score of 0.5 points or more after the first year of treatment. Demographic characteristics of MS patients and controls are shown in Table 1. A control group of 210 healthy subjects with similar genetic background was included in the study. Studies were performed after obtaining written informed consent from all participants under protocols approved by the Institutional Review Board of the Hospital Carlos Haya and Blood bank of Málaga, Spain. All the patients and controls were Caucasians from Malaga and Granada, Spain.

Table 1
Baseline demographic characteristics of MS patients and controls

	RR MS $n=100$	SP MS $n=47$	Controls $n=210$
Age, mean (range)	36.22 (17–57)	40.8 (24–65)	34.84 (18–62)
Females (%)	71	63.8	45.71
Duration of MS mean (median)	9.18 (7.0)	12.73 (11.0)	
EDSS score mean (median)	1.98 (2.0)	4.49 (4.0)	

RR, relapsing–remitting MS; SP, secondary progressive MS.

2.2. PCR amplification and restriction fragment length polymorphisms (RFLP)

The DNA was extracted by standard procedures (Ausubel et al., 1990). PCR reactions were carried out in a final volume of 25 μl, in a mixture of 5 pM of each primer, 50 ng of genomic DNA, 250 μM dNTPs and 1 U *Taq* DNA polymerase in the 10× KCl buffer provided by the manufacturer (Roche). For the *IFNAR1* SNP 18417 determination, each DNA sample was PCR amplified with forward primer IP18 (AGAAGTACATTTAGAAGCTG) and reverse primer IP19 (CAATCCTTTCCTATAACA-CAA), yielding a band of 261 bp. The digestion of PCR amplified products with *Dde*I restriction enzyme (Roche) produced two fragments of 155 and 106 bp from the SNP 18417-G allele product.

The *IFNAR2* SNP 11876 was amplified with forward primer IP20 (5′-TCACCTAATGTTGATTTCAG-3′) and reverse primer IP21 (5′-ATCACAGCTTGCTTCTATAA-3′), yielding a band of 158 bp, whose digestion with *Mbo*II (Fermentas) produced two fragments of 110 and 48 bp for the SNP 11876-G allele product and three fragments of 83, 48 and 27 bp from the SNP 11876-T allele product.

Polymorphism of the -408 C/T SNP involved amplification of a 328 bp fragment with primers forward IP22 (5′-TCTCGCCCCTCAGCCAAGTC-3′) and reverse IP23 (5′-CCTTGACCTTCACAGGATCG-3′). The digestion of PCR amplificates with *MvaI* produced two fragments of 204 and 124 bp for the T allele, and three fragments of 124, 106 and 98 bp for C allele. The fragments from each digestion were separated on 12% polyacrilamide gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light.

2.3. Expression of IFNAR1 mRNA

Briefly, total RNA was isolated from peripheral blood mononuclear cells (PBMC) using a classical method (Chomczynsky and Sacchi, 1987) and was reverse-transcribed to cDNA with MMLV reverse transcriptase. *IFNAR1* and a low number copy housekeeping gene porphobilinogen deaminase (*PBGD*) (Chretien et al., 1988) mRNA expression was measured by real time QT-PCR in a LightCycler.

The following primers (Proligo, France), designed using OLIGO6.0 software (Medprobe, Sweden), were used in the reactions: *IFNAR1* forward 5′-AGAAGTACATTTAGAAG-CTG-3′ and *IFNAR1* reverse 5′-AGTGCTGCTTTAACTTT-3′, PBGD forward 5′-TCCAAGCGGAGCCATGTCTG-3′ and PBGD reverse 5′-AGAATCTTGTCCCCTGTGGTG-GA-3′. Reaction mixtures contained 1X LightCycler-Fast Start DNA Master SYBR green I (Roche, Spain), 3 mM MgCl2, 0.2 mM forward and reverse primer and 20 ng of cDNA. PCR reactions were set up in a total volume of 20 μl, in duplicate, and each run included its standard curve. Data evaluation was performed using the LightCycler data analysis software (version 3.5).

IFNAR1–mRNA expression in PBMC was assessed as the *IFNAR1*–mRNA/*PBGD*–mRNA ratio. *PBGD* was used to verify comparability of RNA loading between samples and to normalise PCR products.

2.4. Statistical analysis

Comparisons of genotype frequencies between healthy controls and MS patients were performed by Pearson χ^2 test for the independent variables, using the SPSS 11.5 statistical package. Comparisons between genotypes for SNP 18417, SNP 11876 and SNP-408 polymorphisms were performed by Pearson χ^2 test for the categorical variables of gender, clinical form and response to IFNβ treatment, and by Student-*t*, Mann–Whitney or Kruskal–Wallis tests for the quantitative variables of age at onset of MS, disease duration, EDSS score at entry and *IFNAR1* mRNA expression. We performed haplotype frequency estimation, taking into account *IFNAR1* 18417 and *IFNAR2* 11876 SNPs, and tested for differences between case and controls and responders and non-responders to IFNβ treatment using the FAMHAP software (Becker and Knapp, 2004).

3. Results

3.1. Influence of the polymorphisms in IFNAR1 and IFNAR2 genes on the response to IFNβ in MS patients

We analysed three recently described IFNAR polymorphisms by RFLP as is shown in Fig. 1. One located within the promoter region of *IFNAR1* gene, at -408 bp relative to the transcription start site and another two substitutive polymorphisms, one located in the fourth exon, the *IFNAR1*–V168L and the other in the second exon, the *IFNAR2*–F10V. The patients were classified as responders and non-responders to IFNβ according to the criteria indicated in Materials and methods section which is different from those previously published (Waubant et al., 2003; Villoslada et al., 2004). Thus, 104 patients (70.7%) responded to INFβ and 43 (29.3%) did not. Interestingly, non-responders patients had a longer disease evolution and a higher EDSS score at baseline than responders. Genotype

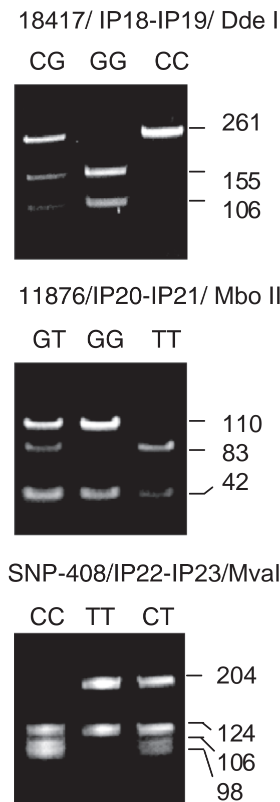

Fig. 1. Detection of *IFNAR1* and *IFNAR2* polymorphisms in samples of MS patients for case–control association analysis. The three panels are ethidium bromide stained polyacrylamide gels of the PCR-restriction enzyme digestion products of DNA from different individuals. On the top of each gel, the polymorphism identification, primer numbers and restriction enzyme used for the RFLP analysis are indicated. The sizes of the resulting bands for each genotype are indicated at the right side.

and allelic distribution of the *IFNAR1* and *IFNAR2* polymorphisms between responders and non-responder to IFNβ are shown in Table 2. No significant associations were observed with any of the genotypes or alleles studied for the *IFNAR1* SNP 18417, SNP-408 of and SNP 11876 in exon 2 of *IFNAR2*, based on the response to IFNβ.

3.2. IFNAR1 and IFNAR2 SNPs association with MS susceptibility

No significant association of *IFNAR1*-408 genotype distribution, allele and carrier frequencies between MS cases and controls were observed. The most common genotype for this polymorphism was the homozygous (C/C) (55.5% of total subjects) followed by the heterozygous (C/T) (32.8% of total subjects) as reflected in Table 3, in agreement with previous findings (Muldoon et al., 2001).

Significant differences on *IFNAR1* 18417 genotype and allele frequencies were found by χ^2 analysis in MS cases and controls ($p=0.001$ and $p<0.001$, respectively). A significant increase in the C allele carrier frequency in MS patients (OR = 1.87, $p=0.008$) was also observed. Viewed as a positive risk factor, the *IFNAR1* 18417 C/C genotype

Table 2
Genotype and allele distribution of the *IFNAR1* and *IFNAR2* polymorphisms in responders and non-responders to IFNβ

Polymorphism		Responders $n=104$ (70.7)	Non-responders $n=43$ (29.3)
IFNAR1 18417	C/C	12 (11.5)	4 (9.3)
	C/G	33 (31.7)	13 (30.2)
	G/G	59 (56.7)	26 (60.5)
	C	57 (27.4)	21 (24.4)
	G	151 (72.6)	65 (75.6)
IFNAR1-408	C/C	63 (60.6)	28 (65.1)
	C/T	28 (26.9)	12 (27.9)
	T/T	13 (12.5)	3 (7)
	C	154 (74.1)	68 (79.1)
	T	54 (25.9)	18 (20.9)
IFNAR2 11876	G/G	16 (15.4)	5 (11.6)
	G/T	43 (41.3)	19 (44.2)
	T/T	45 (43.3)	19 (44.2)
	G	75 (36.1)	29 (33.7)
	T	133 (63.9)	57 (66.3)

p-values >0.05 and therefore are non-significant; *n*, number of subjects genotyped; percentages shown in parenthesis.

was associated with a fivefold increased risk of MS in subjects from the south of Spain ($\chi^2_{df1}=9.81$, $p=0.002$; OR = 5.008, 95% CI 1.79–13.99) compared with carriage of the *IFNAR1* 18417G allele (Table 3).

The most common genotype for *IFNAR2* 11876 was the homozygous (T/T) (47.05% of total subjects), followed by the heterozygous (G/T) (43.41% of total subjects). Genotype frequencies at *IFNAR2* 11876 differed between MS patients and controls ($p=0.035$). Carriage of the SNP 11876 T allele was inversely associated with MS (OR = 0.396, $p=0.017$). Viewed as a positive risk factor, the *IFNAR2* 11876 G/G genotype was associated with a twofold increased risk of MS in Spanish subjects ($\chi^2_{df1}=5.67$, $p=0.017$; OR = 2.53, 95% CI 1.22–5.22,) compared with carriage of the *IFNAR2* 11876T allele (Table 3). No significant associations in the distribution of neither of these genotypes in relation with the sex ratio, clinical form, age at onset of disease, disease duration, and disease progression were observed (data not shown).

The results of the Hardy–Weinberg Equilibrium (HWE) calculations for MS patients and controls population revealed that all polymorphisms were in HWE with the exception of the polymorphisms -408. This polymorphism showed a heterozygote deficit only in the MS population, with a χ^2 value of 10.26.

3.3. Haplotype analysis and interaction between IFNAR1 18417 and IFNAR2 11876 alleles

Potential genetic interactions between the two subunits of the heterodimer receptor were assessed by performing an analysis that included the *IFNAR1* 18417 and *IFNAR2* 11876 SNPs. The frequency of these genotype combinations is shown in Table 4. Significant differences in the distribution of the nine genotype combinations were found between patients and controls by the χ^2 test.

Haplotypes were analysed using the *IFNAR1* 18417 and *IFNAR2* 11876 data to identify those associations with susceptibility to MS. As shown in Table 5, the haplotype *IFNAR1* 18417G/*IFNAR2* 11876T was found to be less frequent in MS patients (0.48) than in controls (0.63). The simulation based test performed by FAMHAP software for the haplotypes resulted in *p* value of 0.0002 for MS versus control population. However, haplotype frequencies were not significantly different between responders and non-responders to the IFNβ therapy (data not shown).

Table 3
Genotype, allele and carrier allele distribution of *IFNAR1* and *IFNAR2* polymorphisms in MS patients and healthy controls

		MS $n=147$	Controls $n=210$	
IFNAR1 18417	C/C	16 (10.9)	5 (2.4)	$\chi^2_{df2}=14.18$ $p=0.001$
	C/G	46 (31.3)	54 (25.7)	
	G/G	85 (57.8)	151 (71.9)	
	C	78 (26.5)	64 (15.2)	$\chi^2_{df1}=13.14$ $p<0.001$
	G	216 (73.5)	356 (84.8)	
	G+	131 (89.1)	205 (98)	$\chi^2_{df1}=9.81$ $p=0.002$
	G−	16 (10.8)	5 (2)	OR = 5.008 95% CI = 1.79–13.99
IFNAR1-408	C/C	91 (61.9)	107 (51)	$\chi^2_{df2}=4.40$ $p=0.111$
	C/T	40 (27.2)	77 (36.7)	
	T/T	16 (10.9)	26 (12.4)	
	C	222 (75.5)	291 (69.3)	$\chi^2_{df1}=3.01$ $p=0.083$
	T	72 (24.5)	129 (30.7)	
	C+	131 (89.1)	184 (87.6)	$\chi^2_{df1}=0.07$ $p=0.791$
	C−	16 (10.9)	26 (12.4)	
IFNAR2 11876	G/G	21 (14.3)	13 (6.2)	$\chi^2_{df2}=6.70$ $p=0.035$
	G/T	62 (42.2)	93 (44.3)	
	T/T	64 (43.5)	104 (49.5)	
	G	104 (35.4)	119 (28.3)	$\chi^2_{df1}=3.67$ $p=0.055$
	T	190 (64.6)	301 (71.7)	
	G+	83 (56.4)	106 (50.5)	$\chi^2_{df1}=5.67$ $p=0.017$
	G−	64 (43.6)	104 (49.5)	OR = 2.53 95% CI = 1.22–5.22

n, number of subjects genotyped; percentages shown in parenthesis.

Table 4
Genotype combination distribution of *IFNAR1* 18417 and *IFNAR2* 11876 polymorphisms in MS patients and controls

Polymorphisms 18417–11876	MS $n=147$	Controls $n=210$
C/C–G/G	1 (0.7)	0
C/C–G/T	8 (5.4)	2 (1)
C/C–T/T	7 (4.8)	3 (1.4)
C/G–G/G	6 (4.1)	4 (1.9)
C/G–G/T	23 (15.6)	28 (13.3)
C/G–T/T	17 (11.6)	22 (10.5)
G/G–G/G	14 (9.5)	9 (4.3)
G/G–G/T	32 (21.8)	63 (30)
G/G–T/T	39 (26.5)	79 (37.6)
$\chi^2_{\rm df8}=22.063$ and $p=0.005$		

n, number of subjects genotyped; percentages shown in parenthesis.

3.4. Expression levels of IFNAR1 in PBMCs from MS patients and relation with the IFNAR1 expression pattern to genotypes

Though *IFNAR1*-408 SNP did not map to any recognised transcription factor binding sites in the promoter (Muldoon et al., 2001), we tested the possible impact of this polymorphic site on the regulation of *IFNAR1* transcription levels in 113 MS patients (34 C/T, 66 C/C and 13 T/T). As even modest doses of systemic IFNβ are known to induce a significant decrease of IFNAR1 cell surface expression (Dupont et al., 2002), blood was drawn immediately before the administration of IFNβ to ensure a time lapse of 48 h following the last dose, and minimize variations in *IFNAR1* mRNA expression due to IFNβ treatment. There was a great individual heterogeneity in *IFNAR1* mRNA expression. In the patients investigated here, we detected no significant differences in the expression at the RNA level among the different genotypes studied for *IFNAR1*-408 when analyzed by the Kruskal–Wallis test ($\chi^2_{\rm df2}=0.530$ and $p=0.767$) (Fig. 2). Also no significant difference was observed at *IFNAR1* transcriptional level between MS patients who responded to IFNβ treatment and those who did not (Fig. 3), when assessed by the Mann–Whitney test ($p=0.362$).

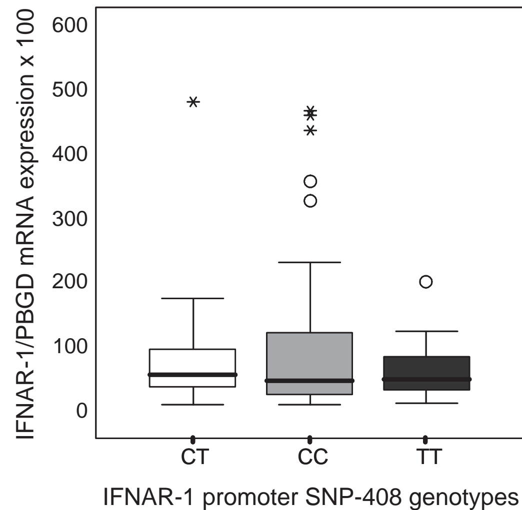

Fig. 2. Box plot showing the difference in *IFNAR1* gene expression between MS patients with different genotypes at the -408 promoter polymorphism.

4. Discussion

This study was aimed to determine the role of *IFNAR1* and *IFNAR2* genetic heterogeneity in the susceptibility to MS, their correlation with the positive or negative response to IFNβ immunotherapy and the effect on the *IFNAR1* expression. We have found that genomic variations in *IFNAR1* and *IFNAR2* genes were not relevant to the IFNβ therapy responsiveness in accordance with a previous report (Sriram et al., 2003). To improve fidelity of predicting therapeutic response to IFNβ it would be useful to assess additional genes of proteins involved in the IFNβ signalling cascade. On the other hand, this is the first study to report an association between allelic variation at *IFNAR1* 18417 and *IFNAR2* 11876 and MS. The *IFNAR1* 18417 C/C genotype was associated with a fivefold higher risk of MS than carriage of the G allele, and the *IFNAR2* 11876 G/G genotype was associated with more than a twofold risk of this disease than carriage of the T allele. Interestingly, in cerebral malaria, a complication of *Plasmodium falciparum* infection characterized by a reversible encephalopathy, a similar association of the *IFNAR1* 18417 G/G genotype to the reduction risk of suffering this complication, compared

Table 5
Haplotype frequency estimation for MS and control population obtained with FANHAP software

Haplotype 18417–11876	MS	Controls
C–G	0.092558	0.052990
C–T	0.169347	0.095336
G–G	0.254381	0.214953
G–T	0.483714	0.634329
Global $\chi^2_{\rm df3}$ 19.7490 $p=0.0002$		

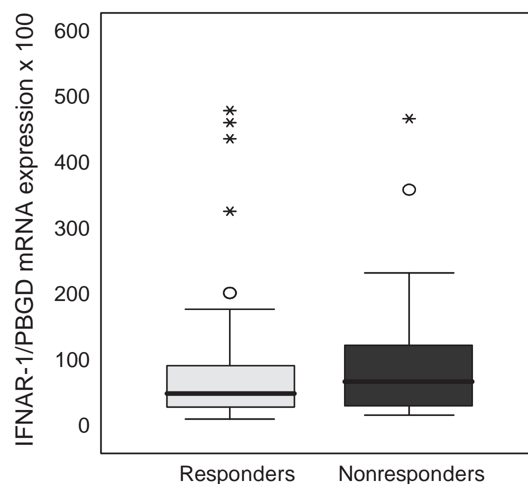

Fig. 3. Box plot showing the difference in *IFNAR1* mRNA expression between 104 IFNβ responders and 43 non-responder patients.

with individuals sharing at least one C allele, has been described (Aucan et al., 2003; Maneerat et al., 1999). Several cytokines associated with cerebral malaria have also been associated with the MS. Irrespective of the initiating stimuli, both pathologies seem to share some characteristics of inflammation at the brain that could explain the confluence of susceptibility genes in both diseases, that not only affect *IFNAR* genes but *IL10*, *IFNG* and *TNFA* genes (He et al., 1998; de Jong et al., 2002; Martinez et al., 2004; Takahashi et al., 2003).

The *IFNAR* receptors are localised on a cluster of immune response genes on chromosome 21q22.11 containing *IFNAR1*, *IFNAR2*, *IL10RB* and *IFRGR2*. Therefore, the association of the *IFNAR* polymorphisms to MS could be due to functional effects on the molecule or linkage to polymorphisms of other members of the cluster. The functional significance of these associations is unclear. However, in the IFNAR1 chain, the variation of Val to Leu at position 168 of the peptide sequence, increasing hydrophobicity of the amino acid residue, could alter the binding affinity of the molecule for the ligand. This polymorphic amino acid position is located at the extra-cellular region at the subdomain 2 (SD2) which together with SD3 seems to constitute the core of the ligand-binding determinants of IFNAR1 (Kumaran et al., 2000; Cutrone and Langer, 2001).

A C/T polymorphism at position -408 in the promoter region of the *IFNAR1* gene was also used as a marker to test for an association with MS finding no significant allelic association with disease. Therefore, our work does not provide evidence in favour of *IFNAR1*-408 as a candidate for conferring genetic susceptibility to, or protection against, MS in the South of Spain. Furthermore, this polymorphism did not show any correlation with the transcriptional levels of *IFNAR1* on MS patients nor with response to IFNβ treatment. However, the patients included in our study have been treated with IFNβ for at least 2 years. IFNβ therapy could lead to down-regulation of *IFNAR1* expression in responders but fail to do it in non-responders, probably due to a primary defect in production of *IFNAR1* transcripts (Massirer et al., 2004). Therefore, because of this problem we cannot rule out the possibility that this promoter polymorphism affects the transcription level of *IFNAR1* and *IFNAR2* gene. In summary, this study has demonstrated an association between two *IFNAR1* and *IFNAR2* polymorphisms and multiple sclerosis suggesting a role for these receptors/cytokines and their signalling pathway in MS pathogenesis.

Acknowledgements

This work was supported in part by Grants FIS 02/0671, CP03/00042 to L. Leyva, FIS 02/0698 to O. Fernandez, FIS 02/0920 to A. Alcina and 01/3149 to F. Matesanz. M. Fedetz is a holder of a fellowship from Hospital Carlos Haya Foundation.

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Journal of Neurovirology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713669475

High susceptibility of a human oligodendroglial cell line to herpes simplex type 1 infection

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Online Publication Date: 01 April 2005

To cite this Article Bello-Morales, Raquel, Fedetz, María, Alcina, Antonio, Tabarés, Enrique and López-Guerrero, José Antonio(2005)'High susceptibility of a human oligodendroglial cell line to herpes simplex type 1 infection',Journal of Neurovirology,11:2,190 — 198

To link to this Article: DOI: 10.1080/13550280590924179

URL: http://dx.doi.org/10.1080/13550280590924179

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Journal of NeuroVirology, 11: 190–198, 2005
© 2005 Journal of NeuroVirology
ISSN: 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280590924179

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More than 20 infectious agents, ranging from retroviruses to mycobacteria, have been associated with multiple sclerosis onset or relapses in which oligodendrocytes, the myelin-forming cells of the central nervous system, are the initial target of the pathogenic status. In this work, the nature of the susceptibility of the human precursor oligodendroglial KG-1C cell line to herpes simplex virus type 1 (HSV-1) was investigated. Infection of KG-1C cells was characterized by a high level of virus production and a notable progression of the cytopathic effect. After infection, there was a significant shut-off of host mRNA translation, which was correlated with evident synthesis of viral proteins. An examination by electron microscopy of the infected cells revealed the presence of large clusters of mitochondria located in the proximity of intracellular HSV-1 particle groups. In addition, transmission electron microscopy and nuclear fluorescence analysis showed neither signs of chromatin condensation nor of apoptotic bodies. Furthermore, procaspase-3 remained uncleaved, suggesting that apoptosis does not take place, at least in this system. Finally, expression and localization of MAL2, a subpopulation of detergent-insoluble lipid raft protein, was studied. Detection of MAL2 significantly increased after infection and it was colocalized with HSV-1 proteins. From these findings the authors conclude that human oligodendrocyte-like cells are highly susceptible to HSV-1 infection. The implications of this for central nervous system viral infection are discussed. *Journal of NeuroVirology* (2005) **11,** 190–198.

Keywords: herpes virus; HSV-1; infection; KG-1C; MAL2; oligodendrocyte

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Introduction

Herpes simplex virus type 1 (HSV-1) is an important neurotropic virus that can infect peripheral sensory neurons and, by retrograde axonal transport, reach the central nervous system (CNS) (Immergluck *et al*, 1998). A latent infection can be established once HSV-1 gains access to the neurons in sensory ganglia (Kramer *et al*, 2003). Periodically, HSV-1 emerges to cause acute infection and, in this circumstance, the virus could be responsible for a variety of disease states, such as encephalitis and some chronic and progressive neurodegenerative disorders (Itzhaki *et al*, 1998; Qiu and Abdel-Meguid, 1999). Nevertheless, little is known about the mechanisms underlying these processes of latency and reactivation.

In addition to the acute neurological disease resulting from the infection of the nervous system, HSV-1, as well as other members of the *Herpesviridae* family of viruses, has been included in models of virus-induced demyelination (Tsunoda and Fujinami, 2002; Kastrukoff and Kim, 2002; Fazakerley and

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The authors are indebted to M.T. Rejas and M. Guerra for their help in the preparation and analysis of samples for electron microscopy, to C. Sánchez and R. Ramos for their help with fluorescence analysis, to Y. Uezono for the kind gift of the KG-1C cells, and to M.A. Alonso and M.C. de Marco for helpful discussions. This work was supported by grants PM99/0101 from the Ministerio de Ciencia y Tecnología and PI020920 from the Fondo de Investigación Sanitaria and the Junta de Andalucía. M. Fedetz is a fellow of the Fundación Carlos Haya.

Received 29 March 2004; revised 30 July 2004; accepted 6 December 2004.

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Walker, 2003). There is molecular evidence of the involvement of HSV-1 in clinical acute attacks of multiple sclerosis (MS) (Ferrante et al, 2000). In this study, the prevalence of some herpesviruses in the peripheral blood of MS patients revealed that HSV-1 DNA was only found in acute MS, whereas both stable MS and healthy controls remained negative. Despite these recent data, HSV-1 infection in oligodendrocytes and oligodendrocyte-derived cell lines has yet to be completely characterized.

Differences in susceptibility to HSV-1 among oligodendroglia derived from different sources have been investigated in mice (Thomas *et al*, 1997) and humans (Kastrukoff and Kim, 2002). These differences, which were determined after viral adsorption but prior to the expression of immediate-early genes, were donor dependent and suggest differences in virus–host cell interactions that are probably determined by genetic factors (Thomas *et al*, 1997).

Traditionally, the inhibition of apoptosis has been recognized as one of the major hallmarks of HSV-1 infections (Derfuss and Meinl, 2002; for review see Goodkin *et al*, 2004). Nevertheless, programmed cellular death mechanisms have been implicated in recent investigations about the pathogenesis of herpes simplex encephalitis (HSE) (DeBiasi *et al*, 2002; Perkins *et al*, 2003). In fact, neuronal and glial apoptosis were clearly detected in acute HSE. This apoptosis induction was due to direct viral injury instead of inflammatory T-cell response (DeBiasi et al, 2002). Additionally, and in contrast to what occurs in human fibroblasts, antigen presentation by major histocompatibility complex class I molecules is not blocked after HSV-1 infection of human T-cell lines. Moreover, viral infection results in apoptosis of antiviral T cells (Raftery *et al*, 1999), which seems to be a mechanism of viral immune evasion.

In the present report, we address the characterization of the infection of a human oligodendroglial cell line by HSV-1. The human immature glioma cell line KG-1C, consisting of undifferentiated glial cells, is shown to be highly susceptible to HSV-1 infection. We examine whether this infection takes place through an apoptotic mechanism. Finally, we propose the involvement of a newly described raft protein of the MAL family in the infection of this human cell line.

Results

Susceptibility of KG-1C cells to HSV-1 infection

The human oligodendroglial KG-1C cell line was infected with HSV-1 (F strain) at a multiplicity of infection (m.o.i.) of 1 (plaque-forming unit [PFU] per cell). Infectious virus production was assayed over a time course. Figure 1 shows that virus production (measured as the log of total PFU) was similar to that observed in the highly susceptible Vero cell line. Twenty h post infection (p.i.), HSV-1 infected

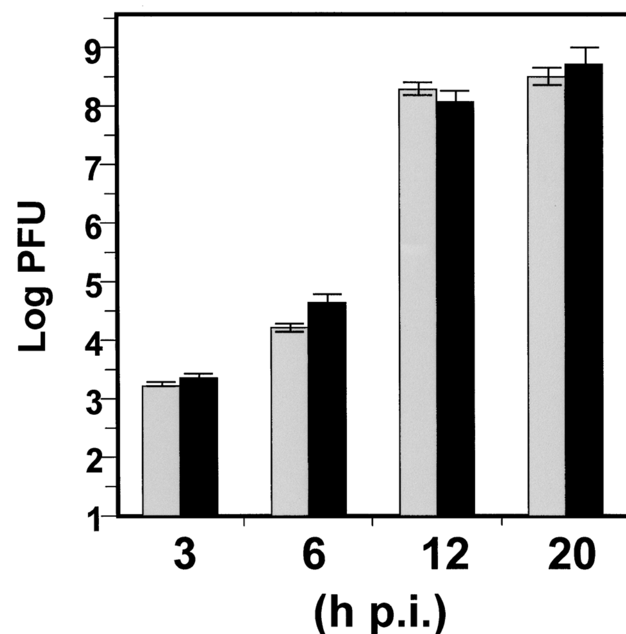

Figure 1 Susceptibility of KG-1C cells to HSV-1 infection. A total of 10^7 Vero (*gray bars*) or KG-1C (*black bars*) cells were infected with HSV-1 at 1 PFU per cell. After the indicated time, cells were harvested and extracts titrated for PFU production. Each value represents the mean of six samples; error bars indicate the standard deviations of the means.

more than 90% of the cells in both KG-1C and Vero cell cultures (data not shown). Comparable productivity between KG-1C and Vero cells was obtained after infection at lower (0.1) and higher (10) m.o.i. (data not shown). In addition, the cytopathic effect (CPE) could be noted from 20 h p.i. onwards (Figure 2A). The kinetics of protein synthesis in KG-1C cultures after HSV-1 infection showed that viral proteins were clearly detectable after 6 h p.i. by means of polyacrylamide gel electrophoresis (PAGE) analysis, in accordance with the production of infectious viral particles, as shown in Figure 1 (Figure 2B). HSV-1–induced shut-off was detectable from 12 h p.i., and most synthesized proteins were viral. Similar results were obtained when the accumulation of HSV-1 proteins was analyzed by Western blot using an anti-HSV-1–specific polyclonal antibody, providing further evidence of the viral nature of the proteins detected by PAGE analysis (Figure 2C).

Morphological and molecular changes in HSV-1–infected KG-1C cells

Modulation of apoptosis by HSV-1 infection has been extensively described (Derfuss and Meinl, 2002; DeBiasi *et al*, 2002; Perkins *et al*, 2003; Goodkin *et al*, 2004; Irie *et al*, 2004). Although HSV-1 possesses a battery of viral proteins involved in apoptosis inhibition (Derfuss and Meinl, 2002; Goodkin *et al*, 2004; Bloom, 2004), induction of this programmed cell death after CNS infection has also been

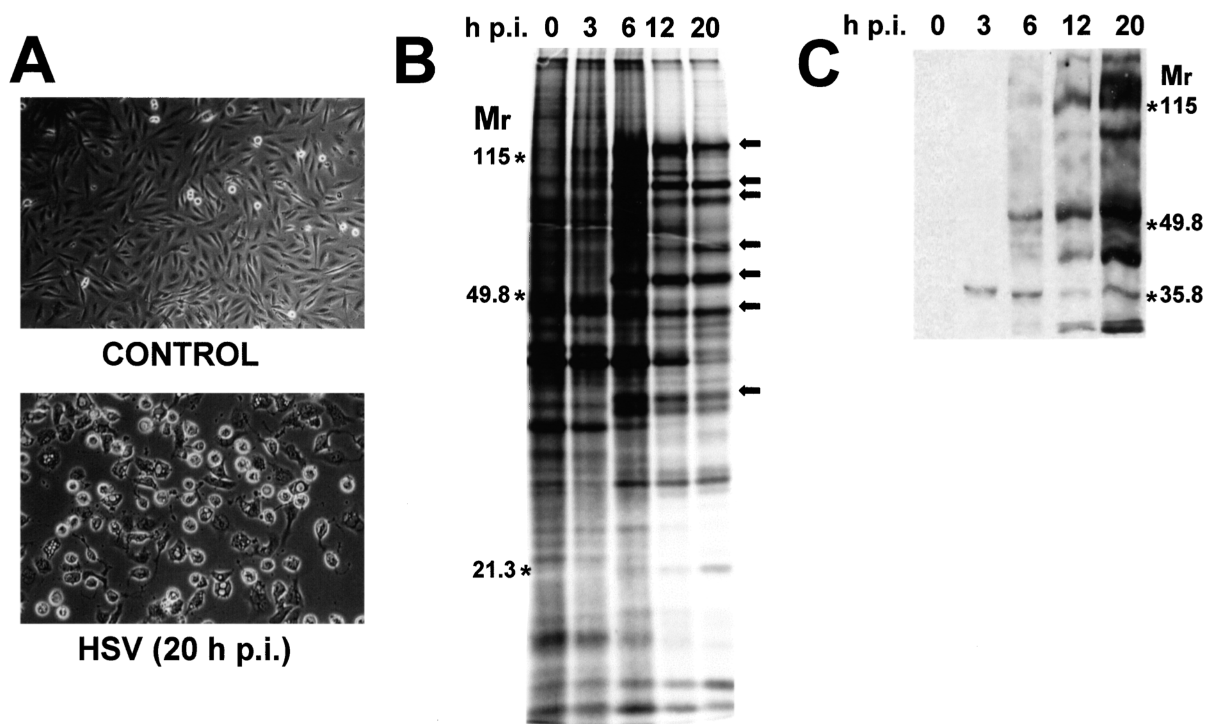

Figure 2 Productive infection of KG-1C cell line by HSV-1. Cultures were mock-infected or infected with HSV-1 at 10 PFU per cell. (**A**) Cytopathology of infected cells was visualized by phase-contrast microscopy. (**B**) SDS-PAGE analysis of ^{35}S proteins from HSV-1–infected and noninfected cells was carried out at the indicated times postinfection. (**C**) sixty microgram of total cell extracts were analyzed at the indicated times postinfection by Western blot using a polyclonal anti-HSV-1 antibody.

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observed (DeBiasi *et al*, 2002; Perkins *et al*, 2003). In the current study, we investigated whether destruction of the oligodendroglial KG-1C cell line by HSV-1 could be due to an apoptosis pathway. For this purpose, nuclear fluorescence staining and the activation of procaspase-3 by means of Western blot was analyzed in HSV-1–infected KG-1C cultures. In our system, no signs of programmed cell death could be detected (Figure 3A and B), suggesting that infection of these glial cells by HSV-1 probably correspond to a necrotizing mechanism. In addition, no chromatin condensation or nuclear budding was produced (Figure 4). Internucleosomal DNA fragmentation assays were also sterile to detect apoptosis induction (data not shown). However, examination of infected cells by electron microscopy produced unexpected results (Figure 4). At 12 h p.i., most viral particles were located outside the nucleus. KG-1C cells infected with HSV-1 featured large clusters of mitochondria located in the proximity of cytoplasmic viral particles. This effect was clearly observed from 12 h p.i. and remained unchanged 8 h later (Figure 4, 2 and 3). Further investigation should determine the function of these large clusters of respiring organelles during the final stretch of the infection's course.

Finally, a recent report has shown that some viral proteins can interact with detergent-insoluble lipid rafts in HSV-1–infected cells (Lee *et al*, 2003). The vhs viral protein appears to be associated with rafts enriched in a cytoplasmic fraction with HSV particles (Lee *et al*, 2003). Of the raft-associated proteins so far studied, MAL2, a novel member of the MAL family (De Marco *et al*, 2002; Wilson *et al*, 2001), has recently been identified as an essential component of the cellular machinery for transcytosis. This molecule is present in specialized polarized cells, but at present it has been detected only in hepatocytes or epithelial cultures. As in the case of oligodendroglial cells, KG-1C was described as bipolar cells (Miyake, 1979). Therefore, we wondered whether MAL2 is expressed in this cell line and if HSV-1 infection could affect such expression. To this end, we used the species-specificity of the monoclonal 9D1 antibody to the human MAL2 protein. Fluorescence microscopy revealed basal expression of MAL2 in KG-1C cells (Figure 5). However, HSV-1 infection induced a significant increase of MAL2 accumulation. Double-label immunofluorescence of MAL2 and HSV-1 proteins (Figure 5) and preliminary confocal microscopy analysis (data not shown) suggested the possibility of colocalization of both components. The precise cellular distribution of this new member of the MAL family is currently being investigated. Finally, two controls of antibody specificity were used, allowing this result to be correctly interpreted (Figure 5, two bottom panels).

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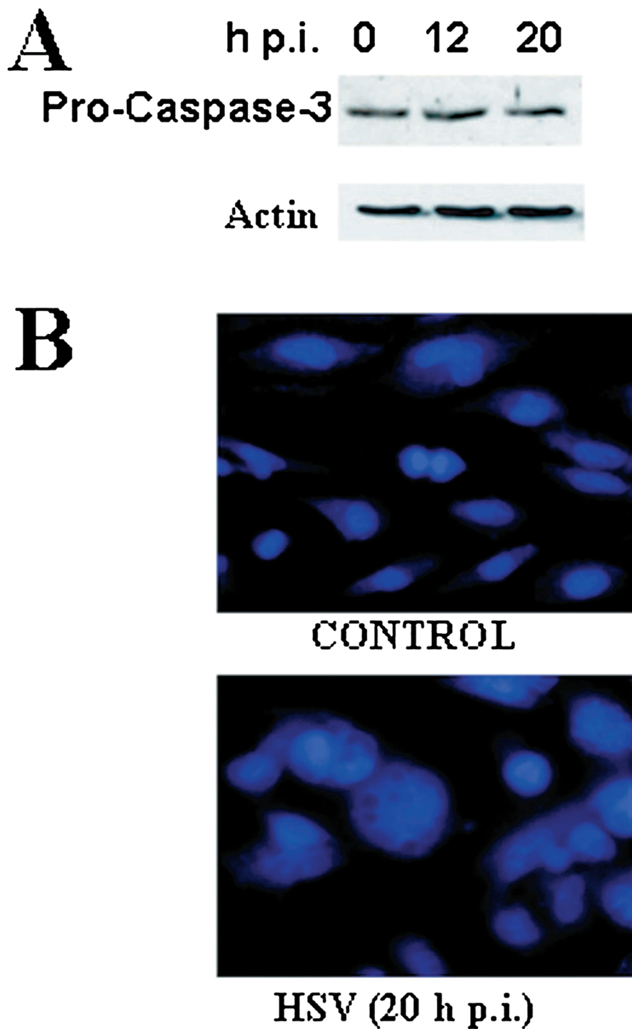

Figure 3 Effect of HSV-1 infection on apoptosis induction in KG-1C cells. Cell cultures were infected for the times indicated in Figure 2. (**A**) Aliquots of 60 μg of protein were analyzed by Western blot with a polyclonal anti-caspase-3 antibody. The band corresponds to full-length (32 kDa) proenzyme. A control with polyclonal anti-actin antibodies is shown. (**B**) Nuclear fluorescence analysis was carried out by staining with DAPI solution and fluorescence microscopy observation.

Discussion

Herein we have determined the level of susceptibility of the human precursor oligodendroglial KG-1C cell line (Uezono *et al*, 1998; Tatewaki *et al*, 1997; Miyake, 1979) to HSV-1 infection. HSV-1 is a neurotropic virus able to induce acute and/or latent infection in peripheral and central nervous systems (Baringer and Pisani, 1994; Schmutzhard, 2001). Nevertheless, although HSV-1 can infect neurons, oligodendrocytes, astrocytes, and microglial cells (Tsunoda and Fujinami, 2002; Kastrukoff and Kim, 2002; Fazakerley and Walker, 2003), and persists in the CNS indefinitely, its role in some chronic degenerative diseases, such as MS, remains a matter for speculation (Ferrante *et al*, 2000). In this sense, herpesvirus 6, another member of the *Herpesviridae* family, has been strongly associated with MS (Challoner *et al*, 1995; reviewed by Berti *et al*, 2000). Although it seems not to be absolutely necessary (Tsunoda and Fujinami, 2002), correlation between neurotropic virus infection and demyelinating diseases could be associated with the viral capacity to infect oligodendrocytes.

In our case, the great susceptibility of KG-1C to HSV-1 and their viral productivity were comparable to that observed in the classically studied Vero cell line (Marconi *et al*, 1996). To date, few publications have reported the study of human oligodendrocyte cell-line infection. Nevertheless, primary cultures of these cells were established from six different donors (Kastrukoff and Kim, 2002). Cultures of oligodendrocytes from donors differed in their susceptibility to HSV-1. Furthermore, it seems that resistance to HSV-1 of human oligodendrocytes could be genetically determined, which may be crucial to the development of CNS infection. Similar results, obtained by the same group, were previously reported with different strains of mice (Thomas *et al*, 1991, 1997). Differences in susceptibility, independent of the immune surveillance, may contribute to virus spread through the CNS and their possibility of demyelinating disease induction. Taking this into account, the molecular characterization of HSV-1 infection of oligodendrocytes or oligodendrocyte-like cells is clearly important.

Having observed the drastic effect on KG-1C cells provoked by HSV-1 infection, we wondered whether activation of an apoptotic mechanism could be involved. Numerous studies have confirmed the capacity of HSV infection to modulate apoptosis with both pro- and antiapoptotic effects (Derfuss and Meinl, 2002; Goodkin *et al*, 2004). Focusing on the CNS, HSV-1 infection has classically been described as a necrotizing process, but significant advances in the past decade have changed this perspective (DeBiasi *et al*, 2002; Perkins *et al*, 2003; Hunsperger and Wilcox, 2003). Apoptotic neurons and glia were detected in significant numbers in acute HSV-1–induced encephalitis (DeBiasi et al, 2002). In fact, a more recent report has associated the apoptotic component involved in viral encephalitis with activation of c-Jun and c-Jun N-terminal kinase (Perkins *et al*, 2003). Furthermore, activation of the proapoptotic proteolytic enzyme, caspase-3, seems to be crucial in both latent HSV-1 reactivation (Hunsperger and Wilcox, 2003) and encephalitic processes (Perkins *et al*, 2003). However, data presented here suggest that, at least in our system, HSV-1–induced CPE is not due to an apoptotic pathway. These results again suggest that the mechanism underlying HSV-1 infection of CNS-derived cells may be complex and differ from one cell type to another.

An important hallmark of apoptosis induction is the condensation of the nuclear chromatin. Consistent with the results presented above, no signal of chromatin condensation could be detected in

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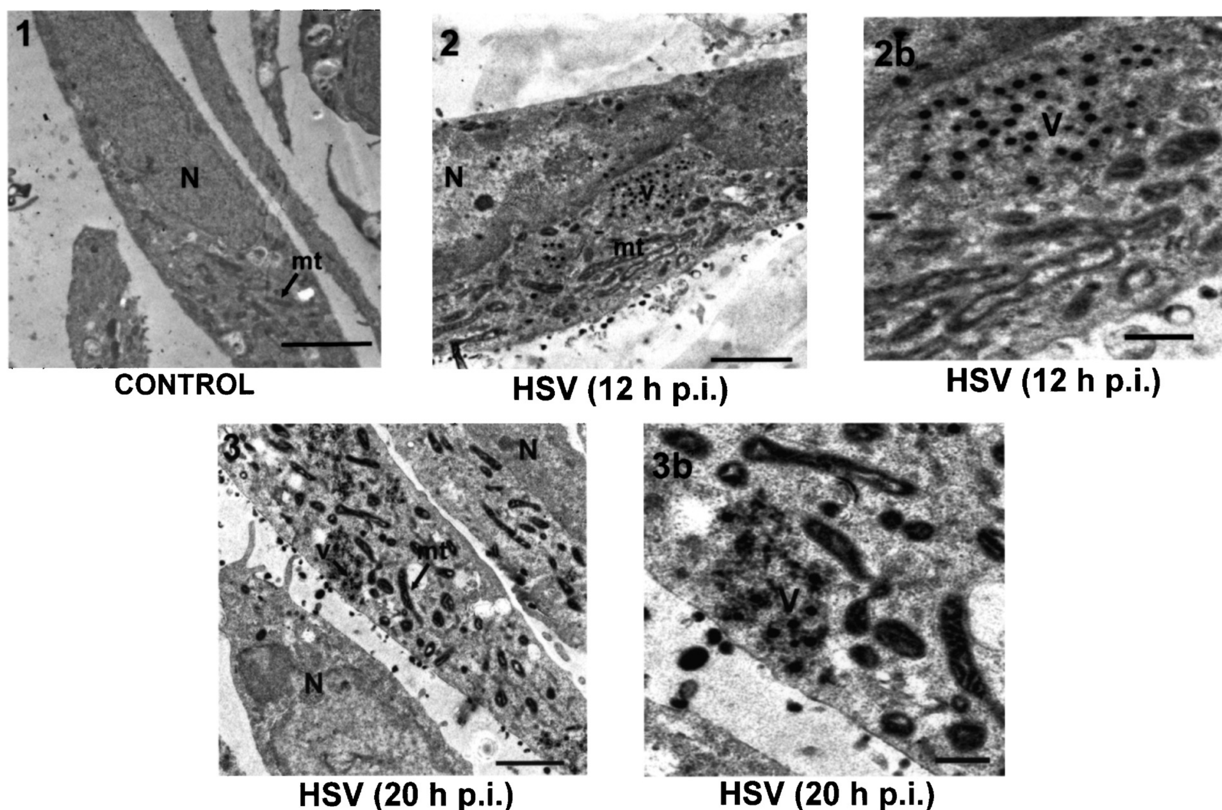

Figure 4 Effect of HSV-1 infection on mitochondrial cluster formation. KG-1C cells were mock-infected or infected for 12 or 20 h with HSV-1 at 10 PFU per cell. After infection, cells were collected by low-speed centrifugation and processed for electron microscopic analysis as described in Materials and Methods. N, nucleus; mt, mitochondria; v, viral particles. (**1**, **2**, and **3**) Bar, 2 μm; (**2b** and **3b**) bar, 0.5 μm.

HSV-1–infected KG-1C cells. Nevertheless, microscopic observation of these cells 12 and 20 h after viral infection (Figure 4) interestingly reveals the appearance of large clusters of mitochondria in the proximity of HSV-1 particles. Most of the mitochondria that surrounded the viral particles had a condensed ultrastructure, with a marked condensation of the cristae, which is a characteristic of the actively respiring mitochondrial state (Hackenbrock, 1966, 1968). Previous results performed in HSV-infected Vero cells showed mitochondrial migration to a perinuclear region in the cytoplasm (Murata *et al*, 2000), suggesting that these organelles respond to the stimulation of HSV infection at least until the middle stage of infection. Further experiments will be focus on the biogenetic or migrational aspect of this accumulation. Migration of actively respiring mitochondria to viral assembly sites has previously been reported in African swine fever virus (ASFV)-infected cells (Rojo *et al*, 1998). However, this complex animal-DNA virus multiplies almost entirely in the cytoplasm of the infected cells, and virus morphogenesis occurs in discrete cytoplasmic areas in the proximity of the nucleus known as viral factories (Breese and DeBoer, 1966). Therefore, it is reasonable to conclude that mitochondria supply the energy that ASFV morphogenetic processes require. In the case of HSV-1 infection, virion entry into, and intracellular transport within, mammalian cells has been widely studied (reviewed by Garner, 2003). HSV capsids are assembled and packaged with DNA in the cell nucleus. They then travel to, and accumulate within, organelles that have the biochemical properties of endosomes and the *trans*-Golgi network (Harley *et al*, 2001). Nevertheless, many aspects of virus morphogenesis and lipid envelope acquisition remain poorly understood and controversial. In this context, recent studies have indicated that some proteins of HSV-1 may be associated with detergent-insoluble lipid rafts. Moreover, this raft population was enriched in a cytoplasmic fraction containing assembling and mature HSV particles (Lee *et al*, 2003). Evidence that lipid rafts play a role in the assembly pathway of some viruses has been widely observed (Martin-Belmonte *et al*, 2000; Briggs *et al*, 2003), although the function of the raft machinery components is completely unknown at present. Thus, we have observed that MAL2, a novel raft protein of the MAL family (Rancaño *et al*, 1994; Wilson *et al*, 2001; De Marco *et al*, 2002), is detectable in the oligodendroglial KG-1C cell line. Although this result is interesting *per se*, because MAL2 has been characterized and is involved in transcytosis initially in hepatoma and epithelial polarized cells, confocal microscopy (data not shown), and

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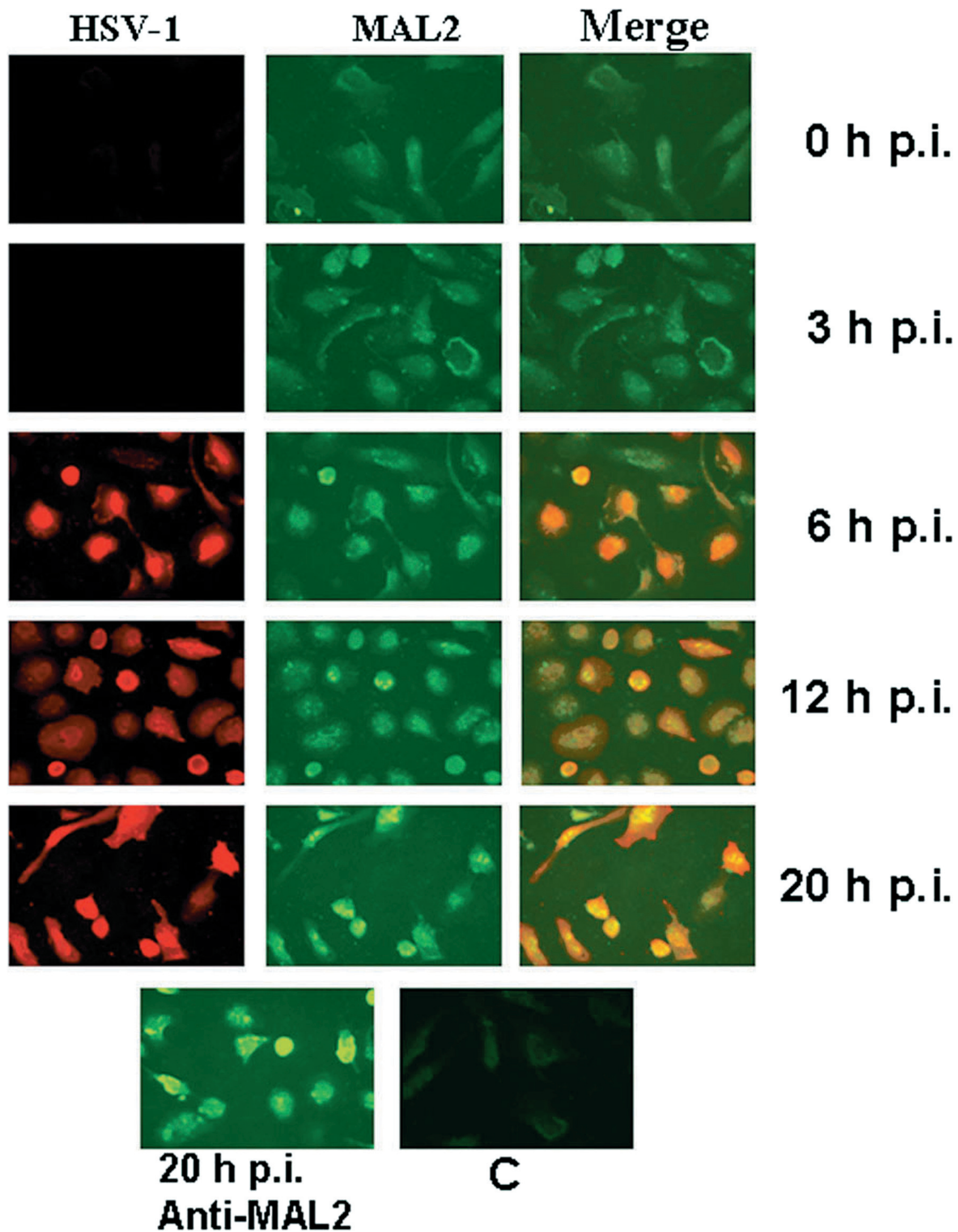

Figure 5 Distribution and increase of MAL2 in HSV-1-infected KG-1C cells. At the indicated times post infection, cultures were double-stained for HSV-1 and MAL2 and analyzed using a conventional fluorescence microscope. A control to assess labeling specificity included incubations with monoclonal anti-MAL2 antibodies alone (shown as anti-MAL2) or omission of the primary antibodies (C).

double-label fluorescence microscopy detected an increase of MAL2 in KG-1C cells after HSV-1 infection. MAL2 signal could not be ascribed to any particular area of the cell, although precise controls assured the specificity of the observations. Further analysis is needed to exclude the possibility that the increase of MAL2 signal is due to the unmasking of an antibody-recognized epitope during infection. Involvement of

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mitochondria and MAL2 raft protein in the molecular mechanism of HSV assembling and trafficking through the infected cells requires further investigation, but our findings represent a first step in this direction.

In conclusion, we have characterized the HSV-1 infection of an oligodendroglial cell line. The high susceptibility of KG-1C to the infection and other results reported here open up new avenues for studying the involvement of HSV-1 on CNS degeneration and demyelinating disease induction.

Materials and methods

Cells and virus
The human oligodendroglial KG-1C cell line was cultured in Dalbecco's modified Eagle medium (DMEM) (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (FCS) in a 5% CO_2 atmosphere at 37°C. HSV-1 (F strain) was propagated in Vero cells. This cell line was used as a control of HSV-1 infection. Virus titration by plaque assay was performed on Vero cell monolayers with a final concentration of 0.8% low-melting-temperature agarose (Gibco BRL, Life Technologies). The m.o.i. was expressed as the number of PFU per cell. Exponentially growing cultures were used for all experiments. After infection, the establishment of cytopathic effects was observed microscopically. Survival of HSV-1-infected cells was determined by the trypan blue exclusion technique.

Protein labeling and PAGE analysis
In vivo labeling of newly synthesized proteins was carried out by giving 1-h pulses with 40 μCi of L-[^{35}S] Pro-mix (approximately 70% L-[^{35}S]methionine [>1000 Ci/mmol] and 30% L-[^{35}S]cysteine; Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom) per milliliter in methionine- and cysteine-free medium at 37°C. After this, samples were washed extensively and subjected to sodium dodecyl sulfate (SDS)-PAGE analysis under reducing conditions, as described previously (López-Guerrero *et al*, 1989).

Western blotting
For immunoblot analysis, cultures (10^6 cells) were infected with HSV-1 (10 PFU per cell) and treated basically as described by López-Guerrero *et al* (2000). Briefly, cells were collected on several occasions post infection and suspended in 370 mM Tris-HCl pH 6.8; glycerol 17%, dithiothreito (DTT) 100 mM, SDS 1%, and bromophenol blue 0.024%. Samples were subjected to SDS-PAGE in 12% acrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Hybond ECL, Amersham Europe). After blocking with 5% nonfat dry milk, 0.05% Tween-20 in phosphate-buffered saline (PBS), blots were incubated with rabbit anti-HSV-1 (Dako, Glostrup, Denmark) or anti-caspase-3 polyclonal antibodies (PharMingen, San Diego, CA, USA). As control, anti-actin polyclonal antibodies were used. After several washes, blots were incubated for 1 h with goat anti-rabbit immunoglobulin G (IgG) antibodies coupled to horseradish peroxidase, washed extensively, and developed using an enhanced chemiluminescence Western blot kit (ECL, Amersham).

Immunofluorescence analysis
Cells were cultured on slides and mock-infected or infected with HSV-1 (10 PFU per cell). At the indicated times after infection, cells were fixed by adding 4% paraformaldehyde (made fresh) for 20 min at room temperature and washed with PBS. Subsequently, cells were incubated for 5 min with 10 mM glycine, rinsed twice with PBS, permeabilized with 0.2% Triton X-100 in PBS, and incubated with 3% bovine serum albumin (BSA) in PBS for 15 min. Cultures were double-stained with a 1:100 dilution of anti-HSV-1 polyclonal (Dako) or anti-MAL2 monoclonal antibodies (the generous gift of Dr. M. A. Alonso, CBM, Madrid, Spain) for 1 h at 37°C. After incubation with the indicated primary antibodies, cells were rinsed several times with 1% BSA in PBS, and incubated for 1 h with the appropriate fluorescent secondary antibodies (Pierce, Illinois, USA). Controls to assess labeling specificity included incubations with control primary antibodies or omission of the primary antibodies. Images were obtained using a conventional fluorescence microscope (Zeiss). Nuclei were stained by incubating cells with 100 ng/ml DAPI (4′,6-diamidino-2-phenylindole; Sigma) in PBS for 30 min following standards protocols, in parallel with immunofluorescence analysis.

Electron microscopy
Cells were processed by freeze substitution. Cultures were mock-infected or infected with HSV-1 (10 PFU per cell). At 20 h p.i., cells were fixed for 60 min with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Cells were then washed and embedded in 10% gelatine in PBS. Subsequently, 0.5- to 1-mm^3 cubes of gelatine-embedded cells were cryoprotected with 2 M sucrose at 4°C overnight, plunge-frozen in liquid propane, and immediately transferred to an Automatic Freeze-Substitution System (AFS, Leica). Freeze substitution was carried out at −85°C in methanol containing 0.5% uranyl acetate for 50 h. After raising the temperature to −35°C at a rate of 5°C/h and washing several times with pure methanol, samples were infiltrated with Lowicryl k4M and polymerized by ultraviolet (UV) light irradiation at −35°C for 2 days. Ultrathin Lowicryl sections were cut in a Reichert-Jung Ultracut E ultramicrotome. Samples were examined at 80 kV under a JEM 1010 electron microscope.

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Tissue Antigens ISSN 0001-2815

BRIEF COMMUNICATION

The 1858*T PTPN22* gene variant contributes to a genetic risk of type 1 diabetes in a Ukrainian population

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Key words
ethnic; gene polymorphism; *PTPN22*; RFLP; type 1 diabetes; Ukraine

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Received 14 December 2005; revised 21 January 2006; accepted 22 February 2006

doi: 10.1111/j.1399-0039.2006.00591.x

Abstract

The 1858*T* variant of the protein tyrosine phosphatase gene, *PTPN22*, is associated with an increased risk of several autoimmune diseases. The aim of this study has been to investigate the possible association of 1858*C*→*T PTPN22* polymorphism and type 1 diabetes (T1D) in Caucasians from Ukraine. Overall, the distribution of 1858 *PTPN22* genotypes differed significantly between the T1D patient group ($n = 296$) and the control group ($n = 242$) ($P = 0.0036$). When both groups were classified according to sex, the *TT* genotype and *T* allele showed a statistically significant higher frequency in T1D female patients (5.9 and 22.8%, respectively) in comparison with the female controls (0 and 11.9%) ($P = 0.008$ for both analyses). The patients with the *TT* genotype were significantly younger at the onset of T1D compared with those with genotypes *TC* and *CC* ($P = 0.035$ and 0.019, respectively). In our Ukrainian Caucasian cohort, we confirmed the association between T1D and the *PTPN22*,1858*T* allele.

Type 1 diabetes mellitus (T1D) is a multifactorial autoimmune disease with a strong genetic component. It constitutes a serious health problem in Ukraine, especially in the eastern region, where the annual incidence of the disease is 28.2 per 100,000 in 2004. Unfortunately, little genetic data concerning T1D in the Ukrainian population have been published to date.

A complex genetic aetiology is thought to underlie susceptibility to T1D. Genome screens have confirmed that the HLA gene region is the major genetic determinant for the risk of developing the illness, accounting for approximately 40% of familial clustering in T1D, and that the insulin gene region contributes a further 10% to genetic susceptibility (1). *CTLA4* polymorphisms are associated with T1D in many ethnic groups (2) but, as with the insulin gene region, they have only a modest impact on T1D risk (3). The linkage to several additional loci, *IDDM4* (11q13), *IDDM5* (6q25), *IDDM6* (18q21), *IDDM7* (2q31), *IDDM8* (6q27), *IDDM10* (10p11-q11), *IDDM13* (2q35) and *IDDM15* (chromosome 6q21) (4–7), has also been demonstrated, although the identity of the susceptibility genes in these regions remains to be discovered.

An association has been reported recently between a functional single nucleotide polymorphism (SNP), C1858T, within the *PTPN22* gene, which encodes lymphoid-specific protein tyrosine phosphatase (Lyp), increasing the risk of T1D and several autoimmune diseases in Caucasians (8–12). Protein tyrosine phosphatases play an important role as negative regulators of stimulatory signalling cascades in the maintenance of immune homeostasis (13). Lyp, through interaction with the SH3-domain of the negative regulatory kinase Csk, participates in the suppression of TCR signalling. The SNP C1858T brings about the substitution of arginine for tryptophan at codon 620 in the protein's SH3-binding domain, thus disrupting the formation of the LYP-Csk complex (8). New data published by Vang et al. (14) show that the 1858T disease-predisposing variant of the protein tyrosine phosphatase gene *PTPN22*, which codifies Trp620, results not in a reduction but in an

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increase in *PTPN22* phosphatase activity in T cells. These authors demonstrated that T cells from T1D individuals who are heterozygous for this allele (1858*CT*) secreted significantly less IL-2 than those from homozygous 1858*CC* T1D patients. This difference was seen in response to TCR stimulation but not in response to pharmacological stimulation that bypasses TCR. By measuring the catalytic activities of two enzyme variants, Vang et al. (14) also found that the specific activity of Lyp-Trp620 was higher despite its not binding Csk. Nevertheless, despite the new data, the whole mechanism underlying the association of *PTPN22* with autoimmune diseases is still not clear.

The frequency of the 1858*T* allele was found to vary in different ethnic groups (15). Because the confirmation of true genetic effects in complex disease is important for association studies, we investigated whether C1858T *PTPN22* gene polymorphism contributes to the genetic risk of T1D in a Ukrainian population. We genotyped C1858T polymorphism (*rs2476601*) in 296 unrelated T1D patients and 242 healthy blood donors with no family history of autoimmune disease. The patients and healthy controls (HC) were Caucasians from the Kharkov region in eastern Ukraine. The age range was from 16 to 65 (mean ± SD 36.0 ± 11.6) for T1D patients and from 18 to 60 (35.4 ± 11.5) for the HC. The male/female ratio was 1.1 in patients and 2.0 in the HC group. The T1D group were recruited from the Department of Endocrinology at Kharkov Regional Hospital (Ukraine) from 2002 to 2004. This group was composed of patients with moderate (20%) to severe (80%) forms of the disease. T1D was diagnosed on the basis of World Health Organization criteria (16). The average age at the onset of T1D was 23.7 ± 12.4 years, within a range of 2–64 years. The study was approved by the local Ethics Committee, and informed consent was obtained from all the participants. The genotyping results are set out in Table 1.

Table 1 Genotype and allele frequencies of 1858 C→T PTPN22 polymorphism in Ukrainian T1D patients and healthy controls

	Patients $n = 296$	Controls $n = 242$	P^{a}	OR (95% CI)[b]
Genotype[c]				
TT	16 (5.4)	2 (0.8)	0.007	6.9 (1.7–27.0)
CT	93 (31.4)	64 (26.4)	0.207	1.3 (0.9–1.9)
CC	187 (63.2)	176 (72.8)	0.024	0.7 (0.5–1.0)
Allele				
C	467 (78 9)	416 (85.9)	0.003	0.6 (0.4–0.8)
T	125 (21.1)	68 (14.1)	0.003	1.6 (1.2–2.3)

n, number of subjects genotyped (percentages shown in parenthesis).
[a]d.f. = 1, Yates corrected.
[b]OR- odds ratio, 95% CI – 95% confidence interval.
[c]χ^2= 11,27, d.f. = 2, $p = 0.0036$.
Genotyping was performed by PCR-RFLP (*RsaI*) methods (17). Statistical analysis of genotype and allele frequencies between the groups was made using the contingency Table 3 × 2 (genotypes) and 2 × 2 (alleles) χ^2 test with Yates' correction.
(http://faculty.vassar.edu/lowry/webtext/html).
(http://www.members.aol.com/johnp71/ctab2 × 2.html).
The power of study to detect the possible contribution of a polymorphism to disease susceptibility was estimated using the Quanto v 0.5 software (Department of Preventive Medicine, University of Southern California, California, USA).

The blood-donor and patient sets were both in Hardy–Weinberg equilibrium. The distribution of genotype frequencies observed in our Ukrainian HC group agreed closely with those reported elsewhere in North American Caucasians (8). Significant differences in *PTPN22* genotype and allele frequencies were found in Ukrainian T1D cases and HC ($P = 0.0036$ and $P = 0.003$, respectively). Viewed as a risk factor, the *PTPN22* 1858 *TT* genotype was associated with a sevenfold increase in the risk of T1D in subjects from the Ukraine ($P = 0.007$, OR = 6.9, 95% CI = 1.7–27.0) compared with bearing the *PTPN22* 1858 *CT+CC* genotype. The minor *T* allele was found at a frequency of 21.1% in TID cases compared with 14.1% in the controls and was significantly associated with the disease ($P = 0.003$, OR = 1.6, 95% CI = 1.2–2.3). The frequency of the *T* allele in both the patients and the controls observed in this study was higher than that found by Zheng et al. (17), (0.141 and 0.211 vs 0.085 and 0.145, respectively) ($P < 0.002$ for both comparisons) but similar to the findings of Bottini et al. (8) and Criswell et al. (12). All these studies were made in North American Caucasian populations. Our data agree with those obtained recently in T1D patients in a Dutch population (18).

When both the TID and HC groups were classified according to sex, the frequency of the *TT* genotype and *T* allele was significantly higher in female T1D patients (5.9 and 22.8%, respectively) compared with female controls (0 and 11.9%) ($P = 0.008$ for both analyses; OR = 2.2 for the *T* allele), while there was no significant difference between the male patients and the male controls (Table 2). No differences were detected either for genotype or for allele distribution between female and male controls. Thus we confirmed the sex-related findings obtained in a German population by Kahles et al. (19). In a separate study, however, Smyth et al. (20) found no evidence for any relationship between the patients' sex and 1858 *PTPN22* polymorphism distribution in a family-based cohort (2061 affected subjects), although in a case-control comparison (1593 case subjects) this effect had a *P*-value of 0.029. In Dutch T1D patients, Zhernakova et al. (18) encountered no gender classification effect either. We share the opinion of Kahles et al. (19) that this inconsistency may be put down to several factors, such as a slightly different 1858T *PTPN22* allele frequency between

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Table 2 Distribution of 1858 C→T *PTPN22* genotype and allele frequencies in Ukrainian patients with T1D and healthy control group classified according to sex and age at T1D onset

	Female		Male		
	Patients[a] $n = 127$	Controls $n = 80$	Patients[a] $n = 136$	Controls $n = 162$	Age at T1D onset[b] $n = 263$
Genotype					
TT	10 (5.9)	0 (0.0)	5 (3.7)	2 (1.2)	17.8 ± 13.7[c,d]
CT	38 (29.9)	19 (23.7)	43 (31.6)	45 (27.8)	24.2 ± 12.7
CC	79 (62.2)	61 (76.3)	88 (64.7)	115 (71.0)	23.8 ± 12.3
p[e]	0.008		0.301		
Allele					
C	196 (77.2)	141 (88.1)	219 (80.5)	275 (84.9)	
T	58 (22.8)	19 (11.9)	53 (19.5)	49 (15.1)	
p[f]	0.008		0.159		
OR	2196		1.358		
(95% CI)[g]	(1.258–3.829)		(0.888–2.078)		

[a]Number of patients available for analysis.
[b]Mean ± SD (years).
[c]$p = 0.019$ (TT vs CC) Mann–Whitney one-sided test.
[d]$p = 0.035$ (TT vs CT) Mann–Whitney one-sided test.
[e]Fisher Exact Probability Test for Table 2 × 3 (http://faculty.vassar.edu/lowry/VassarStats/html).
[f]χ^2 test, Yates corrected
[g]Odds ratio (CI confidence interval) for the T allele.

populations and a smaller sample size in our study. Nevertheless, the fact that autoimmune diseases tend to prevail in women suggests the existence of molecular mechanisms associated with sex hormones affecting the immune response.

The 1858 *PTPN22* genotype distribution was also analysed according to age at the onset of T1D (Table 2). The average age of patients with *TT, CT* and *CC* genotypes at the onset of the disease was 17.8, 24.2 and 23.8, respectively. The patients with the TT genotype were significantly younger when T1D began compared with those with the CT and CC genotypes (*P*-values of 0.035 and 0.019, respectively). Similar findings were obtained by Ladner et al. (21) in a white North American T1D group, but their results did not attain statistical significance. One of the possible explanations for this situation is that our case cohort is fairly homogeneous, made up of patients with a severe clinical form of T1D.

Carlton et al. (22) have recently characterized a linkage disequilibrium across the gene and determined common *PTPN22* haplotypes. The authors support the idea that 620W constitutes a mayor risk haplotype in rheumatoid arthritis (RA) while suggesting that there is at least one other locus predisposing to RA in the same gene.

In conclusion, in a Ukrainian Caucasian cohort, we confirmed an association between T1D and the *PTPN22, 1858T* allele, the gene variant that also confers a risk of several autoimmune diseases such as RA, systemic lupus erythematosus, Graves' disease and Hashimoto's thyroiditis (12). Our data support the idea that *PTPN22* should be considered as a potential therapeutic target in these autoimmune diseases.

Acknowledgments

The authors thank the volunteers for their co-operation in this study. This work was supported by grants FIS PI 020920 to A. Alcina, FIS PI041298 and SAS 304-04 to F. Matesanz. M. Fedetz is a holder of Fundación IMABIS (O. Fernandez) fellowship.

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