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FACTORES INMUNOGENÉTICOS RELACIONADOS CON LA RESISTENCIA/SUSCEPTIBILIDAD A LA INFECCIÓN POR EL VIH-1 Y SU PROGRESION HACIA SIDA.

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Abreviaturas

ADVP :	Adicto a drogas por vía parenteral.
SI:	Cepas inductoras de sincitios.
NSI :	Cepas no inductoras de sincitios.
CTL:	Linfocitos T citotóxicos.
DNA:	Ácido desoxiribonucleico.
EU:	Individuos expuestos al VIH, no infectados.
FH:	Factores del huésped.
HAART:	Tratamiento antoretroviral de alta eficacia.
HC :	Control sano.
HLA:	Antígeno leucocitario humano.
Ig:	Inmunoglobulina.
IL:	Interleucina.
LTNP:	Pacientes infectados por el VIH con progresión lenta de la infección.
MDC:	Quimiocina derivada del macrófago.
MIP:	Proteína inflamatoria del macrófago.
PBMC:	Monocitos de sangre periférica.
RNA:	Ácido ribonucleico.
SDF-1:	Factor derivado de células estromales-1.
SIDA:	Síndrome de Inmunodeficiencia Adquirida.
SNP:	Polimorfismo que afecta a un nucleótido.
Th:	Linfocitos T colaboradores.
UTR:	Región no traducida.
VIH:	Virus de la Inmunodeficiencia Humana.
VIH-TP:	Paciente infectado por el VIH con progresión típica de la enfermedad.

1. Introducción

Los individuos expuestos al VIH-1 y no infectados (EU) y los infectados no progresores de larga duración (LTNP) son situaciones modelo para estudiar factores del huésped (FH) implicados en conferir resistencia a la infección/progresión y su conocimiento puede ser esencial para desarrollar nuevas estrategias preventivas y terapéuticas. Tales FH pueden referirse principalmente a la producción de quimiocinas y/o a la expresión de receptores de quimiocinas que actúan como co-receptores del VIH-1, asÍ como a características de eficacia de la respuesta inmune anti-VIH-1, lo que puede ser dependiente del haplotipo HLA, así como a posibles características polimórficas de otras moléculas del sistema inmunitario que regulan fuertemente la respuesta inmunitaria (por ejemplo CD28 y sus ligandos).

1.1. Interés de los pacientes infectados por el VIH-1 no progresores de larga duración y de las personas expuestas no infectadas.

1.1.1 Interés de los infectados no progresores de larga duración como situación modelo para el estudio de factores del huésped que confieren resistencia a la progresión de la infección por el VIH.

La historia natural de la infección por el virus de la inmunodeficiencia humana (VIH-1) incluye la infección aguda que va seguida de una fase asintomática de infección crónica, la cual finalmente desemboca en la fase de crisis o síndrome de la inmunodeciencia humana adquirida (SIDA) que se inicia con el declive (por debajo de 500/μl) rápidamente progresivo de las células CD4+ circulantes (1). Hay una gran variabilidad interindividual en la duración de la fase asintomática y en su ritmo de progresión hacia SIDA: frente a la "progresión típica" (SIDA a los 8-10 años de la infección aguda), hay una pequeña

proporción de individuos (5-11%), denominados "progresores lentos" o "no progresores de larga duración " (LTNP), que permanecen asintomáticos y con células T CD4+ >500/μL durante más de 8-10 años o incluso 16 años, lo que contrasta con la "progresión rápida" que se da también en otra minoría de infectados(un 10%; SIDA a los 2-3 años)(1). El gran pico plasmático de RNA VIH (viremia plasmática) que caracteriza la infección aguda refleja la intensa replicación vírica que tiene lugar en los órganos linfoides, y unas semanas mas tarde disminuye para alcanzar, a los seis meses de la seroconversión, un "nivel basal de equilibrio" (viremia basal) que permanecerá estable durante la fase crónica asintomática, para incrementar de nuevo en la fase de SIDA(1-5). Los ganglios linfáticos y demás órganos linfoides periféricos constituyen el sitio de replicación vírica y principal reservorio vírico durante toda la enfermedad. La viremia basal de la fase asintomática es representativa de dicho reservorio, siendo siempre este último muy superior a la primera, y su valor marca y predice el futuro curso evolutivo de progresión rápida o lenta hacia SIDA(2). Es decir, la condición de progresores rápidos o no progresores viene en realidad determinada por la forma en que se controla la replicación vírica durante la infección aguda. Aunque en algunos casos la no progresión puede ser debida a la infección por un virus defectuoso (6), se cree que principalmente refleja la existencia de una conjunción óptima de factores del huésped capaces de controlar o frenar la replicación vírica, factores que no se hallarían en los progresores rápidos (1).

1.1.2 Interés de las personas expuestas no infectadas como situación modelo para el estudio de factores del huésped implicados en la resistencia/susceptibilidad a la infección por el VIH.

En la actualidad está claro que existen individuos que han estado altamente expuestos (E) al VIH y que sin embargo no están infectados (EU, por "Exposed Uninfected) , esto es,

permanecen persistentemente seronegativos, sin manifestaciones clínicas y sin VIH detectable ni por cultivos, ni mediante detección de VIH RNA plásmatico o VIH DNA en células mononucleares de sangre periférica (PBMC) (7,8). Tales individuos incluyen drogadictos que han compartido repetidamente jeringuillas con otros drogadictos infectados, hombres homosexuales con relaciones sexuales continuadas no protegidas con hombres infectados, recién nacidos de madres infectadas, receptores de sangre infectada o de derivados plasmáticos infectados, trabajadores sanitarios accidentalmente inoculados con sangre de pacientes infectados, y prostitutas practicantes de sexo no protegido. La no detección del virus en sangre periférica no significa que no puedan existir células infectadas o con DNA vírico integrado acantonadas en algún órgano linfoide. De acuerdo con WE Paul, director de "Office of AIDS Research" de "National Institutes of Health" (USA), el estudio de factores del huésped que conceden protección contra la infección y contra la progresión hacia SIDA de la infección, debe considerarse como una de las líneas de investigación prioritarias en el campo de la infección por el VIH, por cuanto puede desvelar claves esenciales para la comprensión de la patogenia de la infección y por tanto, para el desarrollo de nuevas estrategias preventivas (vacuna) y terapéuticas (9). A este respecto hay que enfatizar que datos recientes indican que la erradicación o remisión del VIH usando sólo la terapia antiretroviral potente (HAART) resultará ser un objetivo inalcanzable, incluso si se aplica durante 5-7 años (10,11), siendo posiblemente necesario, para poder conseguirlo, combinar HAART con alguna forma de terapia inmunopotenciadora de la inmunidad anti-VIH protectiva.

Los EU y los LTNP constituyen modelos excelentes para el estudio de dichos factores como lo ilustra el hecho de que el estudio de individuos EU fue decisivo para el descubrimiento de que el CCR-5 constituye uno de los co-receptores del VIH.

1.2. Principales factores inmunogenéticos conocidos, implicados en la susceptibilidad a la infección por el VIH y a la progresión de la misma.

Los principales factores inmunogenéticos relacionados con la susceptibilidad a la infección por el VIH y la progresión de ésta a Sida están relacionados con 3 familias de genes del huésped, los antígenos leucocitarios humanos (HLA clase I, II y moléculas accesorias), los receptores de quimiocinas y sus ligandos (quimocinas).

Las células presentadoras de antígeno son más o menos capaces de presentar un determinado péptido específico a los linfocitos T dependiendo del tipo de HLA que presentan en su superficie, esto explica la importancia de estas moléculas en la respuesta frente al VIH. Su relevancia en la susceptibilidad al VIH se puso de manifiesto en la cohorte de prostitutas de Nairobi resistentes a la infección por VIH, entre las que predominaba el HLA A2/A28, por lo que los autores sugieren que este HLA supone una mejor presentación de los péptidos del VIH a los linfocitos CD8 (77). Posteriormente se demostró que la concordancia del HLA entre madre e hijo, favorecía la transmisión perinatal del VIH (76). También se ha puesto de manifiesto la importancia del tipo de HLA del huésped en la progresión de la infección a Sida y así el HLA B57, B27 y la heterozigosidad de las moléculas de HLA A, B y C (47, 75, 73) se han relacionado con una progresión lenta de la infección, mientras que el HLA A1, B8, B35, Cw4 y la homozigosidad de las moléculas del HLA se han asociado con una rápida progresión (74). Sin embargo, la introducción de esta tesis pretende centrarse en aquellos factores inmunogentéticos relacionados con los receptores de quimiocinas y sus ligandos (quimocinas).

Desde hace años se sabía que, además de su receptor primario que es la molécula CD4, el VIH requería otros co-receptores para fusionarse e introducirse en el interior de las

células CD4+. En los últimos dos años la comprensión de la patogenia de la infección y el diseño de estrategias terapéuticas, se ha visto completamente revolucionada al ser identificados tales co-receptores, que han resultado pertenecer a la gran familia de receptores de quimiocinas CC (por cisteina-cisteina) y CXC (por cisteina-otro aminoácido-cisteina). El tipo de co-receptor utilizado por el VIH-1 difiere según se trate de una cepa inductora (SI) o no inductora (NSI) de la formación de sincitios. Las cepas NSI son las que transmiten la infección y las que se aíslan de los pacientes durante los primeros años de la infección, y utilizan principalmente el receptor CCR-5 (12, 13), aunque algunas pueden usar también el CCR3, CCR2 y CX3CR1 (14). Las cepas SI se aíslan en estadios más avanzados, cuando se inicia la pérdida de las células CD4+, y utilizan el CXCR-4 como co-receptor (15). El uso de uno u otro co-receptor depende de pequeños cambios aminoacídicos de la región V3 de la glicoproteina de la cubierta del VIH, gp120 (figura 1). La aparición de tales cambios a lo largo de la infección, determina el cambio en el uso del co-receptor, desde CCR-5 a CXCR-4 (12,13), lo que se correlaciona con la progresión de la infección (16,17). Además, se pueden también aislar cepas duales capaces de utilizar ambos co-receptores (17). La mayor patogenicidad de las cepas SI respecto de las NSI (16) vendría probablemente determinada por el diferente patrón de expresión de sus respectivos co-receptores en las células T CD4+. La expresión de CXCR4 es notable y ocurre sobre todo en células de fenotipo "naive", mientras que la expresión de CCR5 ocurre sólo en una pequeña fracción de células T CD4+ de fenotipo memoria, y su expresión es muy escasa incluso tras intensa y prolongada activación in vitro (14,18,19).

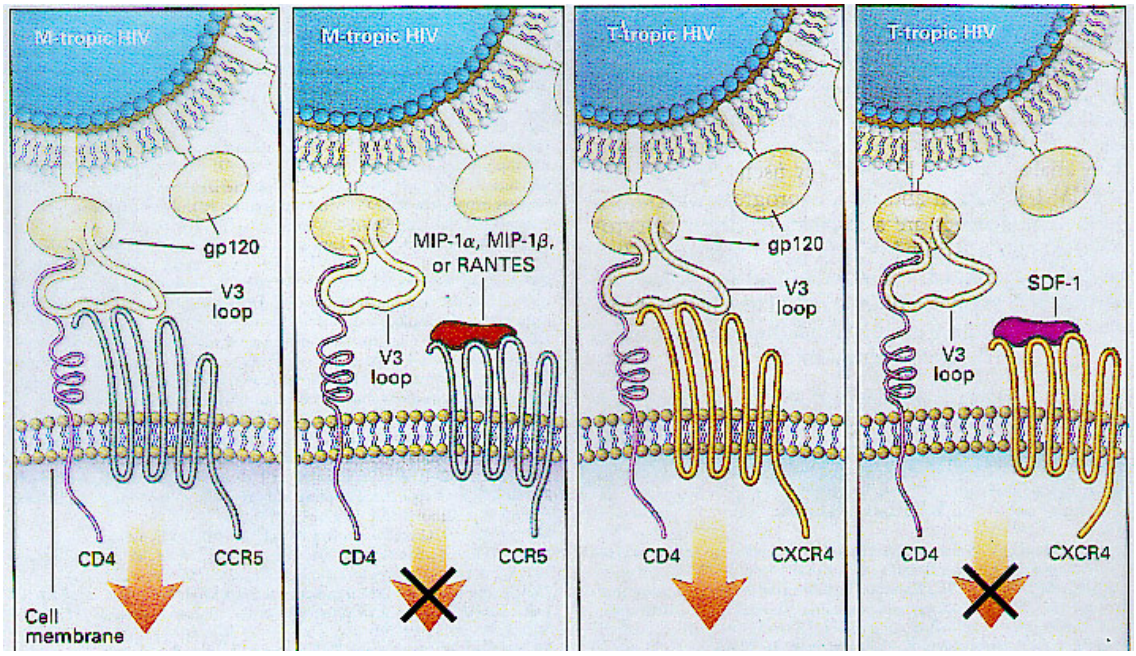

Figura 1. Receptores de quimiocinas que actúan como co-receptores del VIH. La glicoproteína 120 (gp120) se une al CD4 y se produce un cambio conformacional en la región V3 de la gp120 que permite la subsecuente interacción con el receptor de quimiocinas. La variante "M-Tropic" del VIH utiliza el CCR5, mientras que la variante "T- Tropic" utiliza el CXCR4. Los ligandos naturales de estos receptores (MIP-1α, MIP-1β y RANTES para el CCR5 y SDF-1 para CXCR4), son capaces de bloquear la entrada del VIH en la célula. Fuente: New England Journal of Medicine.

1.2.1 Receptores de quimiocinas y resistencia a la infección y a su progresión hacia SIDA.

La importancia del CCR-5 como co-receptor del VIH quedó demostrada al descubrirse que en ciertos EU la resistencia a la infección se debe a que son homozigotos para una variante genética de CCR-5, consistente en una delección de 32bp (Δ32) que determina una proteína truncada incapaz de expresarse en la membrana, por lo que las células CD4+ de esos individuos son altamente resistentes a la infección in vitro e in vivo por cepas NSI (24). Los pacientes heterozigotos para la mutación *CCR-5Δ32* se asocian, además, con la no progresión de la infección (25). Este efecto protectivo está mediado por una menor expresión fenotípica del CCR5 en la superficie de los linfocitos CD4 de

individuos portadores de la mutación *CCR-5Δ32* (26,27,28), lo que impide la propagación del VIH-1 NSI, mostrando estos pacientes una cifra de carga viral menor, mayor número de linfocitos CD4 y en definitiva una progresión más lenta, siempre que el fenotipo viral dominante sea el NSI (29-35,48,49). Pueden existir otras anomalías genéticas de CCR5 protectivas contra la infección como es la mutación (T→A) en la posición 303 (m303) que introduce un "stop codon" prematuro, impidiendo la expresión del receptor (36); hasta ahora sólo se ha descrito en un EU que era heterozigoto tanto para dicha mutación como para Δ32 y, el fenotipo de CCR-5 resultante era equiparable al de un homozigoto para Δ32, es decir, no expresaba CCR5, siendo sus células resistentes a la infección por cepas NSI.

Por otro lado, los pacientes VIH positivos con independencia de la existencia de la mutación CCR-5Δ32 muestran una expresión fenotípica del receptor superior a los controles sanos (26,35) y por tanto sería esperable la existencia de otros polimorfismos que pudieran justificar en parte, las variaciones en la expresión fenotípica del CCR-5, más si tenemos en cuenta la gran variedad de polimorfismos descrita en las regiones reguladoras de este receptor. En este sentido un polimorfismo A/G se ha descrito en la posición 59029 del promotor de CCR5, siendo ambas posibilidades muy frecuentes en la población general. La actividad del promotor G/G es 45 veces inferior a la del promotor A/A, dicho hallazgo, es congruente con la asociación que los autores encuentran entre los portadores del genitipo G/G y una progresión más lenta de la infección respecto a los que tienen un genotipo A/A (37-38). Otros autores han descrito las 4 variantes alélicas más frecuentes del gen promotor del CCR5 (CCR5 P1-P4) y han observado que la presencia homozigota de CCR5 P1/P1 (7-13% en la población general) se asocia con una progresión rápida de la enfermedad, estimándose que el 10-17% de los progresores en 3.5 años o menos se deben a este genotipo (37). Por otro lado se ha descrito una

mutación (G→A) que comporta la sustitución de una valina por isoleucina en la posición 64 (64I) en CCR2 que se asocia con un patrón de no progresión a SIDA a pesar de que no afecta a la función de CCR-2 (40), siendo probable que actúe indirectamente sobre CCR5, como lo sugiere el hecho de que se asocia con una mutación en el promotor de CCR-5 (41,42). Por otro lado Martínez et al. demostraron que la mutación CCR-264I favorece su dimerización con el receptor CCR5, impidiendo de esta forma la unión a sus ligandos, entre ellos el VIH-1, lo que podría explicar una progresión lenta de la infección (43). Por último, se han descrito 3 variantes estructurales de un co-receptor menor del VIH denominado CX3CR1 y un estudio encuentra que los homozigotos para la variante I249M280 presentan una progresión muy rápida hacia Sida (78), aunque un trabajo posterior no apoya estos resultados (79).

Los principales factores genéticos descritos en este apartado, fueron evaluados en: 1) una cohorte de pacientes infectados por el VIH que cumplían criterios estrictos de progresión lenta hacia Sida (Long-Term Non-Progressors, LTNP), 2) en un grupo de individuos altamente expuestos al VIH, por vía sexual o parenteral, y con repetidas determinaciones de ELISA negativas para el VIH (Exponed-Uninfected, EU), 3) en un grupo de pacientes VIH + con una progresión normal de la infección (Typical Progressors, VIH-TP) y 4) en un grupo de individuos sanos (Healthy Controls, HC) (para más detalles sobre las características de los grupos citados, ver artículos publicados). Los resultados se exponen como introducción a la justificación de la presente tesis.

Frecuencia de la delección de 32 pares de bases en el gen de CCR5 (*CCR5Δ32*):

La frecuencia de *CCR5Δ32* en el grupo de individuos sanos (n=215) fue del 16,27%, no encontrándose ningún individuo homozigoto para la delección. El grupo de LTNP es el que presentó una mayor frecuencia de la delección con un 28,8%, frente al 15,9% del

grupo de EU y el 18,3% del grupo VIH-TP (figura 2). Las diferencias no fueron, en ningún caso, significativas.

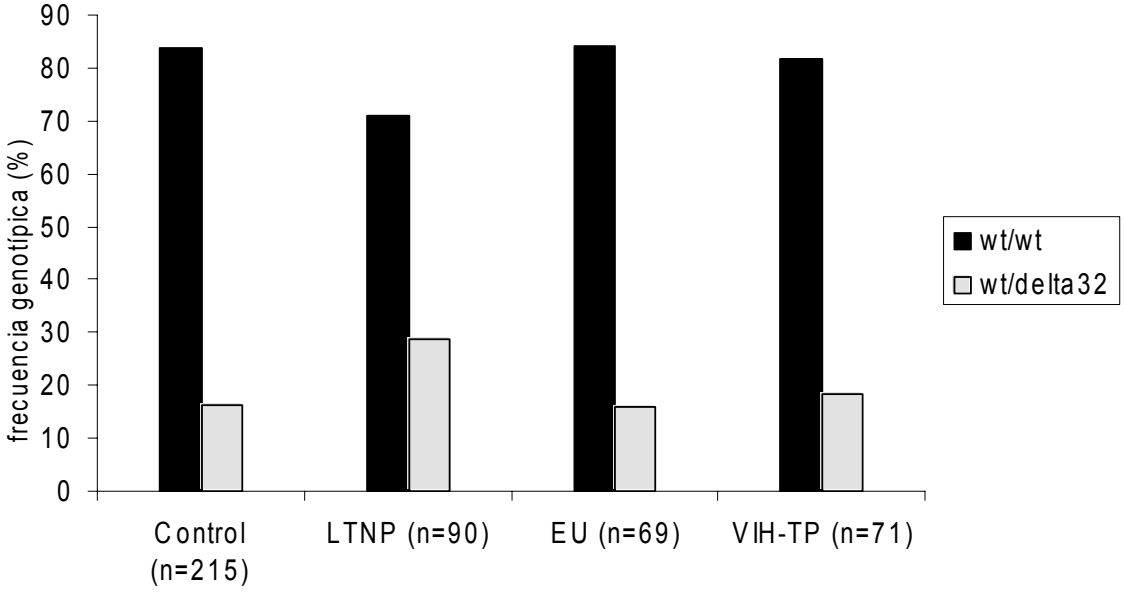

Figura 2. Frecuencia genotípica de CCR5 en los grupos de estudio. wt: alelo no deleccionado (de wild type), delta32: alelo con la delección de 32 pares de bases.

Frecuencia del polimorfismo A/G en la posición 59029 del promotor del gen CCR5:

Para poder estudiar la influencia de este polimorfismo en la susceptibilidad a la infección y/o resistencia a la progresión de la infección, se seleccionaron solo aquellos individuos no portadores del alelo *CCR5Δ32*, puesto que la presencia de una G en la posición polimórfica (relacionada con una progresión lenta de la enfermedad, cuando el individuo es homozigoto, G/G), nunca se encuentra cuando el alelo contiene la delección de 32 pares de bases. En la figura 3 se muestra la frecuencia genotípica en los diferentes grupos de estudio. La distribución en la población control fue similar a la descrita en otras poblaciones y la frecuencia genotípica en los grupos LTNP y VIH-TP fue prácticamente idéntica a la hallada en la población general, mientras que en el grupo EU se apreció un

ligero aumento del genotipo G/G, aunque esta diferencia no alcanzó significación estadística.

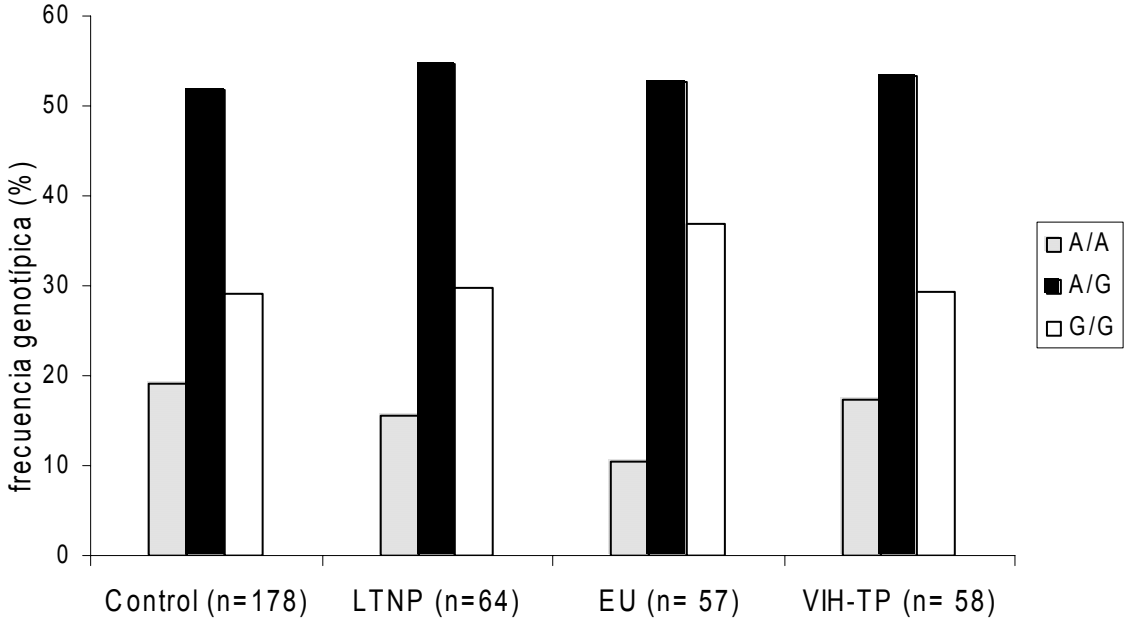

Figura 3. Frecuencia genotípica del promotor de CCR5, en individuos sin delección delta 32 en el gen CCR5. A: alelo con adenina, G: alelo con Guanina.

Frecuencia del polimorfismo G/A en la posición 64 del gen CCR2:

La frecuencia del genotipo heterozigoto *CCR2/CCR264I* en la población general estudiada fue similar a la de otras poblaciones y la frecuencia de la variante homozigota *CCR264I/64I* se situó por debajo del 1%. La distribución genotípica en los grupos de estudio fue prácticamente idéntica (figura 4).

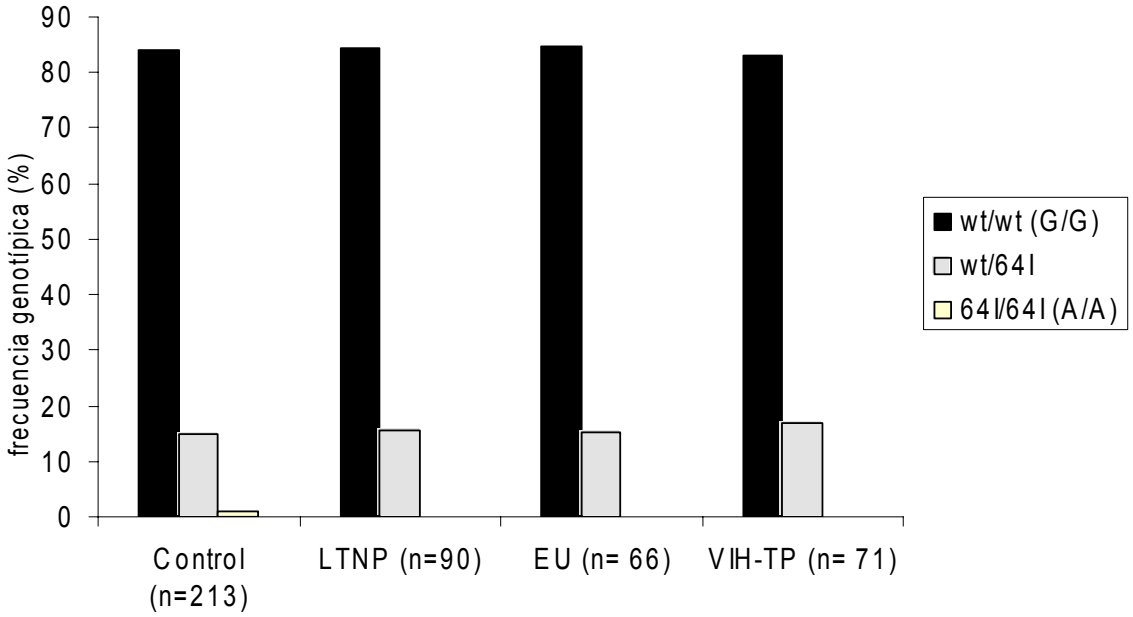

Figura 4. Frecuencia genotípica del polimorfismo 64I en el gen CCR2. A: alelo con adenina, G: alelo con Guanina. wt: alelo salvaje.

Los resultados obtenidos ponen de manifiesto que, salvo la delección de 32 pares de bases en el gen CCR5, la frecuencia de dichas variantes genéticas, previamente asociadas a una progresión lenta de la infección, no se encontraban incrementadas en los pacientes de nuestra cohorte que cumplían las criterios de progresión lenta. Además, de acuerdo con la literatura previa, las variantes estudiadas no estaban asociadas a una mayor o menor susceptibilidad al VIH, puesto que los pacientes EU presentaron frecuencias muy similares a la del resto de los grupos. La explicación a estos datos no puede buscarse en la selección de los pacientes, puesto que los criterios empleados fueron muy estrictos. Tampoco podemos explicar los resultados como una consecuencia de la población estudiada, ya que en todos los casos las frecuencias en el grupo control fueron similares a las descritas en otras poblaciones caucásicas. En nuestra opinión los resultados ponen de manifiesto que las variantes descritas explican un porcentaje de los

pacientes con progresión lenta de la enfermedad y por ello, solo se ponen de manifiesto cuando se estudian poblaciones muy grandes. Por otro lado, cabría esperar una mayor frecuencia de *CCR5Δ32* en el grupo LTNP, sin embargo cuando se revisa el papel de esta delección en la progresión hacia Sida, se aprecia que sobretodo se asocia a una carga viral inferior durante las primeras fases de la infección, en las que predominan los virus con tropismo R5, pero posteriormente, cuando se produce el viraje del virus a fenotipo X4, este efecto beneficioso desaparece y la progresión es similar en los pacientes portadores y no portadores de la delección. Además es importante destacar que la asociación con la no progresión se ha descrito principalmente en cohortes de pacientes homosexuales, mientras que en algunas cohortes de pacientes adictos a drogas por vía parenteral (ADVP), no se ha podido demostrar este efecto beneficioso. Este último dato es importante si tenemos en cuenta que más del 70% de los pacientes LTNP que fueron incluidos en este estudio se habían contagiado por vía parenteral (ADVP o hemofílicos).

1.2.2- Quimiocinas y susceptibilidad a la infección por el VIH y a su progresión hacia SIDA.

El punto de unión de CCR-5 y CXCR-4 a sus respectivas quimiocinas y cepas NSI y SI, son distintos pero se solapan, de forma que el virus y las quimiocinas compiten en su unión a dichos receptores. Así, las CC-quimiocinas , RANTES, Macrophage inflammatory protein [MIP]-1α y MIP-1β, que son los ligandos fisológicos de CCR-5, suprimen la infección por las cepas NSI sin inhibir la infección por cepas SI, mientras que la CXC-quimiocina, “Stromal cell-derived factor-1” (SDF-1) , que es el ligando fisológico de CXCR-4, suprime la infección por cepas SI, sin afectar a la infección por cepas NSI (12,13,20) (figura 1). El factor (o factores) soluble(s) secretado(s) por células CD8+ con actividad antivírica (CAF, por “cell-derived antiviral factor”) descrita por Levy hace más de 10 años (21), no se explica sólo por el efecto antivírico de las quimiocinas RANTES , MIP-1α y

MIP1β, como se creyó en 1995 cuando se descubrió tal efecto (20) , ya que éstas inhiben la infección por cepas NSI, mientras que el CAF suprime la transcripción del VIH de ambos tipos de cepas, además recientemente el mismo autor ha descrito la importancia de esta respuesta en la susceptibilidad a la infección (22) . Probablemente existen otras quimiocinas que inhiban tanto a las cepas NSI como SI, como recientemente se ha demostrado con la CC quimiocina, Macrophage Derive Chemokine (MDC), cuyo receptor permanece sin identificar (23).

Hasta este momento se han descrito variantes polimórficas en el gen promotor del RANTES y en concreto una sustitución C→G en la posición 28 de dicho gen promotor (con una frecuencia alélica del 17% en población general) que se ha correlacionado con un incremento de la actividad promotora y por tanto un aumento de los niveles de RANTES, que bloquearían el CCR5 e impedirían la entrada del VIH fenotipo NSI, favoreciendo una progresión lenta de la infección (45).

Dado que la progresión hacia SIDA viene determinada por el cambio de fenotipo de NSI a SI (14,16,17), la no progresión sostenida debería implicar también la existencia, en los LTNP, de mecanismos para controlar o frenar la aparición de cepas SI, lo que podría consistir en una baja expresión de CXCR-4 y/o alta producción de SDF-1 (ligando natural del CXCR4) o de otras quimiocinas tipo CAF (o tipo MDC) que actúen inhibiendo la infección tanto por cepas NSI como SI. SDF-1 es el prototipo de quimiocina homeostática, producida por células estromales de la médula ósea, órganos linfoides y una gran variedad de células y tejidos, excepto los leucocitos (85). A diferencia de CCR5, la función de CXCR4 y su ligando (SDF-1) es fisiológicamente imprescindible ya que de su interacción dependen funciones tan importantes como la maduración de células madre CD34 en la médula ósea y linfocitos B, regula el tráfico linfocitario y su migración transendotelial y participa en la embrioorganogénesis cardiovascular y cerebral. De

hecho, el modelo animal con un déficit de CXCR4 o SDF-1 no es viable (86). A este respecto hay que señalar que se ha descrito una mutación en el gen SDF-1 (mutación puntual G→A en la posición 801 de la región no traducida 3', que denominaremos alelo SDF1-3'A) cuya presentación homozigota se ha relacionado con la progresión lenta, lo que podría sugerir que en tales individuos existiera una alta producción de SDF-1, que bloquearía la utilización de CXCR4 (46) y disminuiría su expresión (90). Los mismos autores encuentran un aumento del genotipo heterozigoto en 75 EU homosexuales y sugieren un posible papel protectivo de este genotipo. Sin embargo, otros trabajos no han confirmado estos hallazgos e incluso algunos relacionan la presencia de dicha mutación con un descenso más rápido de la cifra de linfocitos CD4 y una progresión más rápida de la infección en los homozigotos para la mutación (87-89). Hasta la realización de esta tesis no se han publicado otros trabajos que corroboren el papel protectivo del genotipo heterocigoto descrito por Winckler C, et al. (46). Por último, sería de gran interés conocer la relación entre el genotipo SDF-1, la producción de SDF-1 y la expresión de CXCR4, así como la relación de todos estos factores con la susceptibilidad y/o progresión de la infección por el VIH, pero hasta el momento solo disponemos de información parcial de algunos de estos aspectos, como la relación entre los niveles plasmáticos de SDF-1 y la progresión del VIH (91,92) y la relación entre la expresión de CXCR4 y la progresión a Sida (93-96).

Otros polimorfismos implicados en la progresión de la infección a Sida son los descritos en la región promotora de las interleucinas (IL) 4 y 10. Se cree que estas variaciones modulan la producción de ARNm de ambas IL que son sintetizadas por linfocitos T-colaboradores tipo 2 (Th2) y tienen un amplio efecto en el sistema inmune, por lo que es razonable pensar que puedan influir en la progresión de la infección. Sin embargo, en el caso de la IL-10 se trata de solo 2 estudios (80,84) y en el caso del polimorfismo en el

promotor de la IL-4, diferentes estudios no alcanzan la misma conclusión e incluso son contradictorios (81-84).

El grado de conjunción de todos y cada uno de los factores descritos y de aquellos todavía por conocer, determinará diferentes grados de susceptibilidad a la infección y su progresión (47).

1.3. Respuesta inmune frente al VIH-1.

1.3.1 Respuesta inmune frente al VIH en individuos infectados y sus características en LTNP

La infección aguda por VIH se asocia con una fuerte respuesta inmune tanto de linfocitos T CD8+ citotóxicos VIH-específicos y MHC clase I-restringidos (CTL, por "Cytotoxic T lymphocytes"), como de anticuerpos, y de la llamada actividad no citotóxica anti-VIH de las células CD8 designada CAF, que corresponde a factores solubles como se indicó antes (21). La respuesta CTL y CAF se empieza a detectar al inicio de la caída del pico de viremia de la infección aguda, mientras que los anticuerpos circulantes anti-VIH se empiezan a detectar más tarde e inicialmente son no neutralizantes, aunque posteriormente también aparecen anticuerpos neutralizantes. Se cree que el declive de dicho pico de viremia es resultado de la respuesta CTL y de la secreción de CAF por las células CD8+ CTL. Datos recientes han demostrado que la CTL se correlaciona con la menor carga vírica y menor progresión hacia SIDA (53,54). La mayor actividad CAF también se correlaciona con la no progresión (21). En modelos murinos de infecciones víricas crónicas, se sabe que para lograr una respuesta sostenida de CTL se requiere la presencia de células T colaboradoras (Th) CD4+ virus-específicas. El papel de las Th en las infecciones por lentinvirus se desconoce prácticamente por completo, y es sabido que

incluso en la fase asintomática de la infección crónica por el VIH no se detecta en sangre periférica respuesta Th contra proteínas del VIH. Probablemente ello refleja que las células Th CD4+ VIH-específicas quedan retenidas en los órganos linfoides en las fases de altos niveles de viremia durante la infección aguda, siendo infectadas al contactar, para el reconocimiento antigénico, con macrófagos y células dendríticas infectadas, por lo que en gran parte pueden ser eliminadas al final de la infección aguda y/o permanecer retenidas en dichos órganos aquellas que sobrevivan. Esto es consistente con datos recientes indicativos de que la respuesta Th probablemente sólo puede detectarse en sangre periférica cuando exista un control estricto y persistente de la viremia como ocurre con algunos LTNP y con individuos con infección aguda que controlan la viremia como resultado de terapia antiretrovírica potente instaurada al inicio de la infección aguda (55). Por otro lado, se ignora, por qué la respuesta de anticuerpos neutralizantes aparece tardíamente respecto a los anticuerpos no neutralizantes y respecto a la respuesta de CTL y de la actividad CAF. Una posibilidad sugerida por Zinkernagel (56), en base al modelo de infección murina por el virus de la coriomeningitis linfocitaria, es que los CTL lisen a las células B que producen anticuerpos neutralizantes contra epitopos de las proteínas de cubierta y que corresponderían a células B infectadas por dicho virus vía Igs de membrana que interiorizarían el virus. De hecho, está demostrado que el VIH puede infectar a las células B humanas vía Ig de membrana y receptor de complemento (57).

El papel de los anticuerpos neutralizantes y su correlación con la no progresión ha sido un tema confuso, lo que probablemente se debía a los ensayos de neutralización usando cepas SI adaptadas a líneas de células T (58), pero datos más recientes demuestran claramente que la no progresión se asocia con altos títulos de anticuerpos neutralizantes (1, 59,60). Por otro lado, a partir del conocimiento de la interacción de la gp120/41 de las cepas NSI y SI con los co-receptores respectivos, hay un renovado interés en el análisis

de los efectos de los anticuerpos neutralizantes, tanto de los sueros de pacientes como de anticuerpos monoclonales derivados de hibridomas humanos o murinos, para definir el mecanismo preciso de la neutralización, y la región epitópica sobre la que actúan según sean cepas NSI y SI (58,61,62). A pesar de que existen muchos anticuerpos monoclonales humanos, sólo hay unos pocos dirigidos contra epitopos relevantes para la neutralización, y es posible que en ello influya que no se han derivado de LTNP que son los mejores candidatos para aumentar las probabilidades de que la metodología de hibridomas humanos (63), permita obtener anticuerpos neutralizantes de amplia reactividad, capaces de bloquear a cepas NSI y/o SI de varios subtipos. Tal posibilidad viene demostrada por el anticuerpo monoclonal humano 2F5 que reconoce un epitopo en la proteína gp41 y que neutraliza la infección por cepas NSI y SI (58,61,62,64).

1.3.2 Posibilidad de respuesta inmune protectiva frente al VIH en los individuos expuestos no infectados

La resistencia a la infección de una pequeña proporción de EU puede deberse a las anomalías del gen CCR-5, antes indicadas: homozigosidad para CCR-5Δ32 o combinación de heterozigosidad para m303 y CCR-5-Δ32, u homozigosidad m303, hasta ahora no descrita. Es posible también que existan otras formas de anomalías genéticas de CCR-5 que también impliquen un CCR-5 truncado o no expresado y que concedan resistencia a la infección, por lo que se considera prioritario investigar tal eventualidad en los EU. Por otro lado, hay evidencias de que en algunos EU, la resistencia a la infección revelaría un estado de inmunización anti-VIH protectivo, mediado por células T y sin producción de anticuerpos, y que sería resultado de una "vacunación" natural inadvertida (7-9). Este concepto se fundamenta en que, en algunos EU , se han detectado respuestas mediadas por células Th (en forma de respuesta proliferativa y/o producción de IL-2 frente a péptidos de gp120) así como respuesta de CTL frente a péptidos de proteínas del

VIH-1, incluidas proteínas reguladoras, lo que parece indicar la vía endógena de presentación antigénica, y por tanto sugiere que haya existido un proceso infectivo transitorio, al que la respuesta inmune habría conseguido eliminar o mantener bajo un estricto control. Hay evidencias fuertes de que, en un caso al menos, tal proceso infectivo transitorio probablemente existió, y, por otro lado, en unos pocos casos de EU se ha detectado VIH DNA integrado en células circulantes (véanse 7 y 8). No se sabe por qué y como tal respuesta mediada por células Th y CTL iría unida a falta de respuesta de anticuerpos, aunque resulta verosímil que, dada la importancia del sistema HLA para la eficacia de la presentación antigénica y para determinar la polarización de la respuesta inmune hacia Th1 o Th2 (65), tales EU pudieran presentar factores HLA-dependientes para desarrollar una fuerte respuesta Th1 con actividad citotóxica anti-VIH restringida por moléculas HLA de clase II, que fuera apropiada para sostener una fuerte respuesta CD8+ CTL restringida por clase I , y que tanto las Th1 como las CTL lisaran a las células B específicas contra el VIH vía Clase II y Clase I, respectivamente (56). Tales factores genéticos podrían ir asociados a otros HLA-independientes, indicados antes, que determinaran una alta secreción de RANTES MIP-1α y MIP-1β por parte de las Th1 (24). Es evidente la gran repercusión que, para el desarrollo de una vacuna, tendría determinar que tal respuesta anti-VIH mediada por células T sin producción de anticuerpos, realmente existe y que es capaz de proteger al huésped eliminando o controlando totalmente la replicación vírica.

2. Justificación de la tesis

La expresión fenotípica del co-receptor CCR5 es sin duda uno de los principales factores implicados en la resistencia/susceptibilidad y/o la progresión de la infección por el VIH-1, ya que este es el principal co-receptor del VIH-NSI, cepa viral implicada en la transmisión de la infección y que domina las primeras fases de la enfermedad. La variabilidad interindividual en la expresión de esta molécula es en parte consecuencia de variantes genéticas entre las que cabe destacar la delección de 32 pares de bases (CCR5Δ32) en el gen CCR5, que impide de forma total la expresión del receptor en su forma homozigota y de forma parcial en su forma heterozigota (figura 2). De esta forma los homozigotos son resistentes a la infección por el VIH y los heterozigotos progresan más lentamente. También se han destacar dos mutaciones en el gen promotor del CCR5, una de ellas implicada en la progresión lenta de la infección y otra con el efecto inverso, es decir, asociada a la progresión rápida de la infección. Por otro lado, la mutación en la posición 64I del receptor CCR2, se ha implicado con una progresión lenta de la infección. Hoy día no se conoce bien cual es el mecanismo que media este efecto, pero algunos

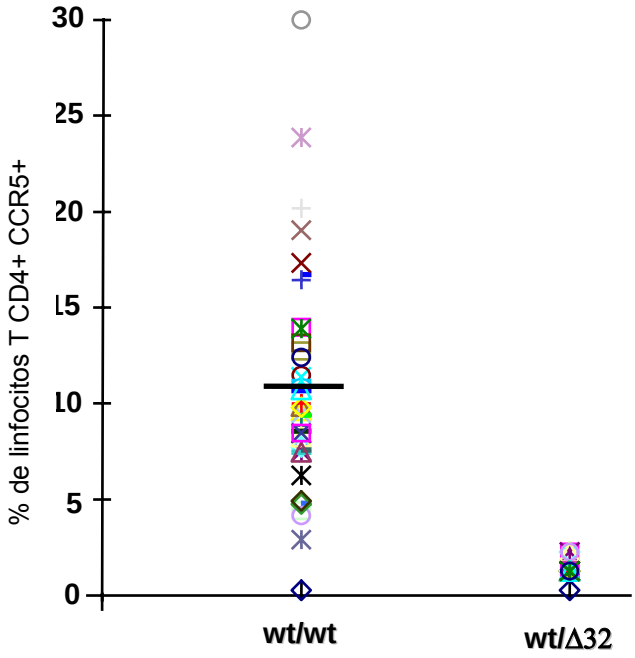

Figura 2. Porcentaje de linfocitos T CD4 que expresan CCR5 en individuos no portadores (wt/wt) y portadores (wt/Δ32) de la delección de 32 pares de bases en el gen de CCR5. La línea negra horizontal representa la media. Fuente: datos elaborados en la presente tesis.

estudios apuntan que actuaría alterando el proceso de dimerización del CCR5 y otros trabajos asocian esta mutación con una mutación en el promotor del CCR5. Por otro lado, en la figura 2 se puede apreciar como la expresión de CCR5 en los individuos con genotipo salvaje (wt/wt) es muy variable, lo cual se debe a que la expresión de CCR5 depende de un complejo sistema de regulación, que apenas estamos empezando a conocer. Por todo ello, consideramos de gran interés la búsqueda de nuevos elementos inmunogenéticos que pudieran influir en la expresión de los principales co-receptores del VIH y en consecuencia estar implicados en la susceptibilidad/resistencia a la infección por el VIH y/o su progresión hacia Sida.

Por otro lado, es preciso detallar la influencia de algunos factores inmunogenéticos previamente descritos, como es el caso de la mutación SDF1-3'A, respecto a la cual se han presentado datos contradictorios respecto a su papel en la susceptibilidad/resistencia y/o progresión de la enfermedad.

2.1. Estudio 1: Polymorphisms in the interleukin-4 receptor alpha chain gene influence the susceptibility to HIV-1 infection and its progression to AIDS.

Este trabajo se sitúa en la línea de establecer nuevos marcadores que influyan en la susceptibilidad y/o progresión a Sida mediante la regulación de los principales co-receptores del VIH (CCR5 y CXCR4). La IL-4 in vitro disminuye la expresión de CCR5 e inhibe la propagación de las cepas R5, mientras que aumenta la expresión de CXCR4 y favorece la diseminación de las cepas X4. Además la IL-4 potencia la diferenciación de los linfocitos Th hacia una respuesta tipo 2 e inhibe la generación de linfocitos Th1, lo cual permite hipotetizar que niveles elevados de IL-4 o una respuesta inadecuada (excesiva) del receptor de la IL-4 podrían acompañarse de una respuesta CD8 citotóxica inadecuada favoreciendo la progresión de la infección. Como se comenta en la introducción, se ha descrito un polimorfismo en la región promotora de la IL-4, aunque diferentes trabajos no se ponen de acuerdo sobre su influencia en la progresión de la infección. Hasta el momento, no se ha evaluado la posible influencia de los diferentes polimorfismos descritos del receptor de la IL-4, en la susceptibilidad y/o progresión de la infección por el VIH. La IL-4 actúa mediante su interacción con el receptor IL4-α, del que se han descrito más de 14 polimorfismos en su región codificada y que comportan el cambio de un solo nucleotido (SNP). Solo un SNP, I50V, se encuentra en el exon 5 que codifica para la región extracelular, mientras que el resto se hallan en el exón 12 que codifica la región intracitoplasmática. En la actualidad existe evidencia de que I50V y 2 SNP situados en el exón 12, afectan la función del receptor, por lo que parece razonable postular que las diferentes variantes del receptor podrían estar relacionadas con la susceptibilidad y/o progresión de la infección por el VIH.

Objetivo concreto:

1.- Determinar la influencia de los polimorfismos del receptor de la IL-4 en la susceptibilidad y/o progresión de la infección por el VIH.

2.2. Estudio 2: A novel polymorphism in the 5' untranslated region (5'UTR) of CD28 gene is associated with susceptibility to HIV-1 infection.

Las principales células productoras de CAF son las células CD8+CD28+ (21) y se relacionan con la no progresión, mientras que el aumento de células T CD8+CD28- es una de las alteraciones más conspicuas y precoces de la progresión de la infección. Estos hechos permiten pensar en una posible relación entre CD28 y expresión de CCR-5, así recientemente se ha demostrado que tras la activación in vitro de las células T usando anticuerpos inmovilizados contra CD28 se impide la activación del promotor de CCR5 (66) y la expresión de CCR-5, CD28-específica y provoca resistencia a la infección in vitro por cepas NSI (18,19), fenómeno que puede estar relacionado además con la activación inespecífica, inducida por CD28 del promotor de RANTES, ligando natural del CCR5, capaz de bloquear, también, la infección por cepas NSI del VIH (67) .

Por otro lado se ha descrito que la activación de linfocitos CD4 inmovilizados con anticuerpos monoclonales frente a CD28 induce una mayor actividad del promotor de CXCR4 (68) y mayor expresión fenotípica de dicho receptor, principal co-receptor de la variante inductora de sincitios del VIH-1(44), que como hemos comentado se asocia a una rápida progresión de la enfermedad. Este efecto junto a la disminución en la expresión fenotípica de CCR5, ejercería una presión selectiva sobre el VIH-1 favoreciendo la emergencia de la variante SI del VIH y con ello una progresión rápida de la enfermedad. Todo ello demuestra la importancia de la molécula CD28 en la expresión de los principales co-receptores del VIH-1, aunque de momento los hallazgos sean contradictorios. Puede postularse que existan variaciones polimórficas en CD28 y/o en CD80 y CD86 (los contrareceptores fisiológicos de CD28), o en CTLA4 (molécula afín al CD28, que actúa como contra-reguladora del efecto del CD28 en la activación linfocitaria), que pudieran asociarse con una menor/mayor expresión de los co-receptores del VIH-1 y la consiguiente resistencia/susceptibilidad a la infección/ progresión. La influencia de

posibles polimorfismos, no se reduce a la regulación sobre los co-receptores del VIH-1, sino que además se conoce que aunque la expresión de CD28 es constitutiva, sus niveles en superficie pueden ser modulados por mitógenos (71,72) y por tanto polimorfismos en CD28 podrían implicar respuestas diferentes a estos elementos reguladores, lo cual es de gran importancia, ya que esta es una molécula co-estimuladora fundamental para la respuesta inmune.

Estos datos permiten postular la posible existencia de polimorfismos en la molécula del CD28 podrían estar implicada en la susceptibilidad y/o progresión de la infección por el VIH. Es conocida la ausencia de polimorfismos en el gen estructural de CD28, pero no se ha estudiado hasta el momento actual el promotor de CD28, por lo que basaremos nuestro trabajo en el estudio de polimorfismos a este nivel y su posible relación con la susceptibilidad y/o progresión de la infección por el VIH.

Objetivos concretos:

1.- Análisis de la existencia de posibles polimorfismos en el promotor del gen CD28 mediante la técnica de SSCP y posterior secuenciación de las variantes halladas.

2.- Analizar la relación con la resistencia/susceptibilidad a la infección y a la progresión de la misma infección por el VIH-1.

2.3. Estudio 3: Plasma Stromal Cell–Derived Factor (SDF)-1 Levels, SDF1-3'A Genotype, and Expression of CXCR4 on T Lymphocytes: Their Impact on Resistance to Human Immunodeficiency Virus Type 1 Infection and Its Progression.

Se ha descrito un polimorfismo en la región no traducida del gen SDF-1 (SDF1-3'A), ligando natural del CXCR4 (co-receptor de la variante SI del VIH, implicada en el descenso de CD4 y la progresión rápida a SIDA). Esta mutación en su forma homozigota se ha relacionado con progresión lenta hacia el desarrollo de SIDA, aunque, como se cita en la introducción, otros 3 trabajos no confirman estos resultados e incluso los contradicen, puesto que asocian la mutación a una progresión más rápida de la infección. Las consecuencias biológicas de esta mutación no se conocen por lo que consideramos como hipótesis de trabajo, que el impacto de la mutación SDF1-3'A sobre la susceptibilidad y/o progresión de la infección por el VIH dependerá de cómo este polimorfismo modifique la producción de SDF-1 que a su vez modulará la expresión de CXCR4. Puesto que SDF-1 es el prototipo de una quimiocina homeostática, producida por un gran número de células y tejidos, podemos postular que en caso de que la mutación SDF1-3'A se asocie a niveles bajos de SDF-1, esto supondrá una alta expresión de CXCR4 y este patrón será propio de los pacientes con una progresión normal o rápida hacia Sida y a la inversa en caso de asociarse a niveles altos. Para ello determinaremos el genotipo SDF-1, los niveles plasmáticos de SDF-1 y la expresión de CXCR4 en los grupos de estudio.

Objetivos concretos

1.- Correlacionar los niveles de SDF-1 con la presencia o no de la mutación SDF1-3'A.

2.- Analizar el genotipo, los niveles de SDF-1 y la expresión de CXCR4 en los diferentes grupos de estudio.

3. Cohortes de pacientes estudiadas:

1.- El grupo de pacientes **"Long-Term Non-Progressors"** (LTNP) se define clásicamente como aquellos individuos que mantienen un número de CD4>500 células/mm^3, tras más de 8 años de infección y sin tratamiento antirretroviral. Con estas características estudiamos un total de 73 pacientes, de ellos 35 se siguen en el Hospital Clínic de Barcelona, 13 en el Hospital la Fe de Valencia y 25 en el Centro Sandoval de Madrid. Pero además es aceptado por todo el mundo que aquellos pacientes capaces de controlar la carga viral sin tratamiento se pueden y deben considerar como auténticos LTNP. Por ello incluimos en este grupo un total de 18 pacientes controlados en el Hospital clínic de Barcelona que tienen un mínimo de 3 determinaciones separadas por 3 meses o más, de carga viral inferiores a 200 copias/ml (límite inferior de la técnica), sin tratamiento antiretroviral y más de 1 año de evolución desde la primoinfección.

2.- El grupo de **expuestos no infectados** (EU) está constituido por un total de 73 personas con exposición repetida al VIH y que a pesar de ello persisten seronegativos y con carga viral indetectable, realizada por lo menos en 4 ocasiones consecutivas con intervalos de 6 meses. 36 de ellos corresponden a hemofílicos expuestos a una media anual de 11.000 UI (intervalo de confianza 95%: 7-15) de concentrados de factor 8 no inactivados entre 1982 y 1985, seguidos en el Hospital la Fe de Valencia (EU-par). Durante este período más del 60% de los pacientes hemofílicos seguidos en esta unidad se infectaron por el VIH. 27 son personas que han mantenido una media de más de 600 relaciones sexuales (anales o vaginales) sin protección con parejas conocidas VIH+, controladas en el Centro Sandoval de Madrid (EU-sex). Por último 11 personas, 3 adictos a drogas por vía parenteral que comparten jeringuillas con pacientes VIH+ (englobados para el análisis en el grupo EU-par) y 8 con relaciones sexuales sin protección con parejas conocidas VIH+ (englobados para el análisis en el grupo EU-sex), todos ellos

identificados en el Hospital Clínic de Barcelona. Para todos los análisis consideramos dos subgrupos dentro de los pacientes EU, aquellos con exposición al VIH por vía parenteral (n=39) y los expuestos por vía sexual (n=35).

3.- Como **grupo control VIH positivo**, estudiamos un total 72 pacientes que presentaron un descenso de CD4 por debajo de 350 células / mm^3, en un tiempo inferior a 8 años, motivo por el cual la mayoría iniciaron tratamiento antirretroviral. 35 eran hemofílicos controlados en el Hospital la Fe de Valencia y 37 pacientes VIH positivos controlados en el Hospital Clínic de Barcelona (VIH-TP, de typical progressors).

Las cohortes de Madrid y Valencia son seroncidentes, mientras que la del Hospital Clínic es seroprevalente y se ha considerado el tiempo de infección en función del inicio de las prácticas de riesgo.

4.- Por último se estudia **grupo control VIH negativo** procedente de una cohorte de donantes de sangre sanos del Banco de Sangre del Hospital Clínic.

4. Copia de los trabajos de investigación

Estudio 1: **Alex Soriano**, Francisco Lozano, Harold Oliva, Felipe García, Meritxell Nomdedéu, Elisa De Lazzari, Carmen Rodríguez, Alicia Barrasa, José I. Lorenzo, Jorge del Romero, Montserrat Plana, José M. Miró, José M. Gatell, Jordi Vives, Teresa Gallart. *Polymorphisms in the interleukin-4 receptor alpha chain gene influence the susceptibility to HIV-1 infection and its progression to AIDS.* Immunogenetics 2005; 57: 644-654

IF: 2.69*

Estudio 2: **Alex Soriano**, Harold Oliva, Montserrat Plana, Felipe García, Meritxell Nomdedéu, Elisa de Lazzari, Francisco Lozano, José M. Miró, Jorge Del Romero, Carmen Rogríguez, Alicia Barrasa, José I. Lorenzo, José M. Gatell and Teresa Gallart. *A novel polymorphism in the 5' untranslated region (5'UTR) of CD28 gene is associated with susceptibility to HIV-1 infection.* Pendiente de ser evaluado para su publicación en el momento de la presentación de la tesis.

Estudio 3: **Alex Soriano**, Catalina Martínez, Felipe García, Montserrat Plana, Eduard Palou, Marylene Lejeune, Juan I. Aróstegui, Elisa De Lazzari, Carmen Rodriguez, Alicia Barrasa, José I. Lorenzo, José Alcamí, Jorge del Romero, José M. Miró ,José M. Gatell and Teresa Gallart. *Plasma Stromal Cell–Derived Factor (SDF)-1 Levels, SDF1-3'A Genotype, and Expression of CXCR4 on T Lymphocytes: Their Impact on Resistance to Human Immunodeficiency Virus Type 1 Infection and Its Progression.* J Infect Dis 2002; 186:922-931.

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ORIGINAL PAPER

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Polymorphisms in the interleukin-4 receptor α chain gene influence susceptibility to HIV-1 infection and its progression to AIDS

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Abstract Interleukin (IL) 4 is a key T helper-2 cytokine that downregulates and upregulates CCR5 and CXCR4, respectively, the main coreceptors for HIV. Our objective is to investigate whether single-nucleotide polymorphisms (SNPs) in the IL-4 receptor α chain gene (*IL4RA*) affect HIV infection and its progression to AIDS. The I50V SNP in exon 5 and the haplotypes of six SNPs in exon 12 (E375A, C406R, S411L, S478P, Q551R, and V554I) were studied by polymerase chain reaction and sequencing in 30 HIV^+ long-term nonprogressors (LTNP), 36 HIV^+ typical progressors (TP), 55 highly exposed but uninfected individuals (EU), 25 EU-sexuals (EU-Sex; mostly women) and 30 EU-hemophiliacs (EU-Hem; hepatitis C $virus^+$), and 97 healthy controls (HC), all Caucasians and lacking *CCR5Δ32* homozygosity. V50 homozygosity was increased in LTNP (44%) compared with the other groups [p=0.005; relative risk ratio=3.4, 95% confidence interval (CI)=1.12–10.6, p=0.03]. The most common (C) exon 12 haplotype, ECSSQV, predominated in all groups, but uncommon (U) haplotypes were increased in HIV^+ individuals (n=64), especially in those (51 of 64) infected via parenteral exposure (35.3%) compared with HC (20.4%) and EU-Hem (18.4%) [p=0.01; odds ratio (OR)=2.14, 95% CI=1.25–3.67, p=0.01]. EU-Sex also had an increased frequency of U-haplotypes (34.8%) (OR=2.10, 95% CI= 1.03–4.21, p=0.01) as well as an increased frequency of CU + UU genotypes (60.9%) compared with HC (38.2%) and EU-Hem (26.6%) (p=0.043). Distributions of genotypes fitted Hardy–Weinberg equilibrium. Data suggest that V50 homozygosity associates with slow progression and that exon 12 U-haplotypes might be associated with both susceptibility to infection via parenteral route and resistance to infection via sexual exposure. Further studies are required to confirm these findings.

Keywords HIV infection · Interleukin-4 receptor α chain · Single-nucleotide polymorphisms · Long-term nonprogressors · Exposed but uninfected

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Introduction

Variants of host genes are important determinants of susceptibility to HIV-1 infection and its rate of progression to AIDS. Thus far, the genes that have been mainly studied are those encoding chemokines and chemokine receptors involved in HIV entry, as well as human leukocyte antigen class I molecules that impact the effectiveness of cytotoxic T cells in recognizing HIV antigens and are ligands of some activating and inhibitory killer cell immunoglobulin (Ig)-

like receptors (KIRs) that are mainly expressed on natural killer cells and in some T-cell subpopulations (reviewed in Kaslow et al. 2005; Nolan et al. 2004; O'Brien and Nelson 2004; Stephens 2005; Tang and Kaslow 2003; Theodorou et al. 2003; Colobran et al. 2005; Gonzalez et al. 2005).

Other components of the innate or adaptive immune system that have been, or are being, investigated as candidate genes impacting on HIV infection susceptibility include KIR (Martin et al. 2002), T helper (Th)-2 cytokines interleukin (IL) 4 (Kwa et al. 2003; Modi et al. 2003; Nakayama et al. 2000, 2002; Singh et al. 2004) and IL-10 (Shin et al. 2000; Vasilescu et al. 2003), mannose-binding lectin (Ji et al. 2005), FAS–FAS-L (Vasilescu et al. 2004), DC-SIGN (CD209) (Martin et al. 2004), and APOBEC3G (An et al. 2004; Do et al. 2005).

IL-4 and its receptor *IL-4R* are very important candidate genes that affect susceptibility to HIV infection and its progression to AIDS due to two reasons. First, apart from its effects on B cells to induce Ig isotype switching to IgE, IL-4 induces differentiation of $CD4^+$ Th cells into Th2 cells, which, in turn, are characterized by production of IL-4 and lack of interferon γ (IFN-γ) production. Th1 cells are characterized by production of IFN-γ and absence of IL-4 production. In addition, IL-4 inhibits generation of Th1 cells, while IFN-γ inhibits generation of Th2 cells (Ho and Glimcher 2002; Robinson and O'Garra 2002). Th1 response is critical to cytotoxic $CD8^+$ T-cell response to viral and other intracellular pathogens and predominates in early stages of asymptomatic chronic HIV infection and in long-term nonprogressors (LTNP), whereas progression to AIDS involves a shift to a response of Th2 or Th0 cells, both of them producing IL-4 (Clerici and Shearer 1994; Klein et al. 1997; Ledru et al. 2003; Maggi et al. 1994; Meyaard et al. 1996; Plana et al. 2000; Torres et al. 1998).

The second reason is that IL-4 regulates the expression of chemokine receptors CCR5 and CXCR4, the main coreceptors used by HIV in entry into $CD4^+$ cells. CCR5 is used by non-syncytium-inducing HIV-1 strains (now designated as R5 strains) that usually transmit infection and predominate in asymptomatic stages of HIV infection, while CXCR4 is employed by more pathogenic syncytium-inducing HIV strains (now designated as X4 strains) that appear later during chronic infection (in 50% of cases), an event associated with rapid decline of $CD4^+$ T cells and fast progression to AIDS (Berger et al. 1999; Connor et al. 1997). In vitro, IL-4 downregulates CCR5 expression and inhibits propagation of R5 strains, while it upregulates CXCR4 expression and promotes spread of X4 strains, while IFN-γ downregulates CXCR4 and upregulates CCR5, and while X4 strains replicate preferentially in Th0 and Th2 cells (Galli et al. 1998; Jourdan et al. 1998; Patterson et al. 1999; Penn et al. 1999; Suzuki et al. 1999; Valentin et al. 1998; Wang et al. 1998; Zoeteweij et al. 1998). In the macaque model, IL-4 also promotes replication of X4 simian × human immunodeficiency viruses in vitro, and IL-4 antisense DNA inhibits viral replication in vitro and in vivo (Dhillon et al. 2005). Together, these data strongly support the hypothesis that polymorphic variants of *IL-4* and/or *IL-4R* genes affecting IL-4 production and/or IL-4R function can influence both the susceptibility to HIV infection and its progression to AIDS.

Regarding IL-4, there was a single-nucleotide polymorphism (SNP) in the promoter region of *IL-4* gene (designated as IL-4 −589T) that was found to be associated with enhanced transcription and elevated serum IgE levels in asthmatic families (Rosenwasser et al. 1995), and its impact on HIV infection and progression was investigated as aforementioned, with divergent results, perhaps depending on the ethnicity of the population studied (Biasin et al. 2003; Kwa et al. 2003; Modi et al. 2003; Nakayama et al. 2000, 2002; Singh et al. 2004). IL-4 operates through IL-4R, which is a heterodimeric complex comprising the IL-4Rα chain (IL-4Rα) and either the common γ chain (type I IL-4R) or the IL-13Rα1 chain (type II IL-4R). Type II IL-4R is also the receptor of IL-13, another key Th2 cytokine that shares most of the IL-4 functions (Hershey 2003; Nelms et al. 1999). To date, more than 14 SNPs have been identified in the coding region of the IL-4 receptor α chain gene (*IL4RA*). Only one SNP, I50V, which encodes for part of the extracellular domain, is found in exon 5, with others being located in exon 12, which encodes for most of the intracytoplasmic region. These exon 12 SNPs are mostly in close proximity to relevant signal transduction motifs. There is evidence that the I50V SNP and two exon 12 SNPs (S478P and Q551R) affect IL-4R function on IgE production and related events and have been extensively studied in the field of IgE-mediated allergic diseases, such as atopic asthma (Deichmann et al. 1997; Hershey et al. 1997; Hoffjan and Ober 2002; Kruse et al. 1999; Lozano et al. 2001; Mitsuyasu et al. 1998, 1999; Ober et al. 2000; Risma et al. 2002; Rosa-Rosa et al. 1999). To our knowledge, no one has so far analyzed the possible influence of *IL4RA* SNPs on the resistance susceptibility to HIV infection and its rate of progression to AIDS. This study was undertaken to investigate this possibility.

Materials and methods

Study subjects Individuals were recruited from "Hospital Clinic" (Barcelona), "Hospital La Fe" (Valencia), two university hospitals, and "Centro Médico Sandoval" (Madrid), which was a specialized center for sexually transmitted diseases. These centers are public, nonprofit, and academic institutions of the Spanish National Health System. Institutional ethical committees approved the study, and patients gave informed consent. All individuals included in the study were European Caucasians and lacked *CCR5Δ32* homozygosity. They were divided into the following groups: (1) HIV-1^+ individuals, subdivided into two categories: (a) a group of LTNP (n=30), most of whom (76.6%) are intravenous drug users, defined as asymptomatic individuals with blood CD4 T-cell counts >500 mm^{-3} after >10 years of known seroprevalence in the absence of antiretroviral therapy (76.6%); (b) a group of 36 typical progressors (TP), mostly (88.8%) corresponding to hemo-

philiacs, defined as patients with a decrease of CD4 T-cell counts below 350 mm^{-3} during the first 7 years from seroconversion. It should be noted that, as indicated in the text, for some comparisons of exon 12 polymorphisms, we used all infected individuals (LTNP + TP) studied for these polymorphisms as one group (n=64); from all these infected individuals, we also segregated as one group (designated as Par) all those who were infected through parenteral exposure (intravenous drug users or hemophiliacs), who were predominant (51 of 64); (2) a group (n=55) of highly exposed but uninfected individuals (EU) (i.e., seronegative and with undetectable plasma viral load after at least four consecutive determinations at 6-month intervals); these EU were divided into two subgroups: (a) 25 individuals (EU-sexuals or EU-Sex) [19 females (76%), 4 heterosexual men, and 2 homosexual men] who have had high exposure through unprotected sex with HIV-infected partners during the last 12 months before the study, with a median of >600 vaginal or anal intercourses with ejaculation during the period of follow-up, which ranged between 1 and 4 years; these EU-Sex and their HIV^+ partners belonged to a large cohort of individuals with a long follow-up ("Centro de Salud Sandoval," Madrid) (Pedraza et al. 1999); and (b) 30 hemophiliacs (EU-hemophiliacs or EU-Hem) who were highly exposed to nonviral inactivated clotting factor concentrates before 1986; they belonged to a large cohort of hemophiliacs, who mostly became HIV-1-infected, and, all of them, including the 30 EU-Hem studied here, also became infected with hepatitis C virus ("Hospital La Fe") (Lorenzo et al. 2001; Montoro et al. 1995); (3) healthy controls (HC) (n=97) matched for sex and age with the HIV^+ and EU groups, who had normal biological parameters, including negativity for HIV-1, HCV, and other infections, as screened for altruistic blood donors. Not all individuals could be assessed for all polymorphisms, with the precise number being indicated in the text.

Detection of SNPs in exons 5 and 12 of IL4RA and CCR5Δ32 polymorphism DNA isolation was performed as reported (Soriano et al. 2002). A polymerase-chain-reaction (PCR)-based analysis and a nucleotide sequence analysis of SNPs in exons 5 and 12 were performed; the positions of SNPs and primers used are shown in Fig. 1 For the analysis of I50V SNP in exon 5, a 275-bp fragment was amplified using the sense 5′-TCTGTCCTCACATCCGTGAT-3′ and antisense 5′-CTCGCTGGGCTTGAAGGAG-3′ primers (Fig. 1). The cycling conditions used were: 95°C for 8 min; 32 cycles of 95°C for 45 s, 55°C for 45 s, 72°C for 1 min; and a final cycle extension at 72°C for 10 min. The analysis of SNPs in exon 12 was performed, as already reported (Lozano et al. 2001). Briefly, a 1,657-bp fragment was amplified using the primers 5′-CAAGTGTCGA AA CTGAACCCT-3′ (sense) and 5′-CCTCATCTGCA GACT CAGCCAA-3′ (antisense) (Fig. 1), with the following cycling conditions: 95°C for 8 min; 32 cycles of 95°C for 45 s, 60°C for 45 s, 72°C for 2 min; and a final cycle extension at 72°C for 10 min. The PCR products of exons 5 and 12 were sequenced either directly or after purification with the QIAquick PCR purification kit (Qiagen, Germany) using the sense primers 5′-TCTGTCCTCACATCCGTGAT-3′ and 5′-GGAGGAGGTAGAGGAAGAA-3′, respectively. Sequencing was performed with the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK), following the manufacturer's instructions. The sequencing reactions were analyzed on an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). To detect *CCR5Δ32*, PCR was performed with primers spanning the 32-bp deletion, as reported (Dean et al. 1996; Soriano et al. 2002).

Statistical analysis The frequency of alleles, haplotypes, and genotypes among groups was compared using chi-square test or Fisher exact test, as appropriate. When a

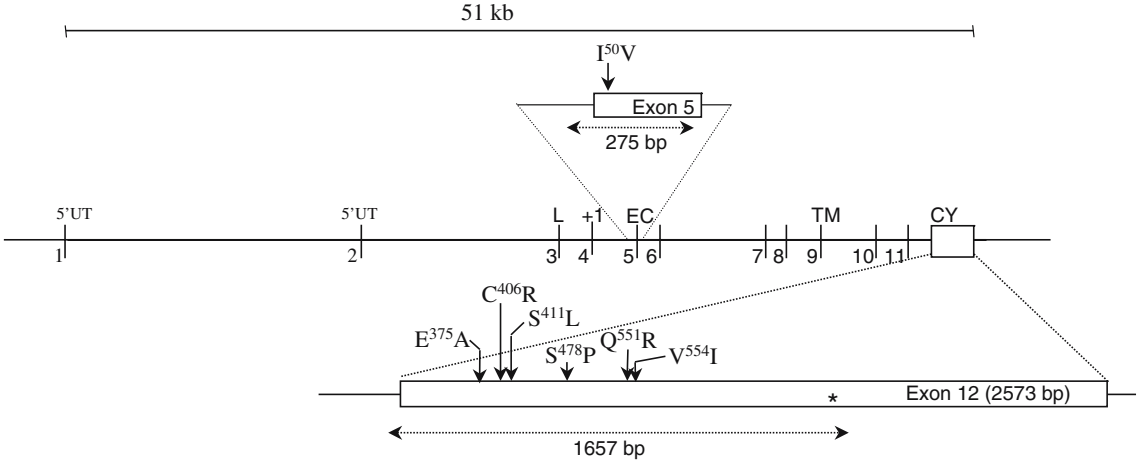

Fig. 1 Schematic representation of the human *IL4RA* gene with exon/intron organization drawn to scale at the top. Exon 5 at the extracellular region (*EC*) and exon 12 at the cytoplasmic region (*CY*) have been magnified. The SNPs analyzed in the current study are indicated with *vertical arrows*. *Arrowhead-ended horizontal dotted lines* show the length of amplified products and the relative position (31) of primers used for amplification. SNPs are numbered by their position in the mature protein and flanked, on the *left*, with the consensus amino acid and, on the *right*, with the amino acid that results from the SNP. A *one-letter code* is used to indicate amino acids. *5′ UT* 5′ Untranslated region, *L* leader peptide, *+1* initiation of mature protein, *TM* transmembrane region. An *asterisk* indicates the relative position of the natural stop codon

significant difference in distribution was found, we applied a logistic regression model for allelic or haplotype comparisons or a multinomial regression model for genotypic comparisons. All tests were two-tailed, with $p \leq 0.05$ considered statistically significant. To evaluate whether the distribution of genotypes fulfilled the Hardy–Weinberg equilibrium (HWE), a goodness-of-fit chi-square test was used to compare the observed number of subjects with the expected number according to the HWE (Xu et al. 2002).

Results

Allele and genotype frequencies of I50V SNP in exon 5 of *IL4RA*

As shown in Fig. 2, the I50 allele was the most frequent in all groups (from 56 to 63.9%) except in LTNP, in whom the V50 allele predominated (60%); these differences did not reach statistical significance (p=0.09). The I50V genotype distribution showed a highly significant difference (p=0.005), which appeared to be mainly due to the higher frequency of V50 homozygotes in LTNP (43.3%) compared with the other groups (14.4% in HC, 16.7% in TP, 28% in EU-Sex, and 16% in EU-Hem). A multinomial logistic regression model used to assess the relative risk ratio (RRR) of a given genotype for each group was applied, considering I50 homozygosity and HC group as reference categories, and showed that LTNP had a significantly higher risk of being V50-homozygous [RRR=3.449, 95% confidence interval (CI)=1.12–10.6, p=0.031]. It should be noted that the allele and genotype frequencies of I50V among HC were entirely consistent with data reported in other European Caucasian populations (Bottini et al. 2002; Howard et al. 2002). The I50V genotype distribution in the different groups was not significantly different from that expected, assuming HWE (Table 1).

It is well-known that *CCR5Δ32* heterozygosity has been found to associate with slow progression in studies with some cohorts (Dean et al. 1996; Huang et al. 1996; Ioannidis et al. 2001; Samson et al. 1996). Although we have found that the frequency of this genotype was slightly increased in LTNP (16.6%), there were no significant differences compared with TP (15%) and HC (15.3%) (p>0.5). Only 1 of 13 V50 homozygotes found in LTNP (13 of 30, 43.3%; Fig. 2) and 2 of 6 V50 homozygotes present in TP (6 of 36, 16.7%; Fig. 2) carried *CCR5Δ32* heterozygosity. Of note, the frequency of *CCR5Δ32* heterozygotes in HC (15.3%) was entirely consistent with that reported by others in other Caucasian populations of Europe (Libert et al. 1998).

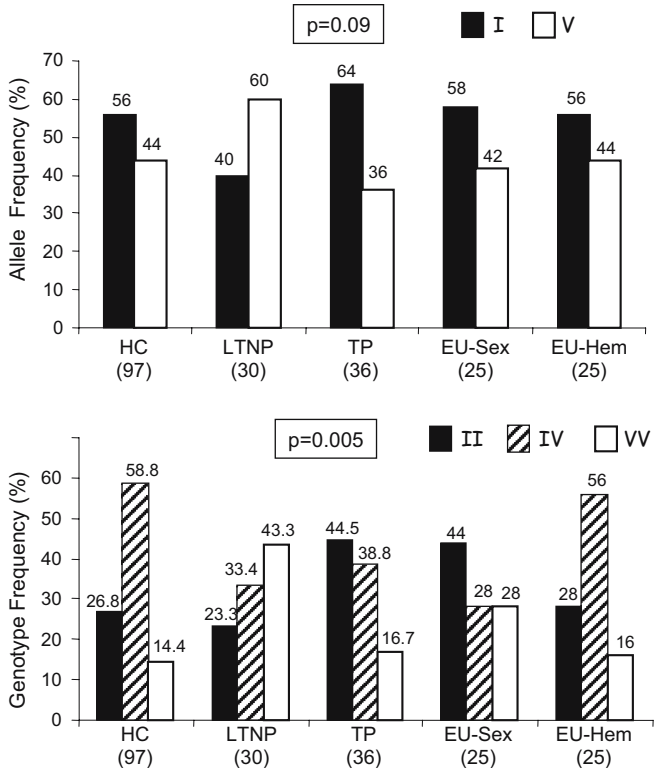

Fig. 2 Allele (*top*) and genotype (*bottom*) frequencies of I50V SNP in the study groups. Whole p values are shown. Above each column, precise percent values are shown. Below each group, the number of individuals is indicated *in parentheses*. *HC* Healthy controls, *LTNP* long-term nonprogressors, *TP* typical progressors, *EU* highly exposed but uninfected individuals, *EU-Sex* EU exposed via unprotected sex, *EU-Hem* hemophiliacs highly exposed to HIV-contaminated clotting factors (all hepatitis C virus^{+})

Haplotype and haplotype–genotype frequencies of exon 12 SNPs of *IL4RA*

The six SNPs in exon 12 of *IL4RA* yielded ten different haplotypes, with the most common (C) haplotype being ECSSQV in all groups, with a frequency of 79.6% in HC, 70.0% in LTNP, 67.6% in TP, 65.2% in EU-Sex, and 81.6% in EU-Hem (Table 2, Fig. 3a). The frequency of U-haplotypes (containing A375 and/or R406 and/or L411 and/or P478 and/or R551 and/or I554) was increased in LTNP (30%), TP (32.4%), and EU-Sex (34.8%), compared with HC (20.4%) and EU-Hem (18.4%), with these differences being at the limit of statistical significance (p=0.050) (Fig. 3a). Of note, the frequency of C- and U-haplotypes in HC was entirely consistent with our previous data in another group (n=45) of healthy Caucasians in the same area of Barcelona (Lozano et al. 2001).

Table 1 HWE of genotype distribution in study groups of I50V SNP in exon 5 of *IL4RA*

Groups	I50I	I50V	V50V	p value
HC (n=96)	26	56	14	0.193
LTNP (n=30)	7	10	13	0.246
TP (n=36)	16	14	6	0.641
EU-Sex (n=25)	11	7	7	0.104
EU-Hem (n=25)	7	14	6	0.793

Shown are the observed numbers of individuals with each genotype for each study group. The p values correspond to the comparison with the expected number of genotypes according to the HWE. The abbreviations of study groups are as indicated in Fig. 2

Table 2 Haplotypes of *IL4RA* exon 12 SNPs and their frequency in study groups

Haplotype							Haplotype frequency				
SNP position and amino acid encoded	375 E/A	406 C/R	411 S/L	478 S/P	551 Q/R	554 V/I	HC	LTNP	TP	EU-Sex	EU-Hem
1. ECSSQV	GAG	TGC	TCG	TCC	CAG	GTA	0.795	0.700	0.676	0.652	0.816
2. ECSPQV	—	–t	—	C–	—	—	0.000	0.050	0.014	0.000	0.000
3. ECSSRV	—	—	—	—	-G-	—	0.015	0.066	0.073	0.065	0.066
4. ECSPRV	—	—	—	C–	-G-	—	0.000	0.016	0.014	0.021	0.016
5. ECLPRV	—	–t	-T-	C–	-G-	—	0.025	0.000	0.044	0.021	0.033
6. ACSSRI	-C-	—	—	—	-G-	A–	0.015	0.000	0.000	0.000	0.000
7. ACSPRV	-C-	—	—	C–	-G-	—	0.000	0.016	0.000	0.043	0.000
8. ARSPRV	-C-	C–	—	C–	-G-	—	0.145	0.150	0.176	0.173	0.066
9. ACSSRV	-C-	—	—	—	-G-	—	0.005	0.000	0.000	0.000	0.000
10. ARSPQV	-C-	C–	—	C–	—	—	0.000	0.000	0.000	0.021	0.000

Nucleotide and deduced amino acid sequences of SNPs in *IL4RA* of exon 12 are shown. Ten different haplotypes are observed, which are named by the one-letter code of the amino acid encoded. Hyphens indicate nucleotide identity with the consensus SNPs that define the most common haplotype 1. Lower-case t in haplotype 2 corresponds to a silent nucleotide change. Abbreviations of study groups are as indicated in Fig. 2

The similarly increased frequency of U-haplotypes in both LTNP and TP suggested that this haplotype lacked influence on the progression of HIV infection and could be perhaps related to the infected status itself. Therefore, we considered all infected individuals (LTNP + TP) studied for C/U-haplotypes as one group (n=64, U-haplotype frequency=32.1%) for comparison with the other groups, and a significant difference was observed (p=0.026) (Fig. 3b). Given that EU-Hem showed the lowest frequency of U-haplotypes (18.4%) [even lower than that of HC (20.4%)], that most LTNP and TP patients (51 of 64) acquired HIV infection through parenteral exposure (intravenous drug users and hemophiliacs, respectively), and that EU-Sex also had an increased frequency of U-haplotypes (34.8%), we hypothesized that C/U-haplotypes might perhaps differentially affect the susceptibility to HIV infection via parenteral or sexual exposure. To gain insights about this possibility, we considered those individuals who became infected as a group through parenteral exposure (designated as Par, n=51, U-haplotype frequency=35.3%) and compared it with the other groups. We found clearly significant differences (p=0.010), which likely reflected the higher frequency of U-haplotypes in Par and EU-Sex groups compared with HC and EU-Hem (Fig. 3c). Accordingly, both Par and EU-Sex were more likely to be U-carriers than C-carriers compared with HC [odds ratio (OR)=2.14, 95% CI=1.25–3.67 for Par, p=0.010; OR=2.10, 95% CI=1.03–4.21 for EU-Sex, p=0.010], whereas EU-Hem individuals did not differ from HC. When the comparison excluding the EU-Sex group (n=23) was made, the highly significant difference was not affected (p=0.011) (Fig. 3d). In contrast, when EU-Sex and EU-Hem were

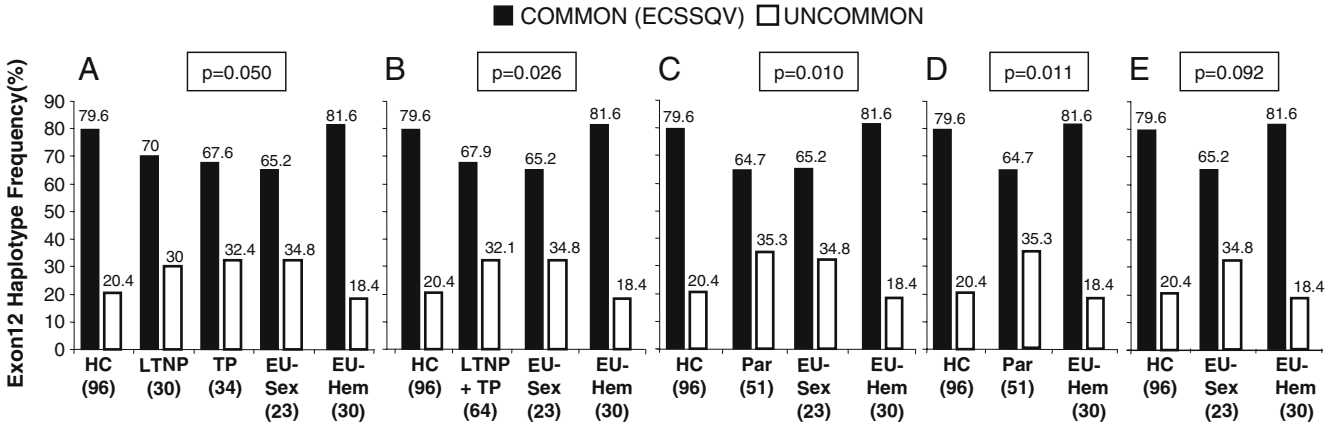

Fig. 3 Frequencies of the exon 12 most *COMMON* haplotype ECSSQV and any of the *UNCOMMON* haplotypes in the different groups. **a**–**e** Comparisons with the groups indicated; for each comparison, the whole p value is shown (Fisher exact test). Below each group, the number of individuals is indicated *in parentheses*. Above each column, the precise percent value is shown. **b** All infected individuals (LTNP+TP=64) are considered as one group. **c** The group named Par (n=51) corresponds to all LTNP + TP subjects infected through parenteral exposure (51 of 64; intravenous drug users or hemophiliacs). Other abbreviations of study groups are as indicated in Fig. 2 and Table 1

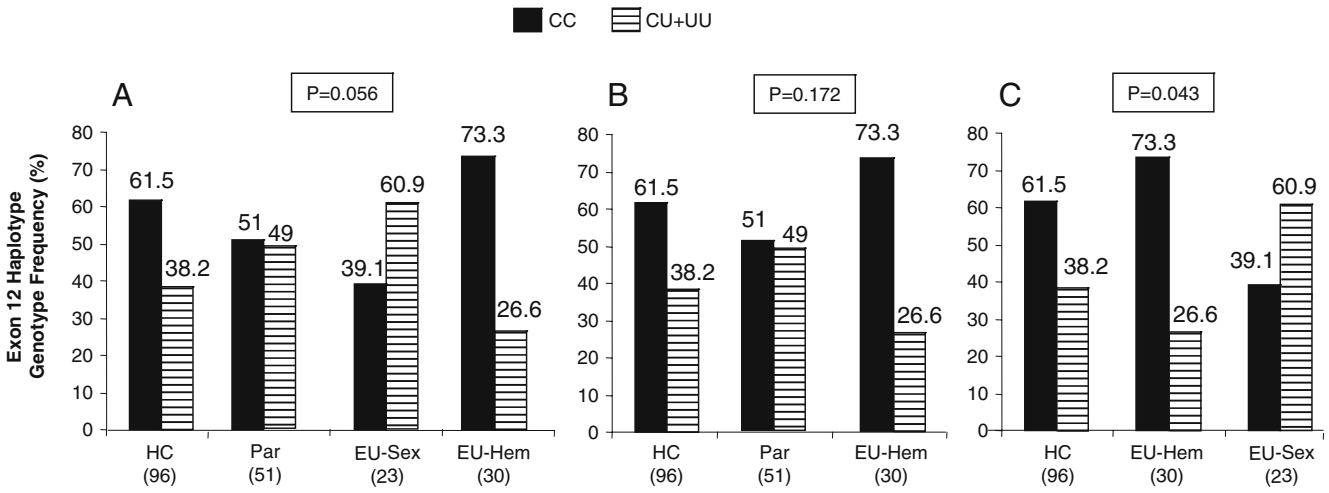

Fig. 4 Genotypic frequencies of exon 12 C- and U-haplotypes. CU and UU genotypes are analyzed together (CU + UU) due to the low frequency of the latter. **a**–**c** Comparisons of different study groups; for each comparison, the whole *p* value is shown (Fisher exact test). Below each group, the number of individuals is indicated *in parentheses*. Above each column, the precise percent value is shown. Abbreviations of groups are as indicated in Figs. 2 and 3

compared with HC, the differences did not reach statistical significance (p=0.092), presumably reflecting the low number of individuals in the EU-Sex group (Fig. 3e).

The distribution of CC, CU, and UU genotypes was also analyzed. U in these genotypes meant any of the nine uncommon haplotypes (see Table 2). The frequency of UU genotype was very low (2.0% in HC, 6.6% in LTNP, 5.88% in TP, 7.84% in Par, 0.0% in EU-Sex, and 3.33% in EU-Hem); therefore, CU and UU genotypes were analyzed together (CU + UU). The CC genotype was more frequent (≥51%) than CU + UU genotypes in all groups, except in EU-Sex in whom the CU + UU genotypes predominated (60.9 vs 38.2% in HC, 26.6% in EU-Hem, and 49.0% in Par); these differences were at the limit of statistical significance (p=0.056) (Fig. 4a). No significant differences were observed between HC, EU-Hem, and Par (p=0.172) (Fig. 4b). In contrast, there was a significant difference between HC, EU-Sex, and EU-Hem (p=0.043), which presumably reflected the increased frequency of CU + UU in EU-Sex (60.9%) compared with EU-Hem (26.6%) and HC (38.2%) (Fig. 4c). In accord with this, EU-Hem and HC were more likely than EU-Sex to carry the CC genotype with respect to the CU + UU genotypes (OR=4.27, 95% CI=1.33–13.70 and OR=2.48, 95% CI=0.976–6.305, respectively; p=0.046). The distribution of these genotypes fitted the HWE (Table 3).

Table 3 HWE in the study population groups of the genotype distribution of C- and U-haplotypes of SNPs in exon 12 of *IL4RA*

Groups	CC	CU	UU	*p* value
HC (*n*=96)	59	35	2	0.466
LTNP (*n*=30)	17	11	2	0.993
TP (*n*=34)	18	14	2	0.945
EU-Sex (*n*=23)	9	14	0	0.111
EU-Hem (*n*=30)	27	7	1	0.793
Par (*n*=51)	26	21	4	0.996

Shown are the observed numbers of individuals with each genotype for each study group. The *p* values correspond to the comparison with the expected number of genotypes according to the HWE. Abbreviations of study groups are as indicated in Fig. 3

Discussion

This study was undertaken to test the hypothesis that *IL4RA* gene SNPs can influence the susceptibility to HIV infection and its progression to AIDS, since hyperresponsiveness or hyporesponsiveness to IL-4 can greatly affect two of the main regulators of this susceptibility, such as the occurrence of an HIV-specific Th1- or Th2-oriented response and the degree of expression of CCR5 and CXCR4 HIV coreceptors (see Introduction). As an approach to test this hypothesis, we investigated seven SNPs in the IL-4Rα chain gene, all of them involving amino acid replacements, in a cross-sectional study with HIV-infected individuals with different patterns of progression, as well as with highly HIV-exposed but uninfected individuals. To the best of our knowledge, this is the first study to investigate the relationship between the *IL-4A* SNPs and the susceptibility to HIV infection and its progression to AIDS.

We investigate three of the SNPs that have been found to be able to affect the functional responsiveness of IL-4R to IgE production and related events in B cells: I50V in exon 5 (extracellular region) and S478P and Q551R in exon 12 (cytoplasmic region) (Hershey et al. 1997; Kruse et al. 1999; Mitsuyasu et al. 1998, 1999; Risma et al. 2002). In addition, we also investigate four additional SNPs in exon 12 (E375A, C406R, S411L, and V554I), as there is increasing evidence that the combination of multiple SNPs or haplotypes (complex allele), but not one SNP alone, can provide information about associations with IgE-related pathological conditions (Mirel et al. 2002; Ober et al. 2000). It should be noted that this type of study usually

individually assesses each SNP, and the haplotype is inferred from bioinformatic algorithms. In contrast, we have employed an experimental sequence-based typing strategy that allows a direct analysis of the whole coding region of exon 12, which encodes most of the cytoplasmic region and holds most of the SNPs of *IL4RA* (Lozano et al. 2001).

We found that V50 homozygosity was significantly overrepresented in LTNP compared with the other groups, suggesting that this genotype was associated with slow progression. Although divergent data regarding this issue exist (Franjkovic et al. 2005), there are several studies reporting evidence that I50 and V50 homozygosities involve the hyperresponsiveness and hyporesponsiveness of IL-4R to IL-4 concerning IgE production and related events in IgE-mediated allergic diseases, respectively (Mitsuyasu et al. 1998, 1999). Our finding that V50 homozygosity associates with slow progression would be consistent with this genotype involving the hyporesponsiveness of IL-4R to IL-4, which could favor an HIV-specific Th1 response, coupled with a reduced expression of CXCR4 that would contribute to delay the emergence of X4 HIV strains—an event that is associated with rapid progression to AIDS. It should be noted that the association of slow progression with HIV-specific Th1 response has been demonstrated (Clerici and Shearer 1994; Klein et al. 1997; Ledru et al. 2003; Maggi et al. 1994; Meyaard et al. 1996; Plana et al. 2000; Torres et al. 1998). In addition, we have also previously found that reduced CXCR4 expression on T lymphocytes is a feature of the same LTNP studied here (Soriano et al. 2002).

A number of gene variants of immune system components have been found to delay progression to AIDS (reviewed in Kaslow et al. 2005; Nolan et al. 2004; O'Brien and Nelson 2004; Stephens 2005; Theodorou et al. 2003). The most important and the first of these variant genes to be described is *CCR5Δ32*, which prevents the expression of the protein on the cell surface; its homozygosity is highly protective against infection, while its heterozygosity delays progression (Dean et al. 1996; Huang et al. 1996; Samson et al. 1996). However, it should be noted that the progression-delaying effect of *CCR5Δ32* heterozygosity is limited and transient, typically by 2 years (Kostrikis et al. 1998), and this occurs in cohorts of homosexual men or in cohorts where homosexual men predominate (Dean et al. 1996; Ioannidis et al. 2001; Kostrikis et al. 1998; Magierowska et al. 1999; Morawetz et al. 1997). This progression-delaying effect is not noticed in cohorts of hemophiliacs (Dean et al. 1996) or intravenous drug users (Ioannidis et al. 2001; Schinkel et al. 1999); therefore, it is not surprising that the frequency of *CCR5Δ32* heterozygosity in our group of LTNP is not significantly increased compared with TP because these groups of patients correspond mostly to intravenous drug users (LTNP, 76.6%) or hemophiliacs (TP, 88.8%).

Regarding the SNPs of exon 12, the data showed an association of U-haplotypes with the infected status, regardless of their rate of progression to AIDS. This association was especially evident for those HIV^+ individuals who were infected via parenteral exposure, which predominated in the LTNP and TP groups, while EU-Hem were indistinguishable from HC. We also found a similarly increased frequency of U-haplotypes in EU-Sex. This latter group also had a significantly increased frequency of CU + UU genotypes with respect to the CC genotype, compared with HC and EU-Hem. These data suggest that U-haplotypes might associate with both susceptibility to HIV infection via parenteral route and resistance to infection via sexual exposure. This notion predicts that sexually infected HIV^+ patients would exhibit a reduced frequency of U-haplotypes compared with EU-Sex and VIH^+ patients who were infected via the parenteral route. Unfortunately, a significant group of VIH^+ patients who were infected via sexual exposure is not available for inclusion into this cross-sectional study. Notwithstanding this, a possible explanation for the apparently contradictory effect of U-haplotypes, favoring infection via the parenteral route while protecting against infection via sexual exposure, might be consistent with U-haplotypes involving hyperresponsiveness to IL-4. Several studies have reported that SNPs P478 and/or R551 included in U-haplotypes involved IL-4 hyperresponsiveness for IgE production and related events and were associated with atopy (Hershey et al. 1997; Kruse et al. 1999; Risma et al. 2002; Rosa-Rosa et al. 1999). If U-haplotypes involve such IL-4 hyperresponsiveness, then they may protect against infection through sexual exposure by reducing CCR5 expression on mucosal $CD4^+$ T lymphocytes, macrophages, and dendritic cells and by promoting a local Th2 response that could result in the production of mucosal neutralizing IgA anti-HIV antibodies. The existence of such antibodies in EU-Sex has been reported (Devito et al. 2000). The hyperresponsiveness to IL-4, however, might be detrimental in the case of parenteral exposure because it could prevent the occurrence of a systemic HIV-specific Th1 response, and the IL-4-mediated reduction of CCR5 expression on $CD4^+$ cells might be insufficient to avoid infection through a parenteral viral inoculum that can easily reach lymphoid organs where HIV finds the best scenario to achieve rapid replication.

In vitro studies on the effect of IL-4 in CCR5 and CXCR4 expression on CD4 T cells from healthy individuals homozygous for V50 and I50 genotypes and for carriers of C- and U-haplotypes of *IL4RA* could help to clarify whether or not these *IL4RA* variants involve hyporesponsiveness or hyperresponsiveness of IL-4R to IL-4 regarding the regulation of HIV-1 coreceptors and the facilitation of in vitro replication of R5 and X4 HIV strains. These studies could be important not only from the point of view of immunopathogenesis regarding the hypothetical interpretations indicated above for the associations observed, but could be also of interest from a clinical perspective as pharmaceutically soluble IL-4Rα antagonists are now available and are being used in clinical trials in the context of IgE-mediated allergic diseases (Mueller et al. 2002). These pharmaceutical IL-4Rα products might be perhaps clinically useful in advanced HIV patients who are resistant to conventional antiretroviral agents.

The differences between EU-Sex and EU-Hem regarding exon 12 haplotypes further highlight that host genetic

factors conferring resistance against infection can greatly differ depending on whether the route of exposure to HIV-1 is sexual or parenteral. Indeed, we previously found that, in the same EU-Sex studied here, in whom women who had unprotected vaginal sex with HIV-infected men predominated (76%), there was underrepresentation of SDF1-3′A allele and absence of SDF1-3′A homozygote. This underrepresentation did not occur in the same EU-Hem group studied here, a finding consistent with data from others about the lack of effect of the infant SDF1-3′A allele on perinatal transmission (Soriano et al. 2002 and references therein). Differences in the association of SNPs with the DC-SIGN promoter for infection via parenteral, but not mucosal, exposure have been also recently documented (Martin et al. 2004). Furthermore, differences in the association of some protective genetic factors with susceptibility to infection via vaginal and anal mucosae might also exist—an issue that is usually neglected and even not mentioned—when great differences exist in the biological characteristics of these two types of mucosa. With regard to this, it should be mentioned that Winkler et al. (1998) observed an increased frequency of SDF1-3′A heterozygotes in highly exposed but uninfected homosexual men, suggesting a possible protective role against sexual infection. In contrast, in our EU-Sex, who mostly corresponded to women (76%), the SDF1-3′A allele was clearly underrepresented as aforementioned. This apparent discrepancy might simply reflect that infection-protecting host factors can also differ depending on whether the mucosal exposure to HIV-contaminated semen is vaginal or anal.

The genotypic distributions of I50V and the haplotype of exon 12 SNPs were not statistically different from those expected according to the HWE. This can be considered as an indicator of the technical soundness of our data, since departure from HWE, especially in the control group, is thought to reflect genotypic mistakes (Xu et al. 2002). Nevertheless, it can be argued that the current study has, as its limitation, a low number of individuals in the groups studied. It should be noted, however, that the different study groups correspond to homogenously and rigorously defined extreme phenotypes. In fact, given the strict definition criteria used here, the number of our groups is not so low and is sufficient for statistical comparisons. On the other hand, it is well-known that HIV-related host genetic studies performed with several cohorts with a very high number of individuals were not confirmed by many other studies. Although multivariate analyses in such studies with many cohorts and a high number of subjects would seem powerful influences on the significance of their conclusions, they can involve numerous confounders owing to the great differences between individuals in many variables, including route of exposure, ethnicity, and other biological characteristics, which strongly complicate statistical analyses. Furthermore, the establishment of genetic associations for complex diseases is independent of early study findings; that is to say, a strong statistical significance with high numbers of individuals in the very first reports does not adequately predict eventual establishment of an association, and, conversely, many genuine epidemiological associations would be missed if research were abandoned after early unpowered "negative" studies (Trikalinos et al. 2004).

In conclusion, this study provides evidence suggesting that *IL4RA* SNPs can be important host genetic determinants of the resistance susceptibility to HIV infection and its progression to AIDS. The main findings were that V50 homozygosity in exon 5 appears to be associated with slow progression (at least in intravenous drug users, who predominated in our group of LTNP) and that uncommon haplotypes in exon 12 might be associated with both resistance to sexually transmitted infection (specially in women, who predominated in our group of sexually exposed but uninfected individuals) and to susceptibility to infection via parenteral exposure. Further studies are required to confirm these findings.

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A novel polymorphism in the 5' untranslated region (5'UTR) of CD28 gene is associated with susceptibility to HIV-1 infection

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Abstract

Background: CD28 is a critical co-stimulatory molecule for the optimal activation of T-cells and regulates the main HIV co-receptors.

Objective: to investigate the presence of polymorphisms in 5' Untranslated Region (UTR) of CD28 and their possible role in susceptibility to HIV-infection or its progression to aids.

Methods: The 5' UTR of CD28 was amplified using appropriate primers and the presence of polymorphisms was checked using Single Strand Conformation Polymorphism (SSCP). The SSCP patterns were sequenced and cloned. Population analysed were 67 HIV+ long-term nonprogressors (LTNP), 66 HIV+ typical progressors (TP), 58 highly-exposed uninfected (EU) individuals, 27 EU-sexual (EU-sex) and 31 EU-haemophiliacs (EU-hem), and 92 healthy controls (HC), all Caucasians and lacking *CCR5Δ32* homozygosity. Proportions were compared using chi-squared or fisher-exact test and a logistic regression model was applied for allelic and genotype comparisons.

Results: We found 3 alleles differentiated by the presence of 3, 4 or 5 tandem repeats (TR) of 5 nucleotides (CTTTT) in the 5' extreme. Allele with 4 TR was the most common and we found 4 genotypes, one common (homozygous of 4 TR) and 3 uncommon (homozygous of 5 TR and heterozygous of 4 and 5 TR or 3 and 4 TR). Since the allelic and genotypic frequencies of LTNP and TP were similar, they were considered as one group (HIV+). The frequencies of uncommon alleles (5TR and 3TR) in HC, HIV+, EU-sex and EU-hem were 15.2, 20, 7.4 and 13%, respectively (p=0.09). The frequency of uncommon genotypes in HC, HIV+, EU-sex and EU-hem were 28.2, 38.8, 30.8, 11.1 and 25.8%, respectively (p=0.07). A logistic regression model showed that the risk of uncommon alleles and genotypes were more than 3 times higher in HIV+ than in EU-sex (OR= 3.11, 95%CI=1.07-8.99 and OR=4.37, 95%CI=1.25-15.28, respectively). Distribution genotypes fitted Hardy-Weinberg equilibrium.

Conclusions: We found new polymorphisms in CD28 5' UTR that could be implicated in susceptibility to HIV infection after sexual exposure.

INTRODUCTION

Since CCR5 is the main co-receptor of non-syncitial inducing (NSI)-HIV, its expression on lymphocytes surface is one of the most important factors implicated in susceptibility and/or progression of HIV infection. The wide range of CCR5 expression in different individuals is, in part, due to genetic variants such deletion of 32 pb in CCR5 gene (1-3), polymorphisms in CCR5 promoter (4) or in CCR2 (5). The link between CCR2 point mutation (G→A in 64 position, CCR264I) and CCR5 expression is not clear, some authors have found an association between CCR264I and CCR5 promoter polymorphisms (6), while others have described an alteration of CCR5 dimerization in CCR264I carriers (7). However, the expression of CCR5 is under a complex control (8) and these genetic variants only explain the low CCR5 expression in a sub-group of patients.

CD28 plays an essential role in determining the effectiveness of T-cell immune responses as it is a critical co-stimulatory molecule for the optimal activation of CD4+ and CD8+ T-cells via CD3-T-cell receptor complex. In addition, CD28 co stimulation of CD4+ T-cells confers resistance to in vitro infection by R5-tropic HIV-1 strains, due to a down-regulation of CCR5 molecule and an up-regulation of CCR-chemokines (9-11), while it increases cell-surface CXCR4 expression, facilitating the infection by X4 strains (12). For these reasons, we hypothesized that immunogenetic variants in CD28 may exist and that they could influence resistance/susceptibility to HIV-1 infection and its progression. We show in the present article non-previously described polymorphisms in the 5' untranslated region (5'UTR) of CD28 and their relationship with resistance/susceptibility to HIV infection and its progression.

MATERIAL AND METHODS

Study subjects. Individuals were recruited at “Hospital Clinic” (Barcelona), “Hospital La Fe” (Valencia), two University Hospitals and at “Centro Médico Sandoval” (Madrid), which is a specialized center for sexually transmitted diseases. These centers are public, non-profit and academic institutions of the Spanish National Health System. Institutional ethical committees approved the study and patients gave informed consent. All individuals included in the study were European Caucassians and lacked CCR5Δ32 homozygosity. They were divided in the following groups. A) HIV-1+ individuals subdivided into 2 categories: (i) a group of LTNP (n=67), defined as asymptomatic individuals with blood CD4 T-cell counts >500/mm3 after >10 years of known seroprevalence in the absence of antiretroviral therapy; (ii) a group of 66 typical progressors (TP), defined as patients with a decrease of CD4 T cell counts below 350/mm3 during the first 7 years from seroconversion. It should be noted that, as indicated in the text, to evaluate the role of CD28 5’ UTR polymorphisms in susceptibility/resistance to HIV infection, we used all infected individuals (LTNP +TP) as one group (HIV+, n=133). B) A group (n=58) of highly exposed but uninfected individuals (EU), i.e. seronegative and with undetectable plasma viral load (PVL) after at least 4 consecutive determinations at 6-month intervals; these EU were divided into two subgroups: (i) 27 individuals (EU-Sex), 19 females (76%), 5 heterosexual men and 3 homosexual men, who had had high exposure through unprotected sex with HIV-infected partners during the last 12 months before the study with a median of >600 vaginal or anal intercourses with ejaculation during the period of follow-up, which ranged between 1-4 years; these EU-Sex and their HIV+ partners belong

to a large cohort of individuals with a long follow-up ("Centro de Salud Sandoval", Madrid) (13); and (ii) 31 hemophiliacs (EU-Hem), who were highly exposed to nonviral inactivated clotting factors concentrates before 1986; they belong to a large cohort of hemophiliacs, who mostly became HIV-1-infected and all of them, including the 31 EU-Hem studied here, also became infected with hepatitis C virus (Hospital "La Fe", Valencia) (14;15). C) Healthy controls (HC, n=92) matched in sex and age with the HIV+ and EU groups, who had normal biological parameters, including negativity for HIV-1, HCV and other infections as screened for altruistic blood donors.

Detection of polymorphisms in 5' UTR of CD28. DNA isolation was done as reported (16). We analyzed whole 5' UTR based on Lee et al. description (17). This sequence contains an "ALU" repeat element and a regulator motive, used by transcription factors of AP1 family. The length of amplified fragment was 558 bp, from the beginning of transcription to known 5' extreme (figure 1). Afterwards, Vallejo A, et al (18) better described the CD28 promoter, including 3 regulator motives at -153 to -220 pb from the beginning of translation (these motives bind transcriptional factors, Elk1, Ets1 and AP3), that were not included in the fragment amplified with our primers (figure 2).

Primers design were (figure 2): CD28-PR-FW: CCC TTT CCT TTT TTC TCT CTC and CD28-PR-RW: CGT TGT TTT GAT GAC TTT AGA CT. Amplification was made with 100 ng of genomic DNA and 0.5 units of Taq Polimerase (Expand^{tm} High Fidelity PCR system, Boehringer Mannheim, Ottweiler, Alemania). PCR conditions were: 30 cycles of 95ºC 5 minutes, 94ºC 30 seconds, 55ºC 30 seconds and 72ºC 1 minute. Single Strandt Conformation Polymorphism (SSCP) was used as screening method to identify

polymorphisms. 10μl of denaturalizing buffer (95% Formamide, 0.05% blue Bromofenol, 0.05% Xilencianol and 0.02M EDTA and pH 8.0) were added to amplified DNA samples (20 μl) and the final volume was denaturalized at 95ºC, 10 minutes. The samples were analysed using an electrophoresis temperature control system (ThermoFlow^{tm}, Novex, San Diego, USA) and pre-fabricated gels (Novex pre-cast TBE gels, San Diego, USA). The gels were revealed with silver stain. Those polymorphisms identified by SSCP were confirmed by sequencing (Applied Biosystems, Gene Amp PCR system 2700) and cloning (pGEM® Easy Vector System) PCR products.

Statistical analysis. The frequency of alleles and genotypes among groups were compared using a χ^2 or Fisher's exact test, as appropriate. We applied a logistic regression model for allelic and genotype comparisons. All tests were two-tailed, with $p \leq 0.05$ considered statistically significant. To evaluate whether the distribution of genotypes fulfilled the Hardy–Weinberg equilibrium (HWE), a goodness-of-fit chi-square test was used to compare the observed number of subjects with the expected number according to the HWE (Xu).

RESULTS

Polymorphisms in 5'UTR of CD28 gene.

The analysis of the 5'UTR by SSCP identified different patterns that were identified by sequencing and cloning. The most frequent allele was characterized by the presence of 4 tandem CTTTT nucleotide repeats (TR), 28 pb upstream of "ALU repeat" (figure 1). Other 2 alleles were identified, one with 5 tandem repeats (insertion of 5 nucleotides, CTTTT) and the other with only 3 tandem repeats (deletion of 5 nucleotides CTTTT). From these 3 alleles we identified 4 genotypes, homozogous of 4 TR, heterozygous of 4 and 5 TR, homozygous of 5 TR and heterozygous of 5 and 3 TR. However, the last two genotypes were very uncommon.

Allelic and genotypic frequency of CD28 5'UTR polymorphisms.

Since the frequency of 3 TR allele was lower than 2% in all groups, 3TR and 5 TR alleles were considered together (uncommon alleles) and their frequency was compared with 4 TR allele frequency (common alleles). The frequency of uncommon alleles in LTNP (21.7%) and TP (15.4%) were not statistically significant (p=0.48, Chi-squared test), therefore, both groups were considered together as HIV+ group. The frequencies of uncommon alleles in HC, HIV+, EU-sex and EU-hem were 15.2, 20, 7.4 and 13%, respectively (figure 3). EU-sex group showed the lowest frequency of uncommon alleles, but the overall comparison of allelic frequencies did not reach statistical significance (p=0.09, Chi-squared test). However, a logistic regression model showed that HIV positive patients were more likely to carry uncommon alleles than EU-sex (OR=3.11, 95%CI=1.07-8.99). The genotypes were grouped in homozygous of 4 TR (common genotype) and heterozygous of 4 and 5 TR, homozygous of 5

TR and heterozygous of 3 and 4 TR (uncommon genotypes). The frequencies of uncommon genotypes in LTNP (38.8%) and TP (30.8%) were not different (p=0.39, Chi-squared test) and they were considered together (HIV+ group). The frequencies of uncommon genotypes in HC, HIV+, EU-sex and EU-hem were 28.2, 34, 11.1 and 25.8%, respectively (figure 4). Again, the difference was due to the low frequency of uncommon genotype in EU-sex, and the different genotypic distributions were at the limit of significance (p=0.07, Chi-squared test). A logistic regression model showed that HIV positive patients had a significantly higher risk to carry uncommon genotypes than EU-sex individuals (OR=4.37, 95%CI=1.25-15.28). The genotype distribution in the different groups was not significantly different from that expected, assuming HWE (Table 1).

DISCUSSION

CC chemokine receptor 5 (CCR5) is a seven-transmembrane, G protein-coupled receptor (GPCR) which regulates trafficking and effector functions of memory/effector T-lymphocytes, macrophages, and immature dendritic cells. It also serves as the main coreceptor for the entry of R5 strains of human immunodeficiency virus (HIV-1, HIV-2) and owing to its prominent role as fusion cofactor for HIV, CCR5 has become a major focus of research aiming at determining its structure, biological function, and of the mechanisms which regulate signalling and cell surface expression (19).

CD28 is a 90-kDa homodimeric glycoprotein present on the surface of a large subset of T cells that appears to play an important role in the modulation of T cell activation. In addition, the activation of CD4+ T lymphocytes from HIV-1 infected donors with immobilized antibodies to CD3 and CD28 induced a specific R5-HIV resistant state by regulating the main HIV co-receptors (CCR5/CXCR4) (20;21). These results suggested that co-stimulation with CD28 could influence the resistance/susceptibility to HIV infection and its progression to aids and it was plausible to investigate the presence of polymorphisms in the promoter region of CD28 and its role in HIV infection.

Our study shows a non-previously described polymorphisms in 5'UTR of CD28. The 3 different alleles found were characterized by the presence of 3, 4 or 5 tandem repeats (of 5 nucleotides, CTTTT) at 28 bp upstream of "Alu repeat" fragment (figure 1). Although in the population studied we identified 4 different genotypes, homozygosity of 4TR was the most frequent (common) genotype, and its frequency in the studied groups was compared with the frequency of the other 3 (uncommon) genotypes. The statistical analysis did not show

differences between LTNP and TP, suggesting that this variants did not influence the disease progression. In contrast, we found the lowest frequency of uncommon alleles and genotypes in EU-sex individuals (fig. 3 and 4). Although the whole comparison of allelic and genotypic frequencies were not statistically different (p=0.09 and p=0.07), HIV+ patients showed a significantly higher risk to carry uncommon alleles and genotypes than EU-sex (OR= 3.11, 95%CI=1.07-8.99 and OR=4.37, 95%CI=1.25-15.28, respectively). Therefore, our results suggest that homozygosity of 4 TR in 5'UTR of CD28 protects to HIV infection, specially after sexual exposure

In conclusion, we identified novel immunogentic variants in 5'UTR of CD28 that could be implicated in susceptibility to HIV infection. Future studies will be necessary to further investigate the biological effects of the different variants found in CD28 promoter region.

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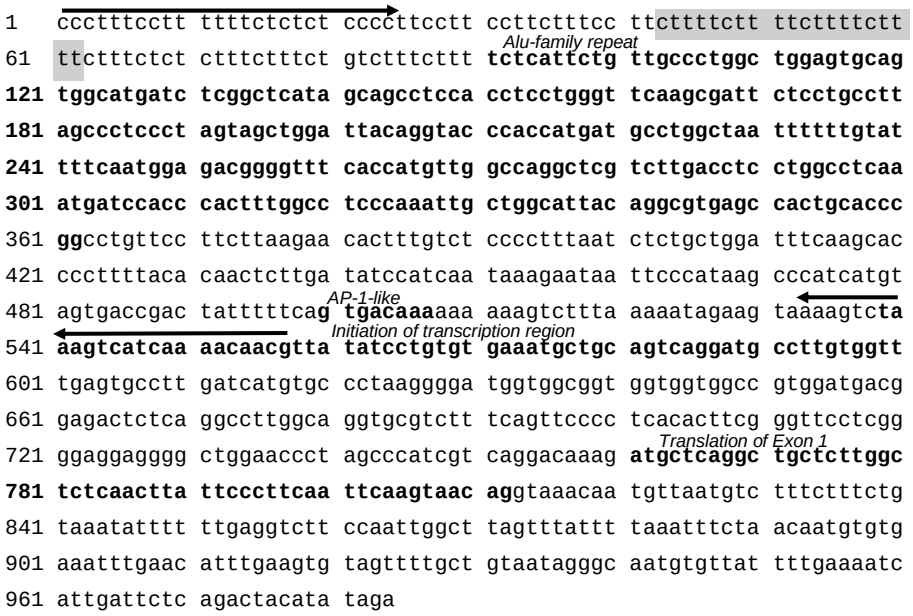
Figure 1.



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61 ttctttctct ctttctttct gtctttcttt **tctcattctg ttgccctggc tggagtgcag**
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301 atgatccacc cactttggcc tcccaaattg ctggcattac aggcgtgagc cactgcaccc
361 **gg**cctgttcc ttcttaagaa cactttgtct ccccttttaat ctctgctgga tttcaagcac
421 ccctttttaca caactcttga tatccatcaa taaagaataa ttcccataag cccatcatgt
481 agtgaccgac tatttttcag **tgacaaa**aaa aaagtcttta aaaatagaag taaaagtc**ta**
541 aagtcatcaa aacaacgtta tatcctgtgt gaaatgctgc agtcaggatg ccttgtggtt
601 tgagtgcctt gatcatgtgc cctaagggga tggtggcggt ggtggtggcc gtggatgacg
661 gagactctca ggccttggca ggtgcgtctt tcagttcccc tcacacttcg ggttcctcgg
721 ggaggagggg ctggaaccct agcccatcgt caggacaaag **atgctcaggc tgctcttggc**
781 tctcaactta ttcccttcaa ttcaagtaac aggtaaacaa tgttaatgtc tttctttctg
841 taaatatttt ttgaggtctt ccaattggct tagtttattt taaatttcta acaatgtgtg
901 aaatttgaac atttgaagtg tagttttgct gtaatagggc aatgtgttat tttgaaaatc
961 attgattctc agactacata taga

Figure 1. CD28 5' UTR and Exon 1 sequence by Lee KP, et al (36). Shadowed area remarks the 4 CTTTT tandem repeats (see text). Arrows indicate the primers designed to amplify the studied fragment.

Figure 2.

Figure 2. CD28 gene scheme and 5' UTR amplified based on Vallejo et al (38).

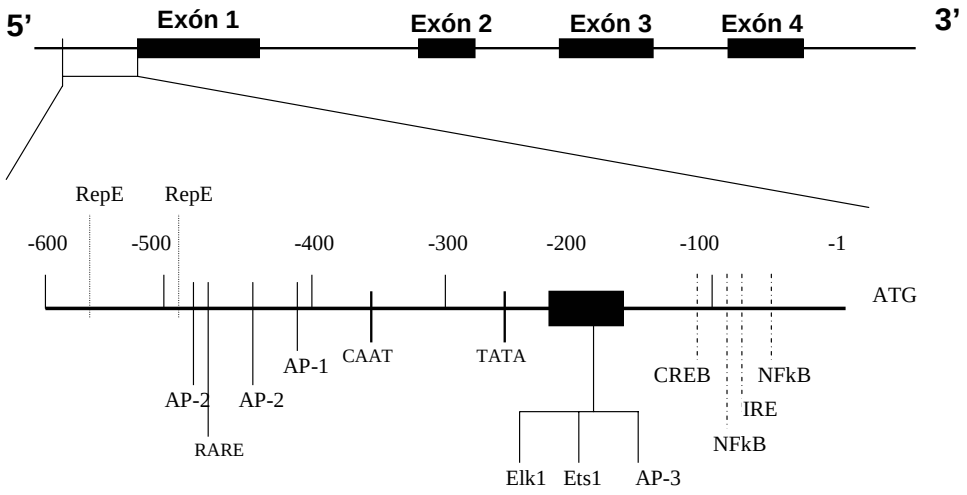

Figure 3.

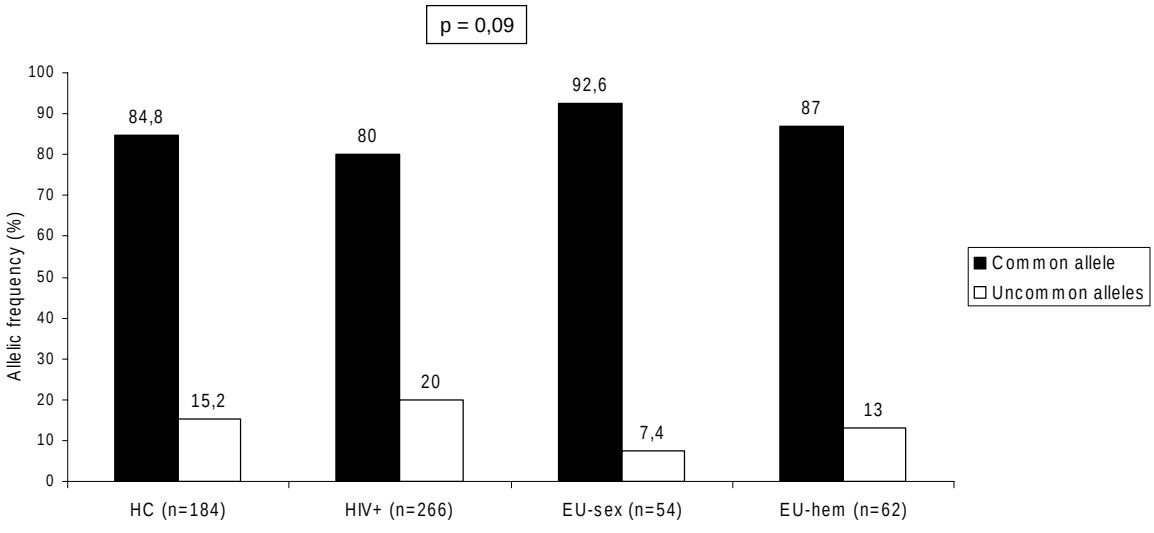

Figure 3. Frequency of CD28 5'UTR alleles in healthy controls (HC), HIV+ patients (HIV+) and sexual and parenteral exposed uninfected individuals (EU-sex, EU-hem). The number of alleles is shown In parenthesis.

Diapositiva 4

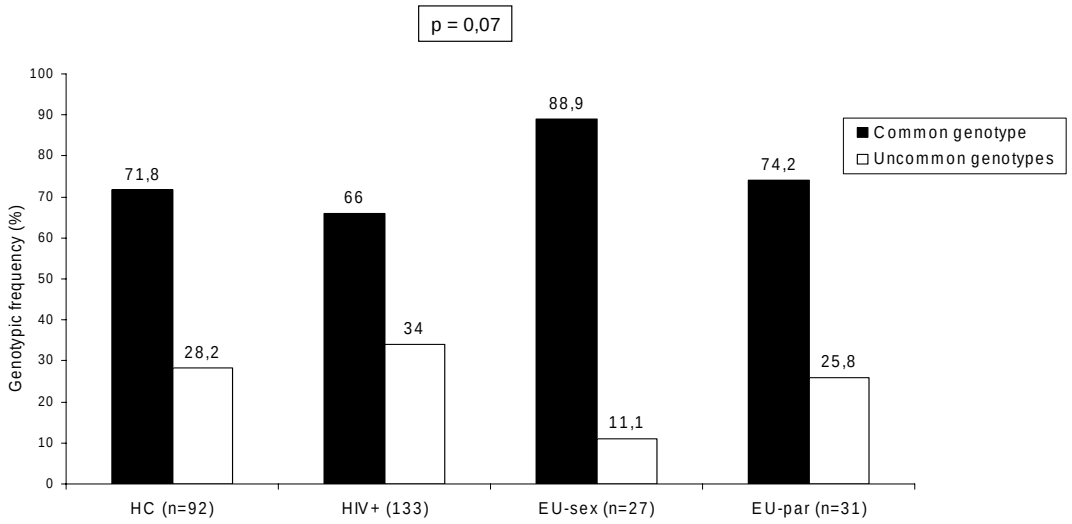

Figure 4 Frequency of CD28 5'UTR genotypes in healthy controls (HC), HIV+ patients (HIV+) and sexual or parenteral exposed uninfected individuals (EU-sex, EU-hem). The number of individuals studied is shown In parenthesis.

Table 1.

Table 1 Hardy-Weinberg Equilibrium in the study population groups of the genotype distribution of C- and U genotypes in 5'UTRCD28.

group	**C**	**CU**	**U**	**p-value**
HC (n=95)	66	23	2	1,00000
LTNP (n=65)	41	21	3	0,98913
EU par (n=30)	23	7	0	0,76977
EU sex (n=26)	24	2	1	0,05746
TP (n=66)	45	18	3	0,79520

Plasma Stromal Cell–Derived Factor (SDF)-1 Levels, SDF1-3′A Genotype, and Expression of CXCR4 on T Lymphocytes: Their Impact on Resistance to Human Immunodeficiency Virus Type 1 Infection and Its Progression

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Plasma stromal cell–derived factor (SDF)-1 levels, SDF1-3′A polymorphism, and CXCR4$^+$ T lymphocytes in relation to resistance to human immunodeficiency virus (HIV)-1 infection and its progression were investigated in a study of HIV-positive patients, exposed but uninfected (EU) subjects, and healthy control subjects, all lacking CCR5Δ32 homozygosity. SDF1-3′A homozygosity was associated with low plasma SDF-1 levels in uninfected persons and was not related to long-term nonprogression. HIV-1 infection involved increased plasma SDF-1 levels, which were not attributable to any kind of chronic viral infection, because all EU hemophiliacs were hepatitis C virus–positive but had normal SDF-1 levels. High plasma SDF-1 levels and low CXCR4 expression on T lymphocytes was associated with long-term nonprogression, whereas in advancing disease expression of CXCR4 increased, accompanied by a decrease in plasma SDF-1 during the more advanced stages of HIV-1 infection. EU subjects with sexual exposure to HIV-1, but not EU hemophiliacs, showed an underpresentation of SDF1-3′A allele frequency, which was coupled with high plasma SDF-1 levels and low CXCR4 expression.

To enter CD4$^+$ cells, human immunodeficiency virus type 1 (HIV-1) requires the use of coreceptors corresponding to chemokine receptors, mainly CCR5 and CXCR4 [1, 2]. CCR5 is used by non–syncytium-inducing HIV strains, which generally transmit the infection (R5 strains) and predominate in nonadvanced stages of HIV-1 infection and in long-term nonprogressors (LTNP). In contrast, CXCR4 is used by syncytium-inducing HIV strains (X4 strains), which appear during chronic infection (reviewed in [1]), an event associated with a rapid decline in CD4$^+$ T cells and progression to AIDS [3]. Although it remains unknown whether the emergence of the highly cytopathic X4 strains is the cause or the consequence of progression to AIDS, there is strong evidence supporting the former possibility [4]. In vitro, CCR5-binding chemokines inhibit infection by R5 strains, whereas the physiologic ligand of CXCR4, the chemokine stromal cell–derived factor (SDF-1), now designated "CXCL12" [2], inhibits infection by X4 strains (reviewed in [1, 5]). CCR5-binding chemokines are typical examples of CC-inducible chemokines, which are mainly produced in response to inflammatory stimuli, whereas SDF-1 is the paradigm of homeostatic or lymphoid chemokines (reviewed in [2, 6]), constitutively produced by bone-marrow stromal cells, lymphoid organs, and a great variety of other cells and tissues, except blood leukocytes [7–13]. Although CCR5 is physiologically dispensable, and its absence in CCR5Δ32 homozygosity provides the host with significant protection against infection by R5 strains (reviewed in [14–16]), SDF-1 and its receptor, CXCR4, which is expressed not only in hematopoietic cells but in a great variety of other cells and tissues, are physiologically indispensable [6, 9, 17–27]. The SDF-1–CXCR4 interaction is essential for the maturation of CD34$^+$ stem cells in the bone-marrow environment, for B cell lymphopoiesis and myelopoiesis, for the physiologic traffic of lymphocytes and their endothelial transmigration, and for car-

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Informed consent was obtained from patients, and the studies were approved by the institutional ethical committees of the public, nonprofit, academic institutions of the National Health Service of Spain, where the study took place.

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Table 1. Demographic, virologic, and immunologic characteristics of human immunodeficiency virus–infected groups when plasma stromal cell–derived factor (SDF)–1 levels and expression of CXCR4 on T lymphocytes were evaluated.

Group	Follow-up, mean ± SD, months	Age, mean ± SD, years	Sex, % male	Risk factor, %			Plasma virus load, mean, copies/mL	$CD4^+$ T cells/mm^3, mean ± SD	$CD8^+$T cells/mm^3, mean ± SD
				Sexual	Intravenous drug use	Hemophiliac subjects			
LTNP (n = 67)	135 ± 12	31 ± 8	69	21	70	9	10,830	696 ± 219	1085 ± 611
LVL (n = 15)	31 ± 20	36 ± 10	56	75	25		299	706 ± 215	985 ± 611
ES (n = 26)	40 ± 25	36 ± 13	72	65	35		99,238	584 ± 171	1014 ± 484
IS (n = 39)	160 ± 10	21 ± 10	60	18		82	58,138	345 ± 180	806 ± 350
AS (n = 15)	81 ± 53	42 ± 8	100	45	55		884,731	61 ± 56	583 ± 340

NOTE. LTNP, long-term nonprogressors; LVL, subjects with low plasma virus load; ES, early stage of HIV-1 infection; IS, intermediate stage of HIV-1 infection; AS, advanced stage of HIV-1 infection.

diovascular and cerebellar embryoorganogenesis, as demonstrated by mice deficient in either CXCR4 or SDF-1 genes, which die perinatally [6, 9, 17–27]. SDF-1 also has T cell costimulatory properties [28] and directs the migration of plasma cells to bone marrow [29].

Apart from CCR5Δ32 deletion, other genetic polymorphisms in chemokine and chemokine receptor genes influencing susceptibility or resistance to HIV infection and its progression to AIDS are known (reviewed in [14–16]). One of these is the SDF1-3′A polymorphism, consisting of a G→A mutation at position 801 (counting from the ATG start codon) in the 3′ untranslated-region transcript of the SDF-1 gene [30]. Its homozygosity, but not its heterozygosity, has been reported to be associated with a delayed progression to AIDS and AIDS-related death [30], and, because the 3′ untranslated region is involved in regulating the stability of mRNA, it has been suggested that such homozygosity might involve an overproduction of SDF-1 that could act by inhibiting the appearance of X4 strains [30]. Other studies, however, have not confirmed the progression-retarding effects of this genotype, and some of them have found that it is, in fact, associated with both a faster decline in $CD4^+$ T cells and progression to AIDS [31–37].

On the other hand, SDF1-3′A heterozygosity was found increased in exposed but uninfected (EU) homosexual men, a finding suggesting that this genotype could play a protective role against HIV infection [30]. Thus far, no other studies have analyzed in detail the SDF1-3′A polymorphism in EU adults, few have investigated plasma SDF-1 levels in relation to HIV disease [38–40], and no data exist on whether there is a relationship between plasma SDF-1 levels and SDF1-3′A genotype. Whether there is a relationship between plasma SDF-1 levels and CXCR4 expression on T lymphocytes is also not known; this issue deserves attention because the SDF-1–mediated down-regulation of surface CXCR4 expression on T lympho-

Table 2. Distribution of stromal cell–derived factor-1 (SDF-1)–3′A polymorphism among human immunodeficiency virus (HIV)–infected subjects, heavily exposed but uninfected subjects, and controls.

Group	Genotypic frequency			Allelic frequency	
	wt/wt	wt/3′A	3′A/3′A	wt	3′A
HIV positive					
LTNP (n = 82)	31 (37.8)	49 (59.8)[a,b,c]	2 (2.4)	111 (67.68)	53 (32.32)
ES (n = 26)	15 (57.7)	11 (42.3)	0 (0)	41 (78.85)	11 (21.15)
IS + AS (n = 44)	22 (50.0)	18 (40.9)	4 (9.1)	62 (70.45)	26 (29.55)
EU					
Sex (n = 30)	25 (83.3)[d]	5 (16.7)	0 (0)	55 (91.67)	5 (8.33)[e,f,g]
Hem (n = 30)	17 (56.7)	9 (30.0)	4 (13.3)	43 (71.67)	17 (28.33)
Healthy controls (n = 88)	49 (55.7)	33 (37.5)	6 (6.8)	131 (74.43)	45 (25.57)

NOTE. Data are no. (%). wt, wild-type SDF-1 allele; 3′A, mutated SDF-1–3′A allele; EU, exposed but uninfected subjects (EU-sex, sexual exposure to HIV-1; EU-hem, hemophiliacs highly exposed to non–virus-inactivated clotting factor concentrates between 1980 and 1985); LTNP, long-term nonprogressors; ES, early stage of HIV-1 infection; IS, intermediate stage of HIV-1 infection; AS, advanced stage of HIV-1 infection (IS and AS can be considered typical progressors and, for this comparison, were considered together). Only P values ⩽.05 are shown.

[a] $P < .005$ vs. EU-hem.
[b] $P < .0001$ vs. EU-sex.
[c] $P < .004$ vs. healthy controls.
[d] $P < .03$ vs. healthy controls.
[e] $P < .001$ vs. HIV-positive individuals.
[f] $P < .05$ vs. EU-hem.
[g] $P < .008$ vs. healthy controls.

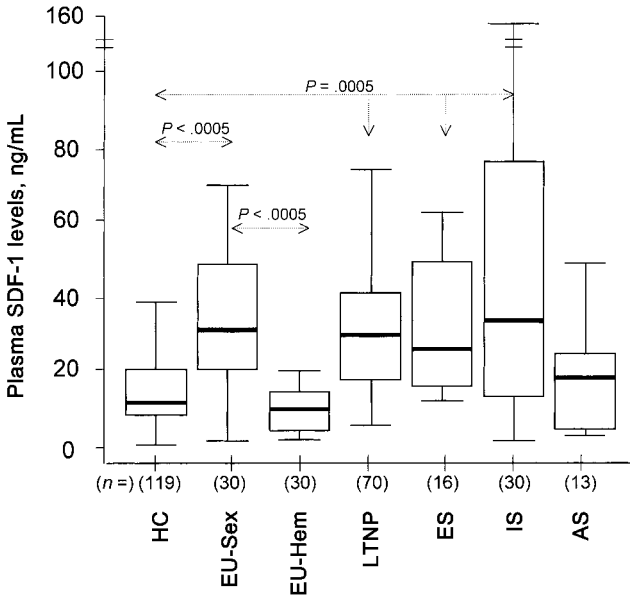

Figure 1. Box-and-whisker plot of concentrations of plasma stromal cell–derived factor (SDF)–1 in human immunodeficiency virus–infected subjects (LTNP, long-term nonprogressors; ES, early stage of HIV-1 infection; IS, intermediate stage of HIV-1 infection; AS, advanced stage of HIV-1 infection), exposed but uninfected subjects (EU-sex, sexual exposure to HIV-1; EU-hem, exposure to HIV-1 via non–virus-inactivated blood products), and healthy controls (HC). Boxes represent interquartile ranges; the horizontal bar within each box indicates the median. Whiskers indicate the 10th and 90th percentiles. Only *P* values ≤.05 are shown.

cytes plays a major role in the in vitro antiviral effects of SDF-1 against X4 strains [41, 42]. The current study was undertaken to gain insight into these aspects.

Subjects, Materials, and Methods

Study subjects. Subjects were recruited at the Hospital Clinic (Barcelona), at the Hospital "La Fe" (Valencia), and at the "Centro Médico Sandoval" (Madrid), a specialized center for sexually transmitted diseases. These centers are public, nonprofit, and academic institutions of the National Health Service of Spain.

Three groups were studied, all of them lacking CCR5Δ32 homozygosity. The first group, HIV-1–positive subjects, was subdivided into the following categories: (1) LTNP ($n = 67$), defined as asymptomatic subjects with $CD4^+$ T cell counts $>500 \times 10^6$/L after >10 years of known seroprevalence in the absence of antiretroviral therapy (15 asymptomatic subjects with low virus load [plasma virus load <500 copies of HIV RNA/mL in 3 consecutive determinations at 5-month intervals], without antiretroviral therapy after ≥1 year of follow-up, included in the LTNP group [$n = 82$], because the plasma virus load set point in seroprevalent subjects is the main predictor of the disease-progression rate and therefore these subjects with low virus loads can be considered as presumably future LTNP [43]); (2) early-stage asymptomatic (ES) patients ($n = 26$), with $CD4^+$ T cell counts $>500 \times 10^6$/L in the absence of antiretroviral therapy, after a follow-up period of <7 years after seroprevalence diagnosis; (3) intermediate-stage (IS) patients ($n = 39$), with $CD4^+$ T cell counts $150–300 \times 10^6$/L after follow-up of <7 years after seroprevalence diagnosis (none of whom had developed opportunistic infections); and (4) advanced-stage (AS) patients ($n = 15$), with $CD4^+$ T cell counts $<100 \times 10^6$/L and who had developed an opportunistic infection after 5–10 years of known seroprevalence.

The second group, EU subjects ($n = 60$), who were seronegative and had undetectable plasma virus loads after ≥4 consecutive determinations at 6-month intervals, were subdivided into the following categories: (1) 30 subjects (EU-sex), including 23 women, 5 heterosexual men, and 2 homosexual men, who during ≥12 months prior to the present study and during the present study, had high exposure to HIV-1, through unprotected sex with HIV-infected partners, with a median of >600 episodes of intravaginal or anal intercourse with ejaculation during the 1–4-year follow-up period; these EU-sex and their HIV-positive partners mostly belong to a large cohort of subjects with high-risk sexual behavior, with a long follow-up ("Centro de Salud Sandoval," Madrid) [44]; (2) 30 hemophiliacs (EU-hem), who were highly exposed to non–virus-inactivated clotting-factor concentrates before 1986; these EU-hem belong to a large cohort of hemophiliacs (Hospital "La Fe," Valencia), who mostly became HIV infected [45, 46], and all of them, including the 30 EU-hem studied here, also became infected with hepatitis C virus.

The third group, healthy controls (HC) ($n = 119$) who were screened as blood donors, were subjects age- and sex-matched with the HIV-positive and EU groups, who had normal biologic parameters, including being negative for HIV-1, hepatitis C virus, and other infections.

Not all subjects in each group could be assessed on all parameters (the precise number for each parameter is indicated). IS and AS patients can be considered as typical progressors, and, for comparisons with regard to SDF1-3′A polymorphism, they were analyzed as a single group. Table 1 shows the demographic, virologic, and immunologic status of HIV-1–infected patients at the time when both the expression of CXCR4 and plasma SDF-1 levels were analyzed.

SDF1-3′A and CCR5Δ32 polymorphism. Genomic DNA was isolated from either peripheral-blood mononuclear cells or whole blood (Qiagen). SDF1-3′A was analyzed by polymerase chain reaction (PCR)–restriction fragment-length polymorphism analysis with primers SDF-F (5′-CAGTCAACCTGGGCAAAGCC-3′) and SDF-R (5′-AGGTTTGGTCCTGAGAGTCC-3′) and the *Msp*I enzyme (Promega), whose restriction site is eliminated by the G→A mutation of the SDF1-3′A polymorphism [30]. Heterozygotes and mutated homozygotes were tested ≥2 different times to ensure soundness of results. PCR analysis of CCR5Δ32 was performed with primers spanning the 32-bp deletion, as reported elsewhere [47].

ELISA quantification of plasma SDF-1. Plasma obtained from an EDTA-anticoagulated blood sample, obtained 16–24 h after extraction of venous blood, was kept at −80°C until required for ELISA determinations. SDF-1 ELISA was developed with appropriate pairs of coating and catcher anti–SDF-1 antibodies, as well as with standard recombinant human SDF-1α (R&D Systems). The ELISA conditions for antibody coating, blocking, reagent for

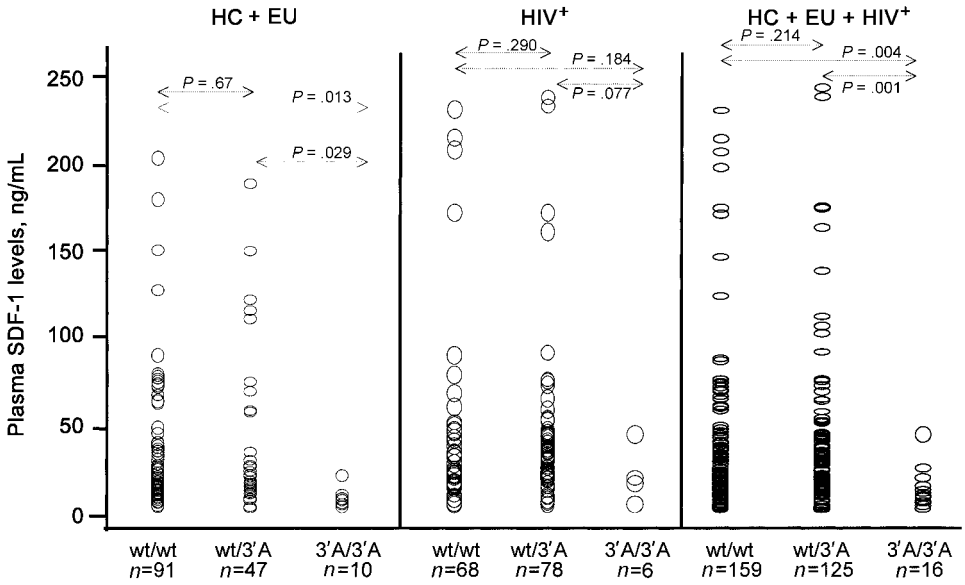

Figure 2. Distribution of concentrations of plasma stromal cell–derived factor (SDF)–1, according to SDF1-3′A genotype, in uninfected subjects (HC [healthy controls] + EU [exposed but uninfected subjects]), human immunodeficiency virus–positive subjects (HIV^+), and the entire population (HC + EU + HIV^+). wt, wild type.

plasma dilution, and enzyme-developing reaction were as recommended by the manufacturer (R&D Systems), with ELISA high–binding capacity plates (MaxiSorb; Nunc Products). All determinations were done in duplicate, and in most cases they were confirmed in an independent experiment.

CXCR4 expression on $CD4^+$ and $CD8^+$ T lymphocytes. From the same sample that had been used to obtain plasma, peripheral-blood mononuclear cells were isolated by standard Ficoll (1.077 density) gradient centrifugation and were cryopreserved ≤4 h after being collected [48]. The expression of surface CXCR4 on $CD4^+$ and $CD8^+$ T lymphocytes was analyzed by flow cytometry using thawed cells and peridinin chlorophyll protein (PerCP; Becton Dickinson)–conjugated CD4 and CD8 with phycoerythrin–conjugated CXCR4 (Becton Dickinson), on a FACSCalibur flow cytometer (Becton Dickinson), as reported elsewhere [48]. With respect to CXCR4 expression, there were no significant differences between fresh and cryopreserved cells.

Statistical analysis. Proportions of different genotypes and allelic frequencies between groups were analyzed by χ^2 test. Plasma SDF-1 levels and percentages of $CXCR4^+$ $CD4^+$ and $CD8^+$ lymphocytes were compared by the Kruskal-Wallis test, with Bonferroni adjustment of P being used for multiple comparisons.

Results

Distribution of SDF1-3′A polymorphism. The genotypic and allelic frequencies of SDF1-3′A polymorphism are shown in table 2. Among HC ($n = 88$), the SDF1-3′A allele frequency was 25.57%, with 6.8% (6/88) and 37.5% (33/88) frequencies, respectively, of SDF1-3′A homozygous and heterozygous genotypes, frequencies that are consistent with data reported in other healthy white populations [30–32, 49, 50]. With respect to SDF1-3′A allelic frequency, there were no significant differences between HIV-positive groups and HC, and this also was true for the frequency of SDF1-3′A homozygosity, although the lowest SDF1-3′A homozygosity frequency occurred in LTNP (2/82 [2.4%]) and in ES patients (0/26), whereas the highest value occurred in patients with a more advanced stage of HIV-1 infection—that is, the IS + AS group (4/44 [9.1%]; $P = .1$, compared with HC). There was a trend toward an increased heterozygote frequency in all HIV-1–positive groups, compared with HC, although this increase was statistically significant only for LTNP (49/82 [59.8%];$P = .004$), whereas in ES patients (11/26 [42.3%] and in the IS + AS group (18/44 [40.9%]) it did not significantly differ from that in HC (table 2).

Among EU subjects, the allelic frequency (8.33%) of SDF1-3′A in EU-sex was clearly lower than that in HC ($P = .008$) and LTNP ($P = .001$); it also was lower than that in EU-hem (28.33%), although at the limit of statistical significance ($P = .05$), probably because of the low number of subjects. In accordance with this decreased allelic frequency, EU-sex lacked mutated homozygotes (0/30), and the SDF1-3′A heterozygote frequency (5/30 [16.7%]) was significantly lower than both that in HC ($P = .03$) and that in LTNP ($P = .0001$) (table 2). In contrast, in EU-hem the SDF1-3′A allele frequency (28.33%), as well as the frequencies of the SDF1-3′A homozygous (4/30 [13.3%]) and heterozygous (9/30 [30.0%]) genotypes, did not differ significantly from those in either HC or the HIV-positive group, except for a significantly decreased heterozygote frequency compared with that in LTNP ($P = .005$). Although it lacked statistical significance, the SDF1-3′A homozygote fre-

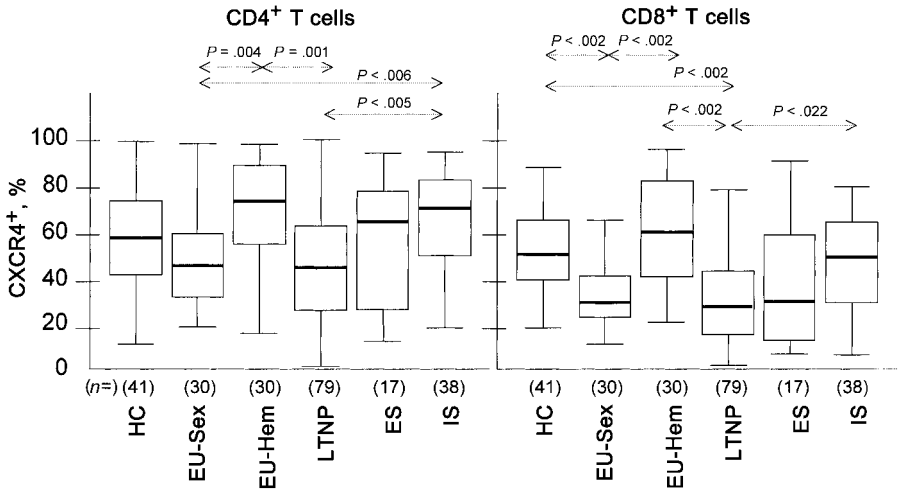

Figure 3. Box-and-whisker plot of percentages of $CD4^+$ and $CD8^+$ T cells expressing surface CXCR4 in human immunodeficiency virus–infected subjects (LTNP, long-term nonprogressors; ES, early stage of HIV-1 infection; IS, intermediate stage of HIV-1 infection; AS, advanced stage of HIV-1 infection), exposed but uninfected subjects (EU-sex, sexual exposure to HIV-1; EU-hem, exposure to HIV-1 via non–virus-inactivated blood products), and healthy controls (HC). Boxes represent interquartile ranges; the horizontal bar within each box indicates median. Whiskers indicate 10th and 90th percentiles. Only P values <.05 are shown.

quency among EU-hem (13.3%) was the highest observed among all groups studied.

Plasma SDF-1 levels. The median and interquartile range (IQR) of plasma SDF-1 concentrations in the different HIV-1–positive groups and in the EU and HC groups are shown in figure 1. The plasma SDF-1 levels in HC ($n = 119$) were 0.0–198 ng/mL (median, 11.2 ng/mL; IQR, 7.85–20.27). In the HIV-positive group, the medians were 29.4 ng/mL (range, 5.4–238.6; IQR, 17.4–40.8) in LTNP ($n = 70$), 25.45 ng/mL (range, 12.0–215.1; IQR, 16.83–46.15) in ES patients ($n = 16$), 33.3 ng/mL (range, 1.0–171.0; IQR, 13.3–69.6) in IS patients ($n = 30$), and 17.8 ng/mL (range, 2.3–65.7; IQR, 4.1–24.4) in AS patients ($n = 13$). Compared with those of HC, plasma SDF-1 concentrations in all these HIV-positive groups were significantly increased ($P \leq .0005$), except in AS patients ($P > .72$) (figure 1).

Among EU subjects, plasma SDF-1 levels in EU-sex ($n = 30$) were 1.2–123.7 ng/mL (median, 30.9 ng/mL; IQR, 20.3–48.6). These values were highly increased compared with those in HC ($P < .0005$) and EU-hem ($P < .0005$). The latter group, EU-hem ($n = 30$), showed a range of 1.3–64.1 ng/mL (median, 9.6 ng/mL; IQR, 4–14.2), which was also decreased compared with that in HC, although without statistical significance ($P = .15$) (figure 1).

Plasma SDF-1 levels and SDF1-3′A genotype. We then analyzed whether there was a relationship between the SDF1-3′A genotype and plasma SDF-1 levels, in uninfected subjects (i.e., the HC and EU groups), in HIV-positive subjects, and in the entire study population. As shown in figure 2, among uninfected subjects there was a clear association between low plasma SDF-1 levels and SDF1-3′A homozygosity. Plasma SDF-1 levels in uninfected SDF1-3′A homozygotes ($n = 10$) clustered within a narrow range, 4.0–23.2 ng/mL (median, 7.0 ng/mL; IQR, 5.1–16.1). These levels were significantly decreased compared with those found in wild-type SDF1 homozygotes ($n = 91$) ($P = .013$) and heterozygotes ($n = 47$) ($P = .029$). Plasma SDF-1 concentrations in wild-type homozygotes and heterozygotes were similar ($P = .67$) and showed a wide range of values, 0.4–198.2 ng/mL (median, 13.4 ng/mL; IQR, 8.2–32.0) in the former and 0.0–170.7 ng/mL (median, 12.4 ng/mL; IQR, 8.85–22.48) in the latter (figure 2).

As in uninfected subjects, among HIV-infected subjects plasma SDF-1 levels in heterozygotes and wild-type homozygotes were similar ($P = .29$) and showed a wide range of values, whereas among SDF1-3′A homozygotes ($n = 6$) they were low and clustered within narrow range, 0–48 ng/mL, although these decreased values did not reach statistical significance when compared with either those found in heterozygotes ($n = 78$; $P = .077$) or those found in wild-type homozygotes ($n = 68$; $P = .184$), likely because of the insufficient number of SDF1-3′A homozygotes in the HIV-positive group. Of the 6 SDF1-3′A homozygote HIV-positive subjects, 2 were LTNP hemophiliacs, and the rest were AS patients.

When the plasma SDF-1 levels of the entire study population (i.e., HC + EU + HIV-positive) were analyzed with respect to SDF1-3′A genotype, the results showed a pattern very similar to that found among uninfected subjects (i.e., the HC + EU group); that is, there were no significant differences between wild-type homozygotes and SDF1-3′A heterozygotes ($P = .21$), whereas each one of these latter two groups showed very significantly increased plasma SDF-1 concentrations compared with those in SDF1-3′A homozygotes ($n = 16$) ($P \leq .0047$) (figure 2).

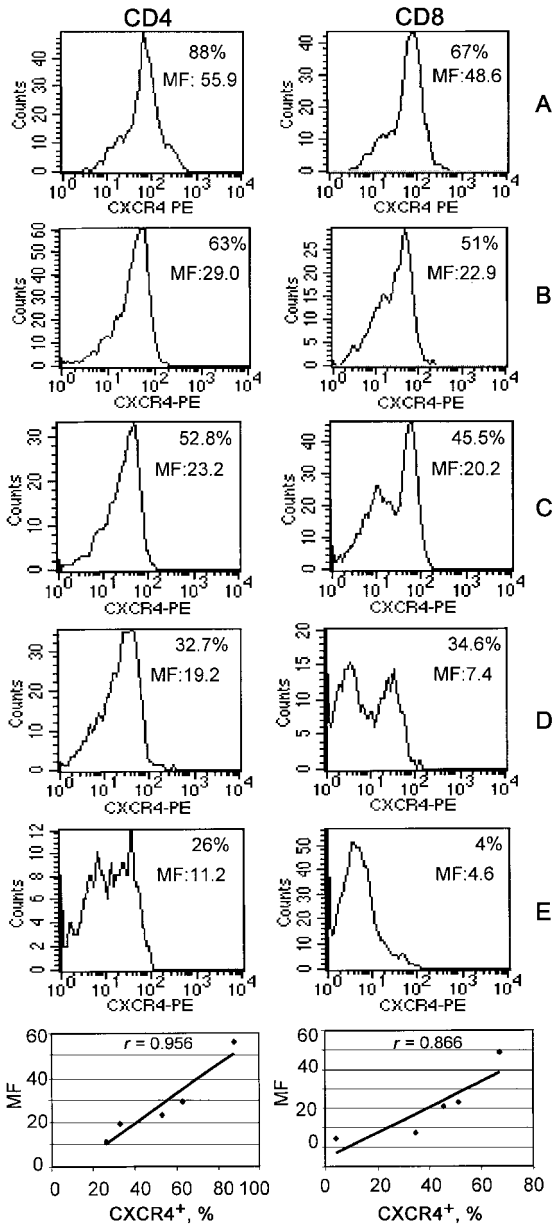

Figure 4. Histograms of expression of CXCR4 on gated $CD4^+$ and $CD8^+$ T cells in 5 representative HIV-positive subjects (A–E) with high, intermediate, and low percentage of $CXCR4^+$ cells. Intensity of mean fluorescence (MF) and percentage of $CXCR4^+$ cells are shown within each pair of panels *A–E;* correlation between the 2 parameters, which was observed in entire study population, is depicted in the bottom pair of unlabeled panels. PE, phycoerythrin.

CXCR4 expression on T lymphocytes. The proportion of $CD4^+$ and $CD8^+$ T cells expressing surface CXCR4 could be analyzed in the different groups—except in the patients with the most advanced stage of HIV-1 infection (i.e., the AS group), because an insufficient number of cells were available (figure 3). In HC, the median percentages of $CXCR4^+$ $CD4^+$ and $CD8^+$ T cells were 57% (IQR, 41.0%–73.0%) and 50.0% (IQR, 39.0%–65.0%), respectively. Among EU subjects, there was a significant difference between the EU-hem and EU-sex subgroups, in their proportions of $CXCR4^+$ $CD4^+$ and $CD8^+$ T cells, which were significantly elevated in EU-hem compared with EU-sex ($P = .004$ for $CXCR4^+$ $CD4^+$ T cells; $P < .002$ for $CXCR4^+$ $CD8^+$ T cells). In EU-hem, the medians were 72.82% (IQR, 54%–88.58%) and 59.49% (IQR, 39.81%–82.84%) for $CXCR4^+$ $CD4^+$ and $CD8^+$ T cells, respectively, whereas in EU-sex they were 45.1% (IQR, 37.2%–52.7%) and 29.1% (IQR, 22.6%–40.39%), respectively. In addition, the levels of $CXCR4^+$ $CD8^+$ T cells in EU-sex were significantly lower than those in HC ($P < .002$), whereas among EU-hem there was a nonsignificant trend ($P = .10$) toward a higher proportion of $CXCR4^+$ $CD4^+$ T cells, compared with that in HC (figure 3).

Among HIV-infected subjects, the levels of $CXCR4^+$ $CD4^+$ T cells in LTNP (median, 45.1%; IQR, 25.53%–63.45%) did not differ significantly from those in either HC or EU-sex but were clearly lower than those in EU-hem ($P = .001$), whereas in ES patients (median, 66.67%; IQR, 26.06%–78.57%) and IS patients (median, 70.04%; IQR, 49.44%–82.35%), the percentages were higher than those in LTNP, HC, and EU-sex, although these increases were statistically significant only for IS patients compared with LTNP ($P < .005$) and for IS patients compared with EU-sex ($P < .006$). As for the percentages of $CXCR4^+$ $CD8^+$ T cells, in LTNP they were similar to those in EU-sex and were significantly lower than those in HC ($P < .002$) and those in EU-hem ($P < .002$); in ES patients these percentages did not differ from those in HC, LTNP, and EU-sex but were lower than those in EU-hem ($P = .072$; i.e., near the limit of statistical significance); in IS patients, they were significantly higher than those in LTNP ($P = .02$) and also were higher, although without statistical significance ($P = .278$), than those in EU-sex.

The histograms and the mean fluorescence intensity of $CXCR4^+$ cells gated on $CD4^+$ and $CD8^+$ T cells in 5 representative HIV-positive subjects with high, intermediate, and low percentages of $CXCR4^+$ cells are shown in figure 4. Note that the mean fluorescence-intensity values and the percentages of $CXCR4^+$ cells are clearly correlated ($r = .965$ for $CD4^+$ cells; $r = .865$ for $CD8^+$ T cells), a result found in the entire study population. This concordance between mean fluorescence intensity and percentage of T lymphocytes positive for surface CXCR4 is entirely consistent with data from other studies in which the in vitro regulation of expression of CXCR4 on normal T lymphocytes has been analyzed [21, 41, 42].

Discussion

This study has shown that SDF1-3′A homozygosity lacks disease progression–retarding effects, because in LTNP the frequency of this genotype is even lower than that in HC and is clearly lower than that in HIV-1–positive progressors. This con-

clusion is consistent with those of other studies [31–37], some of which studies had found that SDF1-3′A homozygosity is, in fact, associated with a faster CD4 T cell decrease and a rapid progression to AIDS. The current cross-sectional study is not informative about this possibility, because rapid progressors were not available. It should be mentioned that the SDF1-3′A allele has also been found (1) to abrogate the progression-retarding effect of CCR5Δ32 heterozygosity in children [51], (2) to be associated with an increased risk for non-Hodgkin lymphoma in HIV-positive subjects [52], (3) to increase the likelihood of perinatal infection in mothers but not in their infants [53], and (4) to be present in nonhuman primates most susceptible to lentivirus-induced AIDS and to be absent in those resistant to the latter [54].

Compared with HC, in HIV-1–infected subjects there was a trend toward an increased frequency of SDF1-3′A heterozygosity, although it was significant only for LTNP, a finding not observed in other studies. This discrepancy is unlikely to be attributable to either (1) methodologic inconsistencies, because genotyping was done by the method originally described elsewhere [30] and was repeated in all cases, or (2) an insufficient number of LTNP and/or a nonrigorous definition of this condition, because other studies, with a lower number of LTNP and with a far less strict definition of LTNP condition, have been used as evidence for an association between SDF1-3′A homozygosity and either slow or rapid progression. Perhaps this discrepancy is related to the fact that in our LTNP group, the risk of intravenous drug use predominated (see table 1), whereas in other studies homosexual or bisexual men predominated. None of the LTNP had evidence of non-Hodgkin lymphoma. Because rapid progressors were not available, no conclusions can be drawn about the possible significance, if any, of the increased SDF1-3′A heterozygosity among LTNP.

Among EU subjects, the SDF1-3′A allelic frequency in EU-sex, but not in EU-hem, was significantly underrepresented compared with both that in HIV-positive subjects and that in HC, with absence of mutated homozygotes and a significantly decreased heterozygote frequency. This indicates that SDF1-3′A heterozygosity not only lacks infection-protective effects, as has been suggested elsewhere [30], but also can somehow act as a factor favoring infection through sexual exposure to HIV-1. Such underrepresentation did not occur in EU-hem, a finding that is in accord with the infant SDF1-3′A allele's lack of effect in perinatal transmission [36, 53]. These data suggest that the multiplicity of infection-protecting host factors [14–16, 55] can greatly differ, depending on whether the route of exposure to HIV-1 is sexual or parenteral [56]. This notion may also apply to HIV-1 progression, because the association between delayed progression and an increased frequency of CCR5Δ32 and CR2-64I heterozygosities was found in homosexual men but not in hemophiliacs, intravenous drug users, or children infected perinatally [36, 37, 57, 58]. On the other hand, most (76.8%) of the EU-sex in the present study were women who, both prior to and during the study, engaged in unprotected vaginal sex with HIV-positive men, whereas all EU subjects analyzed by Winkler et al. [30] were homosexual men. This discrepancy between studies might suggest that infection-protecting host factors could differ depending on whether sexual exposure to HIV-1 is anal or vaginal.

The wide range of plasma SDF-1 levels in HC, which also has been observed in a previous study [38], which has used a similar ELISA, suggests that host factors (genetic or epigenetic) that result in phenotypes for high or low SDF-1 production might exist. The plasma-level differences between the different groups are not attributable to blood and plasma collection and storage conditions, because the latter were the same in all cases. Because SDF-1 is the paradigm of homeostatic chemokines, constitutively produced by a broad range of cells except blood leukocytes [7–13], it is conceivable that plasma SDF-1 levels might reflect the overall SDF-1 production of a given host and/or the half-life of this chemokine in this host, events that likely are dependent on host factors (genetic and/or epigenetic). Therefore, we analyzed the relationship between the SDF1-3′A genotype and plasma SDF-1 levels, because it had been proposed that SDF1-3′A homozygosity could result in a higher SDF-1 production [30]. Our data clearly show, for the first time, that, among uninfected subjects, SDF1-3′A homozygosity, but not heterozygosity, is associated with low plasma SDF-1 levels, and the same result was seen in the entire study population. This association might suggest either that SDF1-3′A homozygosity results in decreased SDF-1 production or that there is a linkage between this genotype and other unknown (genetic or epigenetic) host factors that could decrease SDF-1 production and/or promote faster plasma SDF-1 clearance. An in vitro mutation study did not observe differences, in SDF-1 RNA synthesis, between the unmutated and mutated alleles [59]. However, it is unknown whether this system reproduced the physiologic SDF1-3′A homozygous state, and, therefore, experiments with SDF-1–producing cells from mutated and unmutated homozygous healthy subjects are necessary to assess whether there is a relationship between SDF1-3′A genotype and SDF-1 production. Whatever the case might be, the association between SDF1-3′A homozygosity and low plasma SDF-1 levels seems congruent with those studies reporting that this genotype is associated with a faster $CD4^+$ T cell decline and AIDS progression [31–35].

Among HIV-positive subjects, plasma SDF-1 levels were significantly increased compared with those in HC, a result seen in LTNP as well as in the ES and IS groups but not in the patients with the most advanced stage of HIV-1 infection (i.e., the AS group). To our knowledge, only 2 studies have investigated plasma SDF-1 in relation to HIV-1 disease progression, and the results are consistent with our findings [38, 39], although neither of those previous studies included LTNP or analyzed the relationship between plasma SDF-1 levels, SDF1-3′A genotype, and CXCR4 expression on T lymphocytes. Our

findings suggest that HIV-1 infection involves overproduction of SDF-1, which might act by delaying progression to AIDS, a notion consistent with recent findings that high plasma SDF-1 levels prevent the emergence of X4 strains [40]. It is noteworthy that such SDF-1 overproduction in HIV-positive patients is not attributable to any kind of chronic viral infection and/or chronic antigen stimulation, because all EU-hem were hepatitis C virus–positive and had plasma SDF-1 levels similar to those in HC. Further studies, on plasma SDF-1 levels in other infectious and noninfectious chronic inflammatory diseases, are certainly required.

Among the EU-sex and EU-hem groups, there was a reciprocally inverse relationship between plasma SDF-1 levels and expression of surface CXCR4 on $CD4^+$ and $CD8^+$ T lymphocytes; that is, high plasma SDF-1 levels were coupled with low expression of CXCR4 in EU-sex whereas the opposite situation occurred in EU-hem. Although other, unknown immunologic variables might also result in this decreased CXCR4 expression in EU-sex, it is also conceivable that this association reflects an in vivo down-regulation by endogenous SDF-1, as occurs in vitro. In normal subjects, the expression of surface CXCR4 occurs mainly on resting/naive T lymphocytes, whereas the expression of surface CCR5 occurs in memory/activated CD4 T lymphocytes [60]. However, all blood T lymphocytes constitutively express high intracellular stores of CXCR4, which becomes rapidly expressed on the surface of virtually all naive and memory T cells after a very short culture in the absence of SDF-1, whereas surface CXCR4 remains highly down-regulated in the presence of SDF-1 [21, 41, 42]. Other data suggesting an in vivo down-regulation of CXCR4 by endogenous SDF-1 are the findings that (1) surface CXCR4 is selectively down-modulated on intestinal lymphocytes and is coupled with the prominent expression of SDF-1 in mucosal cells [10] and (2) intravenous injection of SDF-1 into mice causes a rapid loss of surface CXCR4 on CD4 T cells [39].

On the other hand, it is obvious that, in EU-sex, the underrepresentation of the SDF1-3′A allele, coupled with high plasma SDF-1 levels and low expression of CXCR4 on $CD4^+$ T lymphocytes, cannot account for this group's natural resistance to HIV-1 infection, since R5 strains are those which generally transmit the infection, even when X4 strains predominate in the viral inoculum, probably because the mucosa-produced SDF-1 continuously down-modulates CXCR4 on resident HIV target cells [10]. Given (1) the essential role that SDF-1 plays as a homeostatic chemokine governing lymphopoiesis and lymphocyte traffic [6, 23, 61] and (2) SDF-1 production by mucosal dendritic, epithelial, and endothelial cells [10, 11], this association suggests that a phenotype associated with high SDF-1 production is an advantage favoring both optimal maturation of lymphocytes and their migration to vaginal mucosa, which then might be able to "abort" the infection locally. Evidence suggesting a possible immune response–dependent abortion of HIV-1 infection in the vaginal mucosa is available [62, 63].

Among LTNP, high plasma SDF-1 levels were coupled with proportions of $CXCR4^+$ cells that, in $CD4^+$ and $CD8^+$ T cell subsets, respectively, were similar to and lower than those in HC, whereas in patients with advancing HIV-1 infection (i.e., the IS group) (figure 3), the proportions of $CXCR4^+$ cells were significantly increased compared with those in LTNP. These data suggest that high plasma SDF-1 levels coupled with low CXCR4 on T cells is a feature of LTNP and that advancing disease is associated with both enhanced CXCR4 expression on T lymphocytes and a decrease to normal plasma SDF-1 levels in the most advanced stages of HIV-1 infection (i.e., the AS group) (figure 1). This enhanced expression of CXCR4 in advanced disease is in accord with our previously published data [64], as well as with other previously published data, which include reports of an association between enhanced CXCR4 on $CD4^+$ T cells and the emergence of X4 strains [65–67]. It is also congruent with the overexpression of CXCR4 on CD4 T cells in transgenic mice, which causes severe depletion of peripheral $CD4^+$ T cells [68]. Other studies, however, with fewer patients and without LTNP, have found that the expression of CCXR4 on T cells of HIV-positive patients is decreased compared with that in HC [69–71], although a tendency toward greater activation of $CXCR4^+$ $CD4^+$ T cells was found to occur in patients with the most advanced HIV-1 infection [69]. Because advancing disease involves increased activated/memory T cells, these data might be considered to be consistent with the down-regulation of CXCR4 on CD4 T cells activated in vitro by CD3 monoclonal antibody [42, 72]. However, it is now known that costimulation with either CD3 CD28 monoclonal antibodies or CD3 plus HIV Tat protein results in the activation and proliferation of $CD4^+$ T cells and causes CXCR4 up-regulation on both resting and activated $CD4^+$ T cells, concomitant with increased infectivity by X4 strains [72]. Given both the essential role that CD28 plays as a costimulatory molecule for antigen-induced activation of T cells and the fact that the highly cytopathic X4 strains mainly replicate in proliferating cells, these findings suggest a relevant mechanism for the progression of HIV-1 disease [72] and are consistent with our findings, in advancing HIV-1 disease, of an increased expression of surface CXCR4 on T cells.

In conclusion, this study has shown the following: (1) SDF1-3′A homozygosity is associated with low plasma SDF-1 levels in uninfected subjects and lacks progression-retarding effects; (2) HIV-1 infection involves SDF-1 overproduction, which is not attributable to any kind of chronic viral infection, because all EU hemophiliacs whom we studied were positive for hepatitis C virus and had normal SDF-1 levels; (3) high plasma SDF-1 levels and low CXCR4 expression on T lymphocytes are a feature of LTNP, whereas in advancing disease the expression of CXCR4 increases and, in the more advanced stages of HIV-1 infection, is accompanied by a decrease of plasma SDF-1; and (4) EU subjects (but not EU hemophiliacs) with sexual, mostly vaginal, exposure to HIV-1 show an underpre-

sentation of SDF1-3′A allele frequency, which is coupled with high plasma SDF-1 levels and low CXCR4 expression.

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5. Discusión

El objetivo de la tesis presentada ha sido profundizar en los factores inmunogenéticos implicados en la resistencia/susceptibilidad a la infección por el VIH y/o la influencia sobre la progresión de la infección hacia Sida. Para el desarrollo de este estudio se ha elaborado una cohorte de individuos, muy bien definidos, que cumplían con criterios de progresión lenta (LTNP), progresión típica hacia Sida y de expuestos no infectados (EU). Además se definió un cohorte de individuos sanos como grupo control.

Los objetivos recogidos en los estudios 1 y 2 de la tesis pretenden estudiar la presencia de factores inmunogenéticos no descritos previamente, que pudieran influir en la expresión de los principales co-receptores del VIH (CCR5 / CXCR4). En este sentido, el primer estudio de la tesis pretendía demostrar que los polimorfismos previamente descritos en el receptor de la Interleucina 4 (IL4RA) podían influir en la evolución de la infección por el VIH puesto que la IL-4 regula (a través de este receptor) dos aspectos fundamentales de la resistencia al VIH y su progresión a Sida, como son el desplazamiento de la respuesta inmune hacia Th2 y la expresión de los co-receptores del VIH. Entre las variantes descritas en el receptor, destaca la presencia de una posición polimórfica (I50V) en la región codificada extracelular. El principal hallazgo de nuestro estudio fue que la presencia homozigota de la V50 se asociaba con la progresión lenta de la enfermedad, lo que sugiere que esta variación se asocia a una hiporrespuesta a la IL-4 potenciando así la respuesta Th1 y una disminución de la expresión de CXCR4. Otras variantes en la región intracelular del receptor, poco frecuentes en la población general, se hallaron asociadas a una mayor susceptibilidad a la infección por VIH, especialmente tras una exposición por vía parenteral. En este caso, los datos sugieren que variantes polimórficas en la región intracelular del IL4-RA podrían estar relacionadas con una mayor respuesta del receptor tras la unión de la IL-4 (desarrollo de Th2 y aumento de la

expresión de CXCR4). Estos datos son de gran interés si se tiene en cuenta que recientemente se ha comercializado un antagonista de IL4-RA para el tratamiento de enfermedades alérgicas mediadas por IgE.

El segundo trabajo, pendiente de publicación, se basa en descripciones recientes que demostraban que la molécula CD28, esencial en la respuesta inmune, regulaba la expresión de los co-receptores del VIH. Estudios previos no habían demostrado variantes polimórficas en la región codificada de CD28, por lo que orientamos nuestros esfuerzos en el estudio de la región promotora (5'UTR). El análisis mediante SSCP reveló la presencia de varios patrones, que se identificaron mediante secuenciación. Los patrones correspondieron a 3 alelos diferentes, según el número de repeticiones CTTTT halladas en el extremo 5'. De esta forma, hemos descrito alelos con 3,4 o 5 repeticiones CTTTT en tandem y de la combinación de estos, 4 genotipos diferentes. Sin embargo, es necesario destacar que el alelo y el genotipo predominantes fueron el de 4 repeticiones y el homozigoto compuesto por 2 alelos con 4 repeticiones, respectivamente. El segundo alelo más frecuente fue el de 5 repeticiones y el genotipo heterocigoto de 4 y 5 repeticiones, que desde el punto de vista analítico, se consideraron en un mismo grupo junto con otros alelos y genotipos que incluían alelos de 3 y 5 repeticiones (que representaban menos del 1% del total). La caracterización de la región promotora de una molécula tan importante como CD28 es de gran interés, aunque es necesario realizar estudios en el futuro que permitan establecer si estas variantes influyen de alguna forma en la actividad de esta molécula. En la segunda parte del trabajo, se expone que los individuos EU, especialmente los expuestos por vía sexual, presentaban con mayor frecuencia el alelo de 4 repeticiones y el genotipo homozigoto de 4 repeticiones, lo que sugiere que estas variantes podrían proteger de la infección por el VIH.

El tercer estudio de la presente tesis pretendía esclarecer el papel del polimorfismo descrito en el gen SDF-1 y que consiste en la mutación G→A en la posición 801 de la

región 3' no traducida (SDF1-3'A). La presencia homozigota de esta mutación se había asociado con la progresión lenta hacia sida y los autores postulaban que probablemente esta mutación se asociaba a una mayor producción de SDF-1, que bloquearía y disminuiría la expresión de CXCR4, co-receptor del VIH-X4 (productor de sincitios), responsable de una rápida progresión a sida. Sin embargo, estos resultados no fueron corroborados posteriormente por otros autores que incluso exponían resultados opuestos. Por este motivo diseñamos un estudio transversal para analizar el impacto de la mutación SDF1-3'A sobre: i) la susceptibilidad/resistencia a la infección por el VIH y su progresión hacia sida, ii) los niveles plasmáticos de SDF-1 y la expresión de CXCR4. Los resultados mostraron que la mutación SDF1-3'A no se asociaba con una progresión lenta de la infección y si con una menor producción de SDF-1. Como cabía esperar, la expresión de CXCR4 estaba inversamente relacionada con los niveles de SDF-1 y así se dibujaban en el trabajo dos patrones: i) LTNP, con altos niveles de SDF-1 y baja expresión de CXCR4 y ii) pacientes con progresión de la infección, con niveles bajos de SDF-1 y elevada expresión de CXCR4. Aunque se trata de un estudio transversal, los resultados sugieren que a lo largo de la infección se produce una pérdida progresiva de capacidad de síntesis de SDF-1, excepto en los pacientes LTNP, que paralelamente se acompaña de un aumento de la expresión de CXCR4, lo que favorecería el cambio de fenotipo viral R5 a X4. De hecho, en otro trabajo citado en esta tesis, evidenciábamos que una elevada expresión de CXCR4 era un marcador asociado a progresión de la infección.

El estudio que describió por primera vez la mutación SDF1-3'A, estudiaba un grupo de homosexuales expuestos no infectados y apuntaba que la heterozigocia se asociaba a resistencia a la infección por el VIH. Sin embargo, no hay estudios posteriores que corroboren estos hallazgos. En nuestra cohorte de pacientes EU y especialmente entre aquellos con exposición por vía sexual, la mutación SDF1-3'A estaba significativamente infrarrepresentada, contrariamente a lo descrito con anterioridad.

Los datos presentados en esta tesis muestran como los factores relacionados con la resistencia/susceptibilidad a la infección por VIH y su progresión hacia sida son múltiples y su conocimiento es esencial, por que entre otras cosas, pueden ayudarnos a elaborar nuevas estrategias de tratamiento de la infección por el VIH. El ejemplo más claro en este sentido son los inhibidores de la fusión, capaces de bloquear el CCR5, uno de los principales co-receptores del VIH y que actualmente ya son una realidad para el manejo de los pacientes infectados por el VIH con mala respuesta al tratamiento con HAART.

6. Resumen de conclusiones

La presencia de la variante homozigota V50 en el exón 5 del gen que codifica el receptor de la lL-4, se asocial con la progresión lenta de la infección por el VIH. La presencia de haplotipo no comunes en el exon 12 del mismo gen, podrían estar asociados a una mayor susceptibilidad a la infección por el VIH, tras una exposición parenteral.

En la región 5' no traducida del gen que codifica CD28 hemos descrito un polimorfismo no conocido, que consiste en la inserción o delección de 5 nucleótidos (CTTTT). Estas variantes (inserción o delección) se encuentran con más frecuencia en los pacientes infectados con respecto a aquellos pacientes repetidamente expuestos al VIH por vía sexual, pero que permanecen seronegativos. Estos datos sugieren que estas variantes favorecerían la infección por el VIH a través de las mucosas, quizás por modificaciones en la expresión de CD28 o por la modulación de la expresión de los co-receptores del VIH.

La presencia homozigota de la mutación SDF1-3'A, se asocia a concentraciones bajas de SDF-1. La infección por el VIH se acompaña de una elevación de los niveles de SDF-1 y un descenso en la expresión de CXCR4. Este patrón se mantiene en los pacientes LTNP, mientras que en los pacientes con progresión normal de la infección y especialmente en aquellos con una fase muy avanzada de la enfermedad, se observa una baja producción de SDF-1 y una elevada expresión de CXCR4. Por otro lado, nuestro estudio en pacientes expuestos no infectados, no confiere a la mutación SDF1-3'A ningún papel protectivo o favorecedor de la infección por VIH. Todo ello sugiere que SDF1 podría estudiarse como una forma de inmunoterapia en pacientes VIH positivos en fase evolucionada de la enfermedad.

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