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TESIS DOCTORAL

INMUNODEFICIENCIAS PRIMARIAS CELULARES EN LA INFANCIA:
NUEVAS APORTACIONES A SU DIVERSIDAD, PATOGENIA,
DIAGNÓSTICO Y TRATAMIENTO

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TITULO: *Inmunodeficiencias primarias celulares en la infancia: nuevas aportaciones a su diversidad, patogenia, diagnóstico y tratamiento.*

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TÍTULO DE LA TESIS: *Inmunodeficiencias Primarias celulares en la infancia: nuevas aportaciones a su diversidad, patogenia, diagnóstico y tratamiento.*

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INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El presente trabajo compilatorio de publicaciones científicas, representa la suma de tres contribuciones originales al conocimiento molecular, celular y médico en el incipiente campo de la medicina genómica y personalizada. Estos hallazgos, de carácter tanto clínico como básico, constituyen un grupo de aportaciones con impacto en su área científico-médica. Han sido realizadas a lo largo de cinco años de trabajo ininterrumpido y añaden a su valor propio, el hecho de encuadrarse en un ámbito de la patología humana cuantitativamente muy reducido: el de las enfermedades raras. Durante este período de tiempo el doctorando ha realizado con éxito su formación básica y tecnológica que ha aplicado muy acertadamente en el territorio de la patología del sistema inmune y más concretamente en el campo de las inmunodeficiencias primarias. El doctorando ha adquirido una sólida formación conceptual, metodológica y clínica. Su esfuerzo personal le ha permitido traducir esta formación en innovadores logros profesionales como la identificación, diagnóstico, tratamiento y seguimiento de los pacientes. Los resultados tangibles abarcan desde el reconocimiento de nuevas formas de presentación en dos de los síndromes estudiados, hasta la identificación de una nueva mutación patogénica responsable de la enfermedad, pasando por la implementación de novedosas pautas terapéuticas en una patología que cuenta en la actualidad con menos de 40 pacientes descritos en la literatura científica internacional. Todo ello, se ha plasmado en publicaciones en revistas indexadas (JCR) en primer (Pediatr. Blood Cancer 60: 29-31. 2013; Pediatr. Blood Cancer 61:178-179. 2014) y segundo cuartíl (Clin Vaccine Immunol 20: 765-771. 2013) que representan en su conjunto una contribución original al conocimiento de la diversidad, patogenia, diagnóstico y tratamiento en esta familia de enfermedades primarias del sistema inmune.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 7 de Julio de 2014

Firma de los directores

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a. Introducción

Las inmunodeficiencias primarias (IDPs) comprenden un grupo de defectos celulares y moleculares que alteran el normal desarrollo y/o funcionalidad del sistema inmunológico. Sus manifestaciones clínicas aparecen generalmente en edades tempranas de la vida[1]. El aumento de la susceptibilidad a infecciones constituye la característica común en las IDPs [2]. Además de episodios infecciosos reiterados, manifestaciones clínicas de tipo autoinmune, autoinflamatoria, granulomatosa y linfoproliferativa pueden presentarse también durante el desarrollo y evolución de algunas de estas enfermedades [3].

La primera referencia documentada de una inmunodeficiencia data de 1919, al describir alteraciones en el sistema de complemento de cobayas[4]. En humanos, se describieron durante la segunda y tercera década del siglo XX algunos síndromes que fueron posteriormente categorizados como IDPs. Entre ellos una forma severa de neutropenia descrita por Schultz en 1922 y un síndrome consistente en plaquetas de tamaño reducido y otitis recurrente descrito en pacientes pediátricos por Wiskott en 1937[5].

En 1950 Glanzmann y Riniker publicaron un síndrome de curso fatal caracterizado por candidiasis pulmonar e intestinal y linfopenia absoluta, al que denominaron "Linfocitotisis esencial"[6].

En 1952 el Coronel Ogden Bruton describe la agammaglobulinemia, que consideramos como la primera demostración en humanos de la existencia de una inmunodeficiencia primaria (IDP). El paciente, un varón de 8 años, presentó desde el cuarto año de vida 19 episodios de sepsis. En 10 de los cuales se evidenció la presencia de alguna de las cepas 3-8,14 y 33 de neumococo. El análisis electroforético del plasma del paciente mostró la ausencia de fracción gamma. A pesar de este dato y dada la edad de aparición de las infecciones, Bruton consideró la enfermedad como un trastorno adquirido [7].

Un año más tarde Donohue identificó un caso de linfopenia severa en un niño de 2 años con infección respiratoria, aftas orales, amigdalitis y hepato-esplenomegalia que falleció 20 días después de su ingreso hospitalario. El estudio post mortem del paciente evidenció en esófago ulceras invadidas por Cándida albicans. Así mismo, mostró la presencia de un timo hipoplásico, ausencia de placas de Peyer en el intestino y de células linfoides en el bazo. Ante estos hallazgos, Donohue asocia la *"atrofia"* severa de

tejido linfoide, la hipoalbuminemia y las infecciones fúngicas del paciente y propone designar este cuadro como "Alinfocitosis T"[8].

En 1957 se describen cuatro pacientes con *"granulomatosis fatal del la infancia",* actualmente conocida como Enfermedad Granulomatosa Crónica (EGC). La ECG, de herencia ligada al sexo, fue caracterizada inicialmente por linfadenitis granulomatosa supurativa y crónica, elevado numero de infecciones, hepato-esplenomegalia, dermatitis atópica e infiltración granulomatosa especialmente en parénquima pulmonar. Los pacientes con este síndrome no presentaban alteraciones cuantitativas en compartimento humoral, conservando además la capacidad de generar anticuerpos específicos[9].

En 1966 y 1967, Holmes y Quie describen la patogenia de esta enfermedad como un defecto en la capacidad bactericida de los fagocitos, que sin embargo conservaban intacta la capacidad de opsonización [10,11]. En 1968 Baehner y cols. demuestran que en pacientes con ECG los granulocitos marcados con nitro-azul de tetrazolio (NBT) son incapaces de reducirlo a azul formazán, demostrando una alteración en la capacidad oxidativa de los fagocitos dependiente de la enzima NADPH-oxidasa[12].

Hitzig en 1968 designa como "deficiencia combinada humoral y celular" un síndrome caracterizado por linfopenia y agammaglobulinemia, con presentación clínica de evolución fatal[13].

En 1970, un comité de expertos de la Organización Mundial de la Salud designa a las patologías que comprometen ambos componentes, humoral y celular, del sistema inmune como "Inmunodeficiencias Severas Combinadas" (SCID en sus siglas inglesas). La primera evidencia que asociaba defectos genéticos con el desarrollo de SCID fue descrita en 1972 por Giblett , quien demostró la ausencia de la enzima Adenosina DeAminasa (ADA) en eritrocitos de sangre periférica de dos pacientes no emparentados que compartían fenotipo $T^{neg} B^{neg}$[14].

La *International Union of Immunological Societies* (IUIS) revisa cada dos años la clasificación de las IDPs. El objetivo es documentar las bases fisiopatológicas, celulares y moleculares de la clasificación así como la variabilidad fenotípica de las IDPs, pues ambas representan la información clave para su manejo diagnóstico y

terapéutico[3]. La actualización más reciente se publicó en 2014 y añade más de 30 defectos genéticos nuevos a la clasificación publicada en 2011. Así mismo, incluye un nuevo grupo (Grupo IX) en la clasificación[15]:

- Grupo I: *Inmunodeficiencias Combinadas Graves (SCID)*
- Grupo II: *Síndromes bien definidos con inmunodeficiencia asociada*
- Grupo III: *Deficiencias predominantemente de anticuerpos*
- Grupo IV: *Defectos de desregulación inmunológica*
- Grupo V: *Defectos congénitos de numero y/o función de fagocitos*
- Grupo VI: *Defectos de Inmunidad Innata*
- Grupo VII: *Síndromes Autoinflamatorios*
- Grupo VIII: *Deficiencias de complemento*
- Grupo IX: *Fenocopias de IDPs*

En este nuevo grupo (IX) y bajo el epígrafe "*Fenocopias de IDPs*", se ha incluido patología causadas por mutaciones somáticas, o bien por mecanismos adquiridos tales como el desarrollo de autoanticuerpos anti-citoquinas esenciales para el correcto funcionamiento del sistema inmune. Todas ellas, tienen como característica común una expresión clínica similar a las producidas por otras IDPs contempladas en otros grupos de la clasificación [16-19].

Así, la clasificación actual de la IUIS es un catalogo de referencia que incluye todas las variantes de IDPs conocidas. Avanza inexorable hacia su subcategorización en función de las variantes fenotípicas de un mismo defecto molecular, sus distintas formas de evolución, pronóstico y abordaje terapéutico [20]. Así pues, este grupo de patología constituye un campo de estudio en constante desarrollo que estimula la investigación de los aspectos básicos alterados de la respuesta inmune en cada una de ellas, así como en la implementación nuevos métodos diagnósticos y estrategias terapeuticas[1]. En el momento de escribir estas líneas han sido ya identificadas 249 distintas IDPs, 70 de ellas en los últimos 6 años [3,15].

En el aspecto epidemiológico, el acceso a la información de los registros de IDPs tanto de carácter nacional como europeo y norteamericano ha facilitado el desarrollo de estudios multi-centricos de grandes cohortes que han permitido establecer los defectos genéticos responsables, las distintas variantes fenotípicas, las diversas estrategias terapéuticas y el pronostico de las IDPs identificadas en la actualidad[21]. No obstante, aún existen formas de IDPs en las que la caracterización clínico-terapéutica no está completamente dilucidada[15].

El análisis, en 2011, de los datos obtenidos de 8 países europeos con los registros de IDPs más homogéneos en función de la información clínica y de laboratorio documentada, evidencia una gran variabilidad en términos de incidencia, prevalencia y edad de diagnóstico de estas enfermedades. Si bien en Alemania e Italia la prevalencia de IDPs es inferior a 2 habitantes por cada 10, en España y Francia alcanzan a 4 y 5 habitantes por cada 10^5 respectivamente.

Presentamos pues, como tesis doctoral un trabajo compilatorio de 3 publicaciones de carácter clínico experimental, cuya unidad temática la constituye en esencia el ámbito de las inmunodeficiencias primarias. Todas ellas son una contribución original a los mecanismos patogénicos responsables de cada enfermedad objeto de estudio, su diagnóstico y su abordaje terapéutico cuando ello ha sido posible habida cuenta de su evolución extremadamente rápida y grave.

Neutropenia Severa Congénita

La Neutropenia Congénita Severa (SCN) comprende un grupo diverso de alteraciones genéticas que resultan en una drástica reducción en el recuento absoluto de neutrófilos (< 500 células/microlitro). Clínicamente se caracteriza por infecciones bacterianas sistémicas de presentación en la infancia[22]. En 1956, Kostmann describió una enfermedad de herencia recesiva caracterizada por infecciones bacterianas graves secundarias al estatus de neutropenia severa con cifras inferiores a 200 células/microlitro. La causa genética de la enfermedad no fue evidente sino hasta medio siglo después[23]. En la actualidad han sido identificados 5 genes implicados en la patogenia de las SCN.

 La variante SCN1 (OMIM #202700), de herencia autosómica dominante, representa aproximadamente el 70% de los casos de SCN. La gingivo-estomatitis, periodontitis, otitis, abscesos subcutáneos y linfadenitis constituyen las manifestaciones clínicas predominantes de la enfermedad[24,25]. La causa de la SCN1 tiene su origen en alguna de las más de 100 mutaciones descritas que afectan el gen ELA2 que codifica la elastasa de neutrófilos, *ELANE,* serin-proteasa con actividad proteolítica y antibacteriana, presente en el citoplasma de precursores de granulocitos y monocitos[26]. Si bien la fisiopatología de esta enfermedad no está completamente dilucidada, algunos autores sugieren que la acumulación de proteína *ELANE* no plegada e hipofuncionante induce un estatus de estrés del retículo endoplásmico, generando apoptosis acelerada en los precursores de neutrófilos [27,28].

La SCN2 (OMIM #600871), autosómica dominante, es una variante extremadamente rara de SCN en la cual una marcada monocitosis y una moderada linfopenia se suman la neutropenia como características fenotípicas de la enfermedad[29]. Mutaciones en el gen que codifica el factor de trascripción supresor tipo 1 idenpendiente del factor de crecimiento, *GFI1*, han sido descritas como causantes de SCN2. GFI1 es esencial en la homeostasis de las células madre hematopoyéticas, regula el proceso de la diferenciación de los neutrófilos, así como su normal desarrollo y funcionalidad, potenciando la transcripción de genes estrictamente necesarios para estos procesos[30,31].

La SCN3, de herencia autosómica recesiva, se manifiesta con afectación neurológica en forma de retraso cognitivo y de desarrollo, así como episodios de epilepsia a partir de la segunda década de la vida[32]. Esta forma de neutropenia, descrita originalmente por Kostmann, está causada por deficiencia de la proteína HAX1. Esta proteína, expresada de manera ubicua, es esencial en el control del potencial de membrana mitocondrial interna[33]. HAX1 mantiene el balance en los mecanismos de apoptosis de las células mieloides, activando la proteína anti-apoptosis X asociada a BCL2 (BAX) y por otra parte inhibiendo la degradación del inhibidor de apoptosis ligado a X (XIAP)[34,35].

La cuarta variante (SCN4), autosómica recesiva, fue descrita por Boztug y cols. en 2009 como una alteración predominantemente cuantitativa de los neutrófilos. Clínicamente se caracteriza por infecciones bacterianas reiteradas, trombopenia intermitente, recorrido venoso superficial y defectos urogenitales (criptorquidia, fistula de uraco, hernia inguinal) y/o cardiacos (defecto tipo 2 del septum auricular, cor y estenosis pulmonar). La dismorfia facial, híper-elasticidad de la piel, sordera y la disminución del tejido adiposo subcutáneo, constituyen el conjunto de manifestaciones no-hematopoyéticas de este nuevo síndrome[36]. La SCN4 está causada por mutaciones en el gen que codifica la tercera unidad catalítica de la glucosa-6-fosfatasa (G6PC3). Esta enzima, perteneciente a la familia de la glucosa-6- fosfatasa (G6PC1-3), se localiza en el retículo endoplásmico, donde forma un complejo con el transportador de glucosa-6-fosfato (G6PT)[37]. La drástica disminución de G6PC3 induce un importante estrés secundario a la activación de la vía de la proteína quinasa del retículo endoplásmico, aumentando la apoptosis principalmente en neutrófilos y fibroblástos de la piel. La reducción del aporte de glucosa al retículo endoplásmico afecta negativamente la glicosilación de proteínas componentes de la NADPH oxidasa, fundamental en la capacidad funcional de los neutrófilos[38]. El tratamiento de elección es la administración de factor estimulante de colonia granulocítica (G-CSF). El trasplante de progenitores hematopoyéticos queda exclusivamente reservado para los pacientes que no responden al G-CSF o aquellos que desarrollan síndrome mielodisplásico o leucemia mieloide aguda[39].

En 2012 fue publicado el análisis geno-fenotípico sobre la cohorte más grande de pacientes con la variante SCN4. Los resultados de este estudio no permitieron establecer una correlación genotipo/fenotipo definitiva. Sin embargo, el 100% de pacientes portadores de la mutación p.Gly260Arg en el exón 6 del gen G6PC3 presentaron defecto tipo 2 del septum auricular, lo que podría sugerir un posible rol de este gen en la embriogénesis del corazón.

Por todo ello, describimos en 2013 un paciente de 11 años con episodios reiterados de sepsis, dos de ellos en periodo neonatal y cuatro antes de los 6 años. Durante los cuadros sépticos el paciente mostraba una severa neutropenia y trombopenia intermitente. A su ingreso en nuestra unidad se detectan además un llamativo dismorfismo facial, enfermedad periodontal, edema palpebral y un prominente recorrido venoso superficial en tórax, cuello y miembros. Así mismo, fueron detectadas una hernia inguinal derecha y una criptorquidia homolateral, requiriendo corrección quirúrgica de ambos defectos. Ante los hallazgos decidimos realizar la secuenciación genómica del gen G6PC3. El resultado del estudio genético evidenció la presencia homocigótica de la mutación p.Gly260Arg. Esta variante genética solo ha sido descrita a nivel mundial en 9 niños, todos presentando defecto tipo 2 del septum interauricular. Sorprendentemente, los estudios ecocardiográficos por vía trasnesofáfica y transtorácica realizados en nuestro paciente no evidenciaron ningún tipo de anomalía cardíaca, ni estructural ni funcional. Los estudios funcionales realizados en neutrófilos de sangre periférica de nuestro paciente, posterior a la terapia con factor de crecimiento de colonia granulocítica (G-CSF), evidenciaron que estas células, además de aumentar en numero en sangre periférica, se muestran completamente competentes en términos de funcionalidad.

Recientemente ha sido descrita la variante SCN5, de herencia recesiva, clínicamente caracterizada por anemia, trombopenia, nefromegalia, hepatomegalia y poca respuesta al tratamiento con factor estimulante de colonia granulocítica (G-CSF). Este tipo de neutropenia está causada por mutaciones en el gen VPS45, cuyo déficit produce disminución en la motilidad de las células mieloides, así como un aumento en la actividad apoptótica de las mismas. [40].

Síndrome Linfoproliferativo ligado a X (XLP1)

El XLP fue descrito en 1975 por Purtilo y cols., como el primer caso de una inmunodeficiencia caracterizada por susceptibilidad extrema a infecciones por virus de Epstein-Barr (EBV por sus siglas en inglés) que asociaba una triada clínica de mononucleosis infecciosa fulminante, Hipogammaglobulinemia adquirida y linfoma de células B[41]. Desde esta primera descripción hasta hoy, el conocimiento de la patogenia celular y molecular del síndrome linfoproliferativo ligado al X se ha incrementado sustancialmente, sin embargo en el aspecto clínico sigue siendo de gran dificultad la determinación del manejo idóneo y del pronostico para los pacientes a la gran variabilidad de su forma de presentación y a la poca correlación geno-fenotípica. La incidencia estimada de XLP es de 1 por cada millón de nacidos vivos, con un pico de presentación predominante durante la primera década de la vida, así como varios fenotipos clínicos que pueden progresar de uno a otro, teniendo de ejemplo la progresión de Hipogammaglobulinemia a linfoma[42].

En la actualidad se conocen dos formas de XLP: el XLP-1 identificado en 1998 y cuyo gen responsable, localizado en el brazo largo del cromosoma X (Xq25) y designado entonces como SH2D1A, codifica la proteína asociada (*SAP*) a las moléculas de señalización de activación linfocítica (*SLAM*)[43]. La familia SLAM está constituida por nueve moléculas estructuralmente caracterizadas por un dominio amino-terminal Ig-variable-like (V) que carece de los típicos puentes de disulfuro, así como de un dominio carboxi-terminal Ig-constante (C2) en su región extracelular. Los miembros menos caracterizados de esta familia, SLAMF8 y SLAMF9, poseen colas citoplasmáticas cortas que carecen de motivos de señalización y por tanto parece poco probable que intervengan en cascadas de señalización[44,45].

La proteína asociada a SLAM (SAP) interactúa con 6 de los 9 receptores de miembros de la familia SLAM en la superficie celular, interacción facilitada por una arginina conservada en la posición 32 entre el dominio SH2 y el motivo basado en tirosina del dominio citoplasmático[46]. Una vez establecida la asociación SLAM/SAP, esta última recluta de manera selectiva la tirosina-quinasa Fyn, proteína que facilita la fosforilación de los residuos de tirosina del dominio citoplasmático de SLAM, la cual adquiere el

potencial de actuar como sitio de anclaje de diversas proteínas esenciales para el inicio de las cascadas de señalización intracelular[47]. Por este mecanismo, SAP adquiere la capacidad de potenciar: a) el desarrollo de células NKT; b) el normal desarrollo de los centros germinales para una producción robusta de anticuerpos específicos; c) aumentar la capacidad lítica de las células T y NK durante el contacto con células B infectadas por EBV; d) apoptosis celular inducida por re-estimulación[48-50].

La forma descrita recientemente en 2006, XLP-2, está causada por alteraciones en el gen XIAP/BIRC4 localizado en el cromosoma X cercano a SH2D1A. XIAP codifica una proteína de 497 aminoacidos perteneciente a la familia inhibidora de apoptosis, que a diferencia de SAP se expresa de manera ubicua, detectándose en todas las células hematopoyéticas[51]. Entre sus funciones destaca la capacidad supresora de apoptosis a través de la interacción con caspasas vía su dominio de inhibición de baculovirus (BIR) que contiene una secuencia conservada de cisteínas e histidina permitiéndole coordinar interacciones protéicas[52]. La inhibición de las caspasas por XIAP es dirigida por el segundo derivado mitocondrial activador de caspasas (Smac) y por proteínas de apoptosis pertenecientes a la vía de señalización de TGF-Beta[53]. Resulta paradójico que la deficiencia de XIAP pueda inducir fenómenos linfoproliferativos como HLH, de hecho, extisten estudios realizados en ratones deficientes de XIAP que no presentan fenotipo de linfoproliferación, sin bien es cierto que estos modelos animales no fueron infectados por virus[54].

La gran variabilidad en la expresión fenotípica es una característica de ambas variantes de XLP. En el caso del XLP-1, la mononucleosis infecciosa fulminante y la hemofagocitosis aparecen en más de la mitad de los casos descritos. Otros fenómenos como la disgammaglobulinemia, anemia aplásica, granulomatosis, vasculitis y linfomas malignos se han observado hasta en un tercio de los pacientes con deficiencia de SAP[55].

Por otra parte, la deficiencia de XIAP suele producir una clínica más típica de la Linfagiohistiocitosis hemofagocítica familiar (FHLH), esplenomegalia e hipogammaglobulinemia transitoria o secundaria al tratamiento inicial de FHLH. No han sido reportados casos de linfomas en pacientes con mutaciones en XIAP[56,57].

El protocolo terapéutico en XLP es muy similar al utilizado en pacientes con FHLH, siendo el trasplante de progenitores hematopoyético el único abordaje curativo contrastado en la actualidad. Las manifestaciones HLH constituyen el factor más importante en cuanto a la supervivencia post-trasplante, pudiendo reducir su tasa en más de un 50%, por tanto parece recomendable trasplantar a los pacientes, especialmente niños recientemente diagnosticados, que no han presentado hemofagocitosis dado el carácter catastrófico de su aparición[58].

En procesos infecciosos por EBV , principal desencadenante de la mononucleosis fulminante en pacientes deficientes de SAP y XIAP, IL-21 induce la expresión de proteínas de EBV, facilitando su reconocimiento por células efectoras[59,60]. Interleuquina 21 (IL-21) es una citoquina producida principalmente por células T CD4+ y en menor medida por células CD8+ en diferentes enfermedades humanas [61,62]. Entre sus funciones principales destacan su influencia en la supervivencia y la memoria de células T CD8+, así como la promoción de la actividad citolítica de las células NK y T efectoras durante las infecciones virales[63,64]. A pesar de su implicación en el aclaramiento de EBV, no existen evidencias de su implicación en los mecanismos de aparición y mantenimiento del estatus linfoproliferativo de los pacientes afectados por síndrome linfoproliferativo ligado a X, especialmente en el tipo 1.

En 2012 se diagnóstico en nuestra unidad un niño caucásico de 10 meses de edad, portador de la mutación p.R55X en el gen SH2D1A, variante descrita como causante de la forma XLP1. Durante su ingreso en nuestra unidad el paciente desarrolló un estatus de Linfoproliferación masiva y posteriormente se produjo un fallo multiorgánico que causó el fallecimiento del paciente a los 12 días del inicio del cuadro. Por ello, fue posible la realización de estudios *in vivo* en sangre periférica, así como el análisis post mortem de ganglios linfáticos, bazo y órganos afectados como cerebro e hígado [65].

SCID ligado al cromosoma X (XSCID)

La causa más común de inmunodeficiencia severa combinada que afecta a 1 de cada 200,000 nacidos vivos. Está causada por mutaciones en IL2RG, gen que codifica la cadena gamma común ó CD132, subunidad esencial para el desarrollo y homeostasia de las células T[66]. CD132 es una glicoproteína transmembrana tipo I que sirve como subunidad para los receptores de IL-2, IL-4, IL-7, IL-9, IL-15 e IL-21. Las alteraciones en la función de estas citoquinas originan la disminución o ausencia de células T y NK, sin alterar en numero el compartimento celular B[67]. La producción de anticuerpos está severamente reducida en XSCID, secundario a la falta de co-estimulación por las células T, función que es restaurada en cultivos de células B con linfocitos T normales[68].

La clínica predominante en estos pacientes se caracteriza por infecciones severas en los primeros medes de vida, diarrea, perdida de peso, fallo de medro y eccema generalizado, no obstante mutaciones puntuales en el gen IL2RG pueden presentar un amplio espectro de manifestaciones clínicas que van desde la ausencia de alteraciones en el compartimento NK hasta una capacidad proliferativa de las células T conservadas[69,70]. Un fenómeno muy característico, con gran influencia en las manifestaciones clínicas, es la infiltración de células T maternos en tejidos como la piel, observándose hasta en un 40% de los pacientes afectados por deficiencia de cadena gamma común. En un amplio porcentaje de los casos este infiltrado T, de fenotipo predominante CD8+CD45RO+, induce otras alteraciones como citopenias, hepatopatías e incluso cuadros más severos como la enfermedad de injerto contra huésped (EICH)[71,72].

Si bien es cierto que casi la totalidad de los pacientes afectados presentan un fenotipo $T^{neg}NK^{neg}B^{pos}$, algunos pacientes manifiestan formas atípicas de la enfermedad con clínica menos severa debido a la presencia de células T y/o NK residuales que conservan sus funciones[73].

En 1996, Stephan y cols. describieron un XSCID con expresión normal de la cadena gamma común en células T, que a su vez conservaban una discreta capacidad proliferativa frente a estímulos mitogénicos. Los estudios de tipaje HLA y citogenéticos realizados en el paciente permitieron descartar el origen materno de las céluas T en sangre periférica[74].

En 2002, se describió un fenotipo atenuado de la enfermedad que además presentaba un marcado retraso de crecimiento secundario al estatus de resistencia periférica a la hormona de crecimiento y a la disminución del valor sérico del factor de crecimiento Insulin-Like 1[75]. El defecto ontogénico del compartimento NK es característico del XSCID, sin embargo, Ginn y cols. describieron una nueva mutación que derivaba en un fenotipo NK^{pos} cuyas células conservaban la capacidad citolítica, sin embargo carecían de capacidad proliferativa en respuesta a la estimulación con IL-2[76].

La presencia de células T y NK en sangre periférica de pacientes con XSCID dificultan el diagnóstico puesto que introducen en el diagnóstico diferencial algunas formas recesivas de inmunodeficiencia severa combinada (ej. Deficiencia de IL-7Ra, CD45 ó CD3d/e/ζ). En este sentido, nuestro grupo describió un abordaje original que permite dilucidar de forma rápida el origen de las células NK de sangre periférica, evitando así el uso de análisis moleculares que requieren un mayor esfuerzo en términos de personal y de tiempo. El ensayo consiste en la identificación por citometría de flujo de la co-expresión de CD132 y CD360, ambos marcadores presentes exclusivamente en células NK de origen materno[77].

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b. Hipótesis y Objetivos

1.-"A Novel Phenotype Variant of Severe Congenital Neutropenia Caused by G6PC3 Deficiency".

Hipótesis: La tercera unidad catalítica de la glucosa-6-fosfatasa (G6PC3) es una enzima con importantes funciones en el proceso de apoptosis en medula ósea. En humanos se ha identificado una variante de la neutropenia severa congénita producida por mutaciones en el gen que codifica G6PC3. El reducido numero de pacientes con SCN4 no ha permitido aún establecer las relaciones genotipo-fenotipo debidas a la alteración de este gen.

Nuestra hipótesis fue que la deficiencia de esta enzima, en un paciente afectado por la mutación del gen que la codifica, puede alterar la capacidad funcional del reducido numero de neutrófilos que consiguen pasar de la medula ósea a la circulación periférica.

Objetivos:

- Evaluar la capacidad de activación de los neutrófilos de sangre periférica a través de la señalización vía receptores Toll-like.

- Evaluar la capacidad oxidativa de los fagocitos de sangre periférica posterior a la administración de G-CSF.

- Evaluación geno/fenotípica de su relación embriológica con las mutaciones.

2.-"Interleukin-21 Overexpression Dominates T cell Response to Epstein-Barr Virus in a Fatal Case of X-linked Lymphoproliferative Syndrome Tipo 1".

Hipótesis: La Interleuquina-21 desempeña al menos dos funciones fisiológicas esenciales en el sistema inmune. De una parte, destaca su papel en la generación de centros germinales, así como en el desarrollo, diferenciación y supervivencia de células B productora de anticuerpos de clase IgG. Por otra, participa de manera esencial en la respuesta de los linfocitos T citotóxicos frente a diferentes virus. El síndrome linfoproliferativo ligado a X Tipo 1 (XLP1) es un defecto molecular que afecta a la proteína acopladora SAP. La ausencia o no funcionalidad de esta proteína impide el normal desarrollo de respuestas de células citotóxicos (T y NK) a virus como el de EBV, sin afectar a la formación de anticuerpos contra dicho virus.

Por ello, nuestra hipótesis, al tener oportunidad de estudiar un paciente con Síndrome Linfoproliferativo ligado a X, fue que la IL-21 podría tener una participación directa en los mecanismos fallidos de citotoxicidad, así como en la dirección y mantenimiento de la linfoproliferación presente en la enfermedad. Si ello es así, representará una diana terapéutica de primera magnitud para el futuro tratamiento de estos pacientes.

Objetivos:

- Identificar la presencia de células T (CD4 y CD8) productoras de IL-21 en sangre periférica, ganglio linfático, tímo y en los órganos diana: cerebro e hígado.

- Evaluar la capacidad de IL-21 para el mantenimiento de la actividad linfoproliferativa de las células del paciente.

3.- "A novel IL2RG mutation presenting with atypical T(-)B(+)NK(+) phenotype: rapid elucidation of NK cell origin".

Hipótesis: La señalización celular a través de la cadena gamma común del receptor de la IL-2, que recibe este nombre por ser compartido con receptores de otras citoquinas tales como IL-2, IL-4, IL-7, IL-9, IL-15 e IL-21, es esencial en la ontogenia y función de las células T y NK. En más del 95% de los casos el fenotipo celular resultante en los pacientes es B+T-NK-. En raras ocasiones su diagnóstico se ve dificultado por la aparición en sangre periférica del paciente de fenotipos T+, NK+ ó T+ y NK+. La dilucidación del origen, propio o materno de dichas células es una urgencia en estos pacientes, pues el diagnóstico y en consecuencia la actitud terapéutica, pueden cambiar muy significativamente.

Así pues, nuestra hipótesis fue que al ser la cadena gamma común parte esencial del receptor de IL-21, debía estar ausente de las células tanto B como T y NK del paciente, mientras que si pertenecen a la madre, las células NK deben expresarlo constitutivamente, sin necesidad de activación previa.

Objetivos:

- Evaluar la expresión dual de CD132 (IL2RG) y CD360 (IL-21R) en células NK de sangre periférica del paciente.

c. Publicaciones

BRIEF REPORT
A Novel Phenotype Variant of Severe Congenital Neutropenia Caused by G6PC3 Deficiency

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José Peña, MD, PhD,[1,2] and Manuel Santamaría, MD, PhD[1,2]*

Severe congenital neutropenia type 4 (SCN4) is associated with mutations in the *G6PC3* gene. To date, all patients bearing the p.Gly260Arg variant of the *G6PC3* gene show heart defects. Here, we present a case of the p.Gly260Arg variant in a patient who did not have structural or functional heart anomalies. Treatment with granulocyte colony-stimulating factor recovered the absolute neutrophil count and neutrophil functional competence. Pediatr Blood Cancer 2013;60:E29–E31. © 2013 Wiley Periodicals, Inc.

Key words: atrial septal defect type II; G6PC3, G-CSF; SCN4; severe congenital neutropenia

INTRODUCTION

Severe congenital neutropenia (SCN) represents a group of genetic disorders characterized by an absolute neutrophil count (ANC) less than 500 cells/μl. Patients with SCN present with early-onset of recurrent, life-threatening infections and may also develop leukemia or myelodysplastic syndrome. A number of gene defects are associated with SCN [1]. In 2009, biallelic mutations in the gene encoding glucose-6-phosphatase catalytic subunit 3 (G6PC3) were shown to be associated with the occurrence of a complex syndrome known as SCN4 or G6PC3 deficiency. SCN4 is characterized by severe congenital neutropenia, intermittent thrombocytopenia, prominent superficial venous circulation, congenital heart defects and urogenital malformations [2]. Our understanding of the clinical phenotype of SCN4 has been expanded by means of an analysis of the largest reported cohort (n = 31 patients). The SCN4 phenotype is characterized by facial dysmorphism, disrupted bone remodeling, abnormalities in the integument, hydronephrosis, and growth hormone deficiency [3]. However, a definitive SCN4 phenotype/genotype has not been established thus far. Consequently, further information obtained from newly diagnosed patients will be valuable in delineating the SCN4 phenotype. Herein, we present the case of a patient with G6PC3 deficiency. This report expands our understanding of the SCN4 phenotype, because the clinical manifestations of this patient differ somewhat from all described patients carrying the same G6PC3 genotype.

CASE REPORT

An 11-year-old Caucasian male born to a non-consanguineous couple suffered two neonatal sepsis episodes and four pediatric sepsis episodes. During infection episodes, a marked ANC decrease (oscillating from 45 to 1,200 cells/μl; reference range, 1,800–8,000 cells/μl) and intermittent thrombopenia were detected. During the first admission of the patient to our hospital at 11 years of age, fever (39.5–40.5°C), oral thrush, and multiple respiratory, ear, nose, and throat infections were present. A right-sided inguinal herniation and cryptorchidism, which required surgery, were also present. Blepharitis, palpebral edema, and prominent superficial venous circulation in the chest, limbs, and neck were evident (Fig. 1A). Furthermore, bone pain, facial dysmorphism, multiple oral ulcers, and periodontal disease was observed. Abdominal ultrasound revealed splenomegaly (17 cm); however, enlargement of lymphoid tissues and neurological anomalies were not detected. Remarkably, 2D echocardiography displayed normal heart structures without evidence of septal or valve defects (Fig. 1B). Electrocardiogram (ECG) in all derivations was normal. Laboratory analysis showed a marked decrease in ANC (98 cells/μl) and platelet count (87,000 cells/μl) and a normal absolute lymphocyte count (1,750 cells/μl; reference range, 1,500–2,500 cells/μl). Circulating anti-neutrophil and anti-platelet autoantibodies were absent. Based on the clinical similarities of this patient with G6PC3 deficiency, a mutational analysis of the *G6PC3* gene was performed [4] after approval by the Reina Sofia University Hospital Institutional Review Board and written-informed consent was obtained from the patient's parents. A homozygous G-to-C nucleotide transversion at position c.778 (exon 6), which provokes a missense variant, was detected (Fig. 1C). Treatment was initiated using granulocyte colony-stimulating factor (G-CSF) at 5 μg/kg/24 hours [3] that increased ANC from 98 to 2,000/μl over an 8-week period. Next, microbicidal oxidative ability (Fig. 2A) and toll-like receptor (TLR)-mediated response of the neutrophils (Fig. 2B) were determined. Comparable responses were obtained for neutrophils from our patient and normal control neutrophils.

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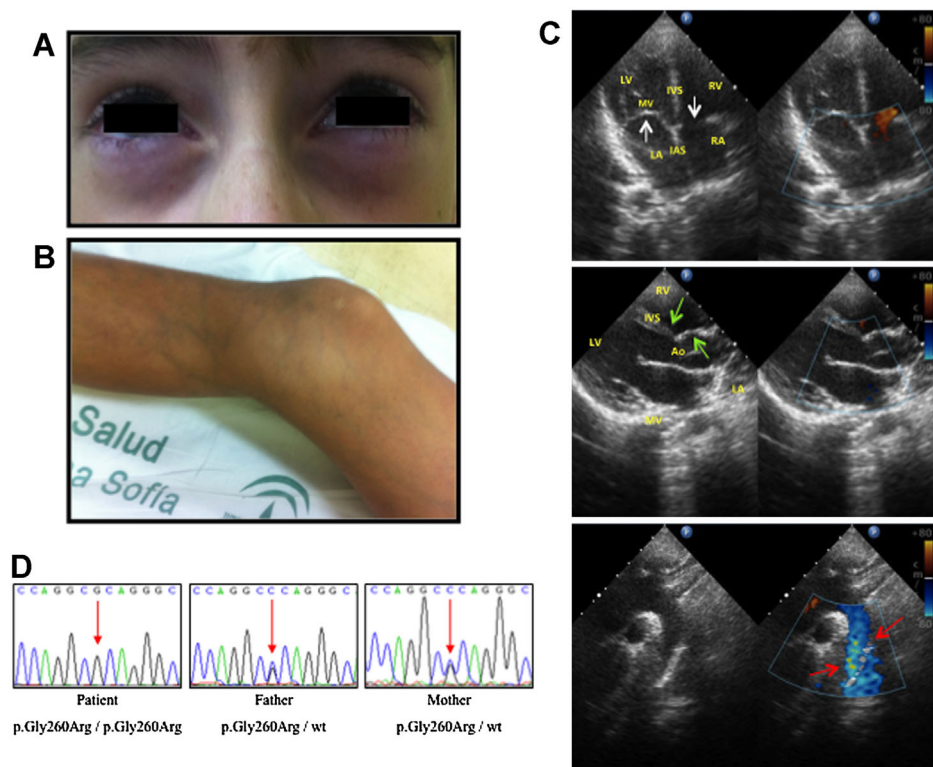
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Fig. 1. **A**: Blepharitis and palpebral edema affecting both eyes before G-CSF treatment. **B**: Detail of the prominent superficial venous circulation. **C**: 2D echocardiography depicting absence of heart defects: White arrows signals a conserved atrial septum (**upper panel**; LV, left ventricle; MV, mitral valve; IVS, interventricular septum; RV, right ventricle; LA, left atrium; IAS, interatrial septum; RA, right atrium). Green arrows points to conserved aortic (Ao) and mitral valves (**middle panel**). No left to right shunt is observed (red arrows, **lower panel**). **D**: Patient G6PC3 biallelic mutation and parents' heterozygous mutations, respectively.

DISCUSSION

The p.Gly260Arg variant has been found in homozygosis and compound heterozygosis in several unrelated patients with G6PC3 deficiency [5]. Gene analyses performed on the patient's parents showed that both progenitors carried 1 mutated G6PC3 allele, which is in accordance with the autosomal recessive inheritance pattern proposed for G6PC3 deficiency. Therefore, these findings indicate a G6PC3 deficiency diagnosis despite the absence of heart defects in our patient.

Twenty-four different disease-causing mutations of the *G6PC3* gene have been reported and different recurrent mutations have been detected. Among these, the p.Gly260Arg mutation has been detected in nine different patients, including the patient described in this report. All reported patients with this mutation have atrial septal defect (ASD) type 2 [2,3,6,7]. Remarkably, the ECG and 2D transthoracic echocardiography in our patient did not show any heart defects or malformations. These findings suggest that the p. Gly260Arg missense mutation may not be responsible for the heart defects associated with SCN4 syndrome. However, the potential hypomorphic nature of the observed mutation may have contributed to the absence of heart defects. Given the strong association of the p. Gly260Arg mutation with ASD in previous reports [3], further investigation of the association between G6PC3 mutations and heart defects is warranted.

Knowledge of this association will be valuable in determining how G6PC3 mutations induce embryological defects in cardiac

formation and what factors may account for patient variability. However, the role of G6PC3 in the embryogenesis of human heart remains to be elucidated yet. Ad hoc animal models and cumulative phenotype/genotype data from newly diagnosed SCN4 patients will be important to these investigations. From a genetic perspective, the fact that this mutation has been detected in patients of different ethnic backgrounds may be explained by several factors, including mutation hotspots in the *G6PC3* gene or a founder character of this particular mutation.

Positive neutrophil and clinical responses outcome (ranging from 1,000 to 5,500 cells/μl) were achieved with recombinant human G-CSF at 5 μg/kg/24 hours. The patient no longer required antibiotics and oral ulcers, bone pain, blepharitis, and palpebral edema disappeared. Palpebral edema and blepharitis are clinical features not previously reported in this syndrome; however, their disappearance immediately after G-CSF administration strongly suggests a relationship with neutropenia. Wright-stained peripheral smears from our patient showed the absence of immature forms of polymorphonuclear (PMN) cells. To examine neutrophil function, oxidative capacity in response to *Escherichia coli* and neutrophil response to TLR agonists (1, 2, 4, 5, 6, 7, 8, and 9) were determined. Due to the severity of the patient's neutropenia at inception, functional studies could not be performed before treatment initiation. Functional studies were performed once ANC reached 1,500 cells/μl. The functional responses to *E. coli* and TLR agonists demonstrated that circulating neutrophils from our patient were functionally competent (Fig. 2A). TLR9-mediated responses
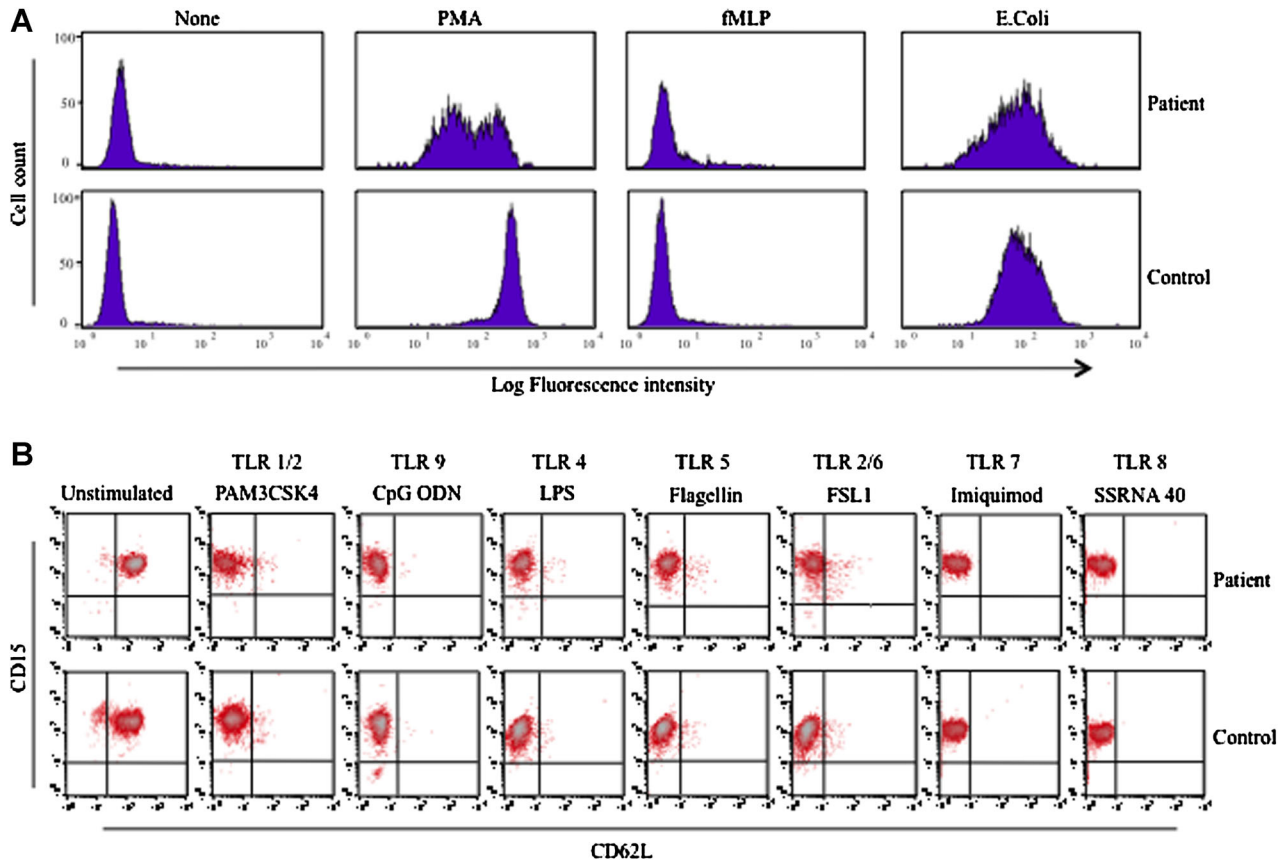
Fig. 2. Functional evaluation of the neutrophils after treatment with G-CSF. **A:** Oxidative response to *E. coli.* Right column histograms show a comparable response from either patient or control neutrophils to *E. coli* challenge. Middle columns show positive (phorbol-myristate-acetate [PMA]) and negative control (*N*-formyl-methionine-leucine-phenylalanine [fMLP]). **B:** Response to TLR agonists' stimulation. Agonist used to stimulate each TL receptor is indicated (bacterial lipopolysaccharide [LPS]). Histograms display CD62L expression in neutrophils from patient and control, which is lost upon TLR-mediated activation, showing the integrity of neutrophil's TLRs-dependent function in our SCN4 patient (a representative experiment of three performed is shown).

require the pretreatment of neutrophils with G-CSF [8]. The positive TLR9-mediated response in our patient supports this fact and further highlights the efficacy of G-CSF treatment (Fig. 2B). However, this is an interesting and controversial finding, given that G-CSF treatment corrects neutropenia and improves neutrophil function in G6PC3$^{-/-}$ mice, but not in all SCN4 patients. In a recent study [2], two SCN4 patients were found to have normal neutrophil oxidative activity. In contrast, others have found impaired neutrophil superoxide generation ability in SCN4 patients [7,9]. Whether this variability in response to G-CSF treatment is the result of a specific feature of SCN4 in humans remains to be determined.

In summary, we present a case of SCN4 not associated with heart defects; heart defects are typically found in SCN4 patients with the p.Gly260Arg *G6CP3* gene variant. ANC recovery, absence of immature PMN in the peripheral blood and clinical improvement in our patient after G-CSF treatment, indicate that granulocytes leaving the bone marrow were mature and functionally competent. Finally, the therapeutic response to G-CSF in our patient is in agreement with the current use of G-CSF as the treatment of choice for SCN4 syndrome [10].

ACKNOWLEDGMENT

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Interleukin-21 Overexpression Dominates T Cell Response to Epstein-Barr Virus in a Fatal Case of X-Linked Lymphoproliferative Syndrome Type 1

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Interleukin-21 Overexpression Dominates T Cell Response to Epstein-Barr Virus in a Fatal Case of X-Linked Lymphoproliferative Syndrome Type 1

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Interleukin-21 (IL-21) is a cytokine whose actions are closely related to B cell differentiation into plasma cells as well as to CD8^{+} cytolytic T cell effector and memory generation, influencing the T lymphocyte response to different viruses. X-linked lymphoproliferative syndrome type 1 (XLP-1) is a primary immunodeficiency syndrome that is characterized by a high susceptibility to Epstein-Barr virus. We observed in a pediatric patient with XLP-1 that IL-21 was expressed in nearly all peripheral blood CD4^{+} and CD8^{+} T cells. However, IL-21 could not be found in the lymph nodes, suggesting massive mobilization of activated cells toward the infection's target organs, where IL-21-producing cells were detected, resulting in large areas of tissue damage.

CASE REPORT

Our patient was a 10-month-old Caucasian male, born full-term to a nonconsanguineous couple, who had a medical record of bronchiolitis and ear, nose, and throat infections. The child was referred to our Pediatric Infectious Disease Unit because of fever (38.5°C to 39.5°C) lasting 11 days, associated with a non-pruritic erythematous rash, tonsillitis, cervical lymphadenopathies, and hepatomegaly (3 cm below the costal edge). Neither splenomegaly nor abdominal lymph node enlargement was detected on admission. At this point, the blood values showed a white blood cell count of 20,900/μl (lymphocytes, 72%), thrombocytopenia (72,000/μl), a raised C-reactive protein level, and a mild elevation of liver enzymes (aspartate aminotransferase [AST], 120 U/liter, alanine aminotransferase [ALT], 111 U/liter, and γ-glutamyl transferase [GGT], 324 U/liter). Specific IgM and IgG antibodies to cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were found, with the latter at a high titer (anti-EBV IgG, >1/640). Viral loads were determined by means of PCRs. PCR values for EBV found in infectious mononucleosis (IMN) patients ranged from 6,541 copies/ml to 11,476 copies/ml. The value detected in the patient was as high as 50,368 copies/ml for EBV (human herpesvirus 4 [HHV-4]) but <600 copies/ml for CMV. EBV infection was diagnosed.

The patient's general condition worsened after 72 h, and he developed splenomegaly (14 cm) and basal right pneumonia, which was treated with 50 mg cefotaxime/kg of body weight intravenously every 12 h in the absence of microbiological culture data. The fever disappeared, and the patient's condition remained stable for a week, but the fever returned (39.5°C) and was accompanied by pronounced jaundice (bilirubin, >4 mg/dl; AST, 843 U/liter; ALT, 339 U/liter; and GGT, 1,233 U/liter). High levels of ferritin (2,682 ng/ml) and plasma triglycerides (240 mg/dl), low levels of hemoglobin (7.3 g/dl), and a lymphocyte count of >50,000/μl were detected. Within the next 24 h, severe thrombocytopenia occurred (<40,000 platelets/μl) along with general tonic-clonic seizures, brain front-lobe bleeding, and generalized cerebral edema (as observed on a computed tomography [CT]

scan), which evolved in the subsequent 16 h to respiratory distress, hemodynamic shock, multiorgan failure, and exitus. The main clinical events that occurred in this X-linked lymphoproliferative syndrome type 1 (XLP-1) patient are represented in Fig. 1.

XLP-1 is a primary immunodeficiency syndrome characterized by a high susceptibility to Epstein-Barr virus (EBV) (1–3). The disease is caused by germ line mutations in the *SH2D1A* gene, which encodes the adaptor molecule signaling lymphocytic activation molecule (SLAM)-associated protein (SAP) (4, 5). This protein modulates the signal transduction of SLAM family receptors in T lymphocytes, natural killer (NK) cells, and natural killer T (NKT) cells (6, 7), influencing their cytotoxic ability and cytokine regulation (8–10). The loss of a functional SAP results in both an impaired ability of cytotoxic cells to clear the EBV infection and overexpression of proinflammatory cytokines by T and NK cells (11).

Interleukin 21 (IL-21) is a cytokine that is produced mainly by CD4^{+} cells but also by CD8^{+} lymphocytes in different human diseases (12, 13). In EBV-infected B cells, IL-21 induces the expression of EBV genes, such as the latent membrane protein 1 (*LMP1*) gene, thus providing viral peptides that are recognizable by the immune system (14–16). Indeed, IL-21 is critical for CD8^{+} T cell survival and memory generation (17–21), as well as for promoting the activity of CD8^{+} T cell effectors during viral infections (22–24), enhancing the cytotoxic response to virally infected cells by NK cells and CD8^{+} T lymphocytes (25–27).
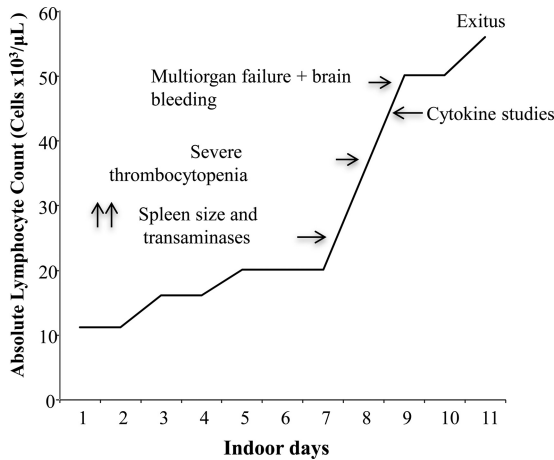
FIG 1 Clinical course of the XLP-1 patient from hospital inception.

However, despite the well-known involvement of IL-21 in EBV infection, direct evidence of its participation in the mechanisms leading to, or maintaining, the lymphoproliferative response to EBV infection in SAP-deficient (SAP[neg]) patients is lacking. We report here the results for the expression, production, and function of IL-21 in a SAP[neg] pediatric patient with a fatal EBV infection.

Human subjects. T cells from a SAP[neg] patient were analyzed during a fatal evolution of EBV infection. In this study, seven pediatric patients diagnosed with infectious mononucleosis and seven age- and sex-matched healthy donors were included to serve as controls. For this purpose, written informed consent from donors' and patients' parents as well as approval from the Institutional Review Board of The Reina Sofia University Hospital was obtained.

Gene analysis procedure. Genomic DNA was extracted from whole peripheral blood using a Maxwell 16 blood DNA purification kit on a Maxwell DNA extraction device (Promega, USA). All coding exons and intronic boundaries of the *PRF1*, *STX11*, *UNC13D*, and *SH2D1A* genes were amplified by PCR. The PCR amplicons were purified with an illustra ExoStar one-step kit (GE Healthcare, USA); bidirectional fluorescence sequencing was performed with an ABI BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA), and samples were run on an automated ABI 3730 XL DNA analyzer.

Flow cytometry. For surface-directed staining, cells were incubated with relevant fluorochrome-conjugated mouse anti-human monoclonal antibodies (MAb) on ice for 30 min in the dark and washed twice before analysis. Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy7-, antigen-presenting cell (APC)-, or Alexa-Fluor 647-conjugated anti-CD3, -CD4, or -CD8 MAb and isotype-matched control mouse IgG1 and IgG2 MAb were used as isotype controls (all from BD Biosciences, San José, CA, USA). For intracellular staining, T cells from IMN patients and controls were activated with a lymphocyte activation cocktail consisting of 25 ng/ml phorbol myristate acetate (PMA) and 1 μg/ml ionomycin (Sigma-Aldrich Spain) for 6 h at 37°C, prior to being stained, fixed, and permeabilized with the Cytofix/Cytoperm kit and a BD Golgi plug (BD Pharmingen, USA), according to the manufacturer's protocol; an inactivated control was included in the assays and treated with brefeldin A (BD Pharmingen, San Diego, CA, USA)

only. For analysis of the XLP-1 patient's T peripheral blood cells, the previous treatment was not performed, as they were found to be already activated. The following anti-human MAb were used: anti-gamma interferon (anti-IFN-γ) and anti-tumor necrosis factor alpha (TNF-α) (both from BD Biosciences, USA), anti-IL-22 (R&D Systems, Minneapolis, MN, USA), anti-IL-17 and anti-IL-21 (eBioscience, USA), and anti-IL-4, anti-IL-5, and anti-IL-10 (BD Pharmingen, USA). Mouse IgG1 and IgG2 were used as isotype controls. Flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences, San José, CA, USA), and data were analyzed using the Cell Quest Pro software (BD Biosciences, San José, CA, USA).

Immune cell isolation and cultures. Single-cell lymphocyte suspensions were isolated from peripheral blood mononuclear cells (PBMC), thymus, and lymph nodes by gradient centrifugation with Ficoll-Paque (Sigma-Aldrich, Spain). Cells were cultured in complete medium, consisting of RPMI 1640 medium containing 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin and supplemented with 10% fetal bovine serum (FBS) (all from BioWhittaker, Belgium) at 37°C in a humidified atmosphere containing 5% CO_2. For some experiments, peripheral blood lymphocytes (PBL) from IMN patients and controls were stimulated in the presence of anti-CD3/CD28-coated beads (1 bead/cell) (human T-Expander CD3/CD28; Invitrogen Dynal Biotech ASA, Norway) for 24 h. An EBV cell line was used as the stimulus in experiments addressing the composition of IL-21-producing T cells in response to prolonged exposure to EBV-infected cells. Briefly, 10^6 PBL/well were cocultured with 10^6 irradiated (3,500 rads) EBV-infected cells/well. Cells were plated at a final volume of 2 ml/well in 24-well culture plates (Nunc, Denmark) for 15 days. At day 7 of incubation, T cells were restimulated with the same number of EBV cells after careful removal of 1 ml of culture medium.

IL-21 production and proliferation assays. The production and role of IL-21 in the proliferative response observed were studied as previously described (13). Briefly, carboxyfluorescein succinimidyl ester (CFSE; 5 μM; Sigma-Aldrich, Spain)-labeled T cells (5×10^4 cells/well) were stimulated with anti-CD3/CD28-coated beads (human T-Expander; 1 bead/cell) in the absence or presence of anti-IL-21 MAb (10 μg) in complete medium. After 3 days, proliferation was determined on a FACSCalibur flow cytometer with CellQuest Pro software. IL-21 and IFN-γ levels were determined in sera and 24-h culture supernatants from all studied subjects using enzyme-linked immunosorbent assays (ELISAs) according to manufacturer instructions (Med Systems, USA).

Immunohistochemistry. Paraffin-embedded sections from brain and liver (4 μm) were deparaffinized and then hydrated with graded alcohol solutions. Endogenous peroxidase was blocked by incubation with 0.75% hydrogen peroxide for 15 min, followed by antigen unmasking with heated citrate buffer (pH 9). Sections were stained separately with anti-IL-21 at a 1/100 optimal dilution (Millipore, USA), mouse anti-human anti-CD4 MAb, or anti-CD8 MAb (Dako, United Kingdom) for 30 min. Slides were developed with the EnVision FLEX+, mouse, high-pH link kit (Dako, United Kingdom) by incubation for 30 min at room temperature with the horseradish peroxidase anti-rabbit/anti-mouse complex (Dako, United Kingdom) as described by the manufacturer (Autostainer Link 48; Dako, United Kingdom). Staining was performed by incubating slides with diaminobenzidine as the chromogenic substrate for 5 min. To counterstain, we used
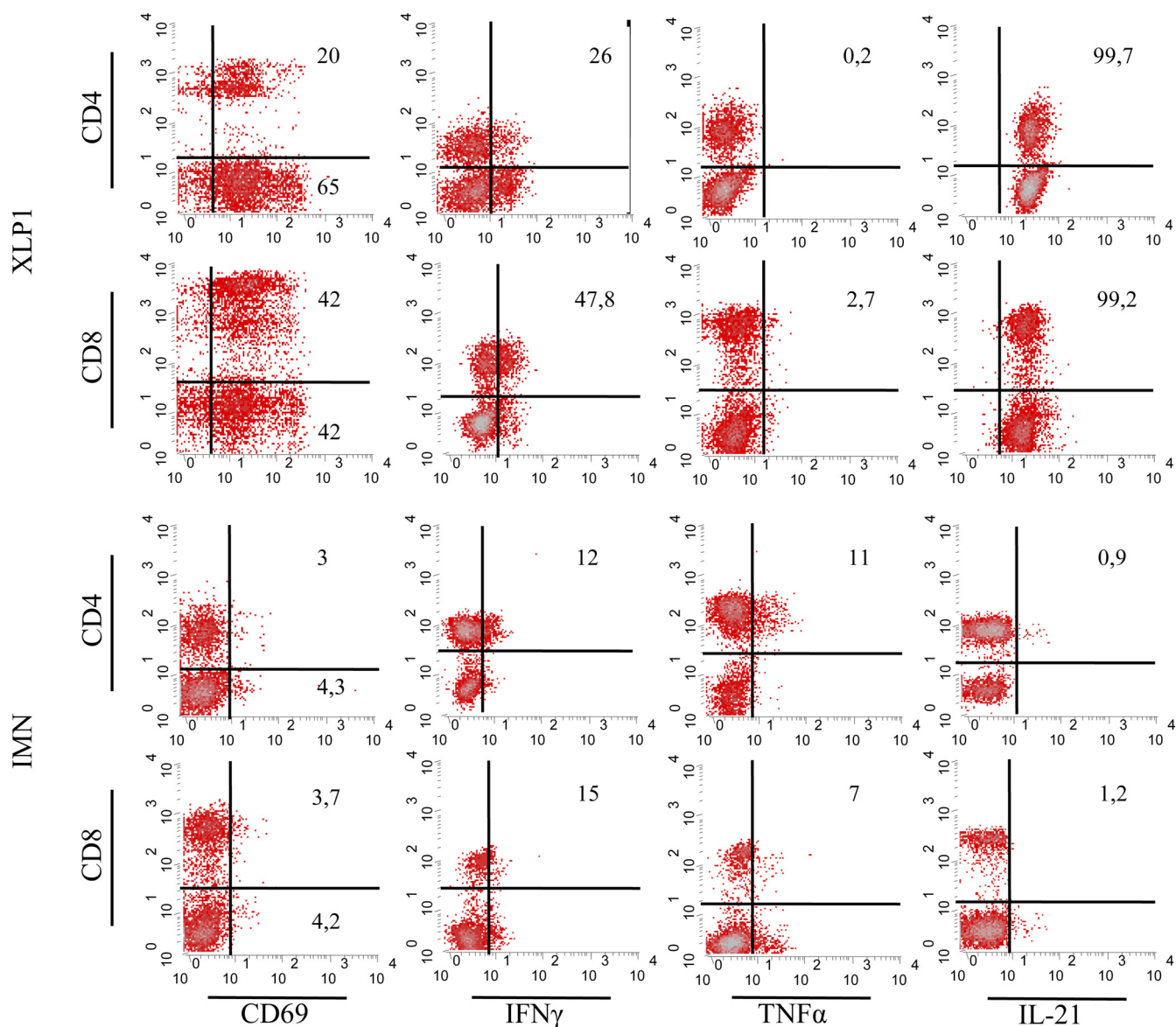
FIG 2 T cell activation was assessed by expression of CD69 (left column). Intracellular IFN-γ, TNF-α, and IL-21 expression levels by CD4⁺ and CD8⁺ T cells from an XLP-1 patient (n = 1) and IMN patients (n = 7) are depicted in the middle and right columns, respectively. The results of a representative experiment are shown.

EnVision FLEX hematoxylin (Dako, United Kingdom). As negative controls for each staining, the same procedure as mentioned previously was followed except that isotype control MAb was used.

On the basis of the clinical evolution as a fatal infectious mononucleosis, mutational analysis of the XLP-1 (*SH2D1A*) gene and of familial hemophagocytic lymphohistiocytosis (FHLH)-related genes (*PRF1*, *STX11*, and *UNC13D*) was performed. No defects were detected in any of the FHLH-causative genes. However, at exon 2 of the *SH2D1A* gene, the nonsense mutation p.R55X was detected, a genetic variant previously reported as a disease-causing mutation (28).

Ex vivo analysis of the T cell compartment of the SAP^neg patient showed that, in addition to the number of the patient's circulating lymphocytes being increased, 85% of his T cells expressed CD69,

denoting a T cell activation status that was not detected in EBV-infected SAP-positive (SAP^pos) patients (Fig. 2, left column). To further analyze this massive activation of T cells, the production profiles of the Th1 (IFN-γ, TNF-α), Th2 (IL-4, IL-5, IL-10), and Th17 (IL-17, IL-21, IL-22) cytokine families were studied. Results showed that 26% of CD4⁺ cells and 47.8% of CD8⁺ T lymphocytes from the XLP-1 patient produced IFN-γ. Notably, T cells from the XLP-1 patient did not produce TNF-α, whereas CD4⁺ and CD8⁺ cells from IMN patients produced TNF-α and, to a lesser extent, IFN-γ (15% of CD8⁺ cells) (Fig. 2, two middle columns). Th2-related cytokine-producing cells were undetectable, except that IL-10 that was found in 7% of the total T cell population of the SAP^neg patient (data not shown). Regarding the Th17 family, expression of IL-21 was detected in most of the peripheral blood T lymphocytes from the XLP-1 patient. IL-21 was expressed
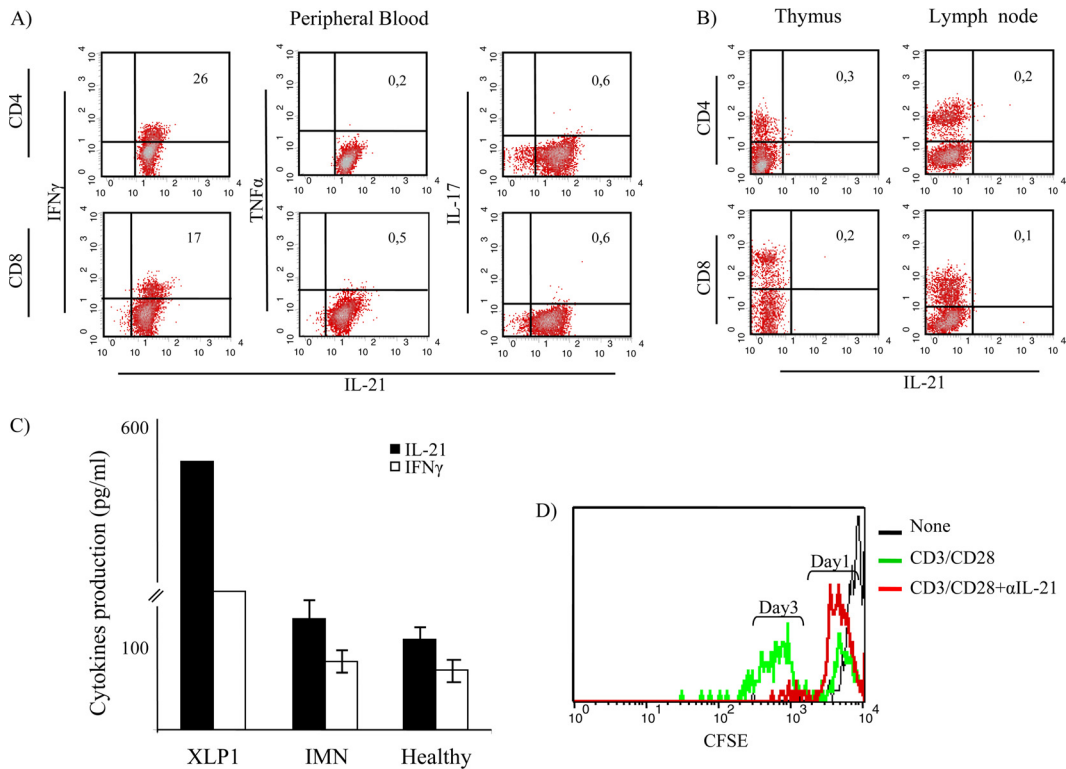
FIG 3 Analysis of cytokine expression by T cell subtypes from the XLP-1 patient. IFN-γ was coexpressed by 26% of CD4⁺ T cells and 17% of CD8⁺ lymphocytes producing IL-21. (A) Peripheral blood T cells from the XLP-1 patient did not produce IL-17A. (B) Expression of IL-21 was not detected in single positive thymocytes or in lymph node T cells obtained from the XLP-1 patient in postmortem studies. (C) IL-21 and IFN-γ production levels by T cells from the XLP-1 patient, IMN patients ($n = 7$), and healthy individuals ($n = 7$) were measured in culture supernatants following stimulation with anti-CD3/CD28-coupled beads for 24 h. Results from the XLP-1 patient are expressed as the means of results from triplicate cultures; in the case of IMN patients and healthy individuals, results represent the means ± standard deviations (SD) obtained from 7 independent experiments. Neutralizing anti-IL-21 MAb inhibited T cell proliferation in all individuals tested. (D) Results obtained from the XLP-1 patient are shown.

in only 2% of T cells from IMN patients (Fig. 2, right column). IL-17-producing T cells were absent in both the IMN patients and the SAP-deficient patient. IL-21 was found to be expressed in CD4⁺ and, remarkably, in CD8⁺ T cells. IL-21 and IFN-γ were coproduced by 26% of the CD4⁺ and 17% of the CD8⁺ T lymphocytes (Fig. 3A).

IL-21-producing cells were absent from the lymph nodes and thymus, as revealed by single-cell suspension flow cytometry postmortem analysis (Fig. 3B). The thymus did not contain IL-21-expressing cells, despite a prominent population of thymocytes producing IL-17A (data not shown). The levels of IL-21 and IFN-γ were measured to assess whether cytoplasmic cytokines found in T cells were secreted following adequate stimulation. Results confirmed that cytokines produced by T cells from both the XLP-1 patient and IMN patients were released into the culture supernatant *in vitro* (Fig. 3C). The concentrations of IFN-γ, TNF-α, and IL-21 were also determined in the patient's serum. Serum from the XLP-1 patient contained elevated levels of IL-21 compared with levels in IMN patients and healthy controls (Table 1). To elucidate whether IL-21 plays a role in the lymphoproliferation of this syndrome, we studied the ability of neutralizing anti-IL-21 MAb to inhibit T cell proliferation. Peripheral blood mononuclear cells (10^6/ml) from the SAP^neg patient, IMN patients, and healthy donors were stimulated with anti-CD3/CD28-coupled beads in the presence or absence of the relevant MAb.

Results showed that neutralization of IL-21 nearly abrogated the proliferation of T cells in all the subjects studied, including T cells from the XLP-1 patient (Fig. 3D), supporting a relevant autocrine role for the cytokine in the lymphoproliferative activity of this disease.

Immunochemistry studies demonstrated lymphocytic infiltration in the brain and liver, the attack-targeted organs in our patient. Infiltrating immune cell populations consisted of CD4⁺ and CD8⁺ T cells producing IL-21. These cells were found to occupy large areas of tissue destruction in both organs, strongly suggesting their participation in the mechanisms of organ damage of IL-21-producing cells in XLP-1 disease (Fig. 4). Finally, we studied the ability of a prolonged EBV cell line stimulation to generate IL-21-producing T cells and the compositions of the responding T cell populations in the IMN patient and controls. We found that in

TABLE 1 Serum cytokines in EBV-infected patients and healthy controls

Patient(s) (no.)	Amt (pg/ml) of:		
	IFN-γ	TNF-α	IL-21
XLP-1	163.2 ± 21.4	17.3 ± 6.5	334.4 ± 24.1
IMN (7)	69.1 ± 14.3	11.5 ± 9.6	45.8 ± 12.6
Control (7)	36.3 ± 8.8	8.6 ± 3.3	34.7 ± 14.2

HE	CD4	CD8	IL-21
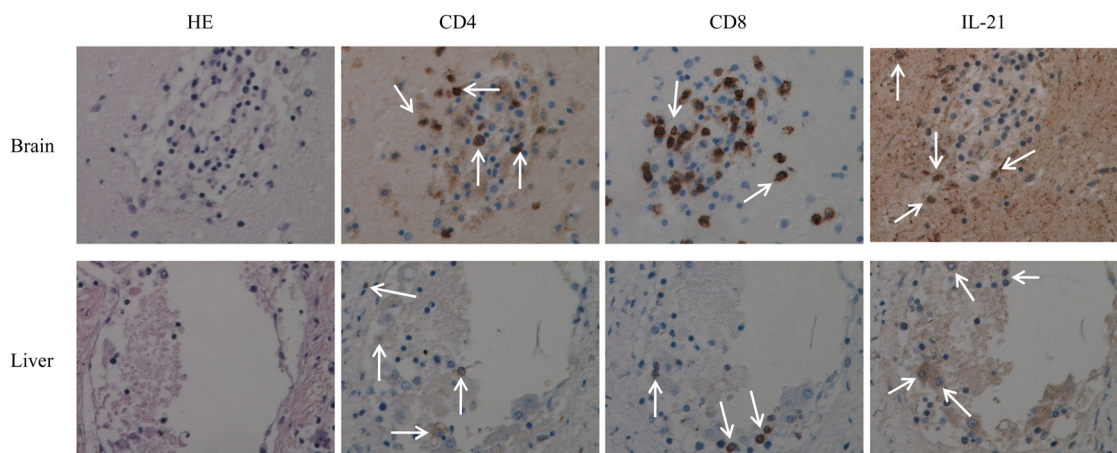
Brain

Liver

FIG 4 Tissue sections from the brain (upper panels) and liver (lower panels) were used in immunochemistry studies to assess the presence of IL-21-producing T cells. CD4$^+$ and CD8$^+$ T lymphocytes were found to infiltrate brain and liver tissues. IL-21-producing T cell subtypes are shown (arrows point to stained cells for each marker). A representative immunohistochemistry (IHC) image (magnification, ×40) is shown. HE, hematoxylin and eosin.

both groups, mainly CD4$^+$ cells produced IL-21. Interestingly, the percentage of IL-21-producing CD8$^+$ cells increased at the end of the culture period, reaching values of 17% ± 1.8% (controls) and 21% ± 2.2% (IMN) after 15 days in culture with EBV-infected cells (Fig. 5).

Discussion. The clinical onset of XLP-1 is triggered by EBV infection. CD8$^+$ T cells from patients with *SH2D1A* gene mutations show a defective lytic activity against EBV-infected cells that may have fatal consequences for the patient (29).

The main features of T lymphocytes from the XLP-1 patient in this study were an activated phenotype, a reduced proportion of cells producing IFN-γ, and the absence of TNF-α-positive cells, clearly pointing to T cell exhaustion (30, 31). Additionally, no IL-4 or other Th2 cytokines except for residual levels of IL-10 seemed to be present in the peripheral T lymphocytes of a patient with fatally evolved XLP-1. In the context of a productive anti-EBV

humoral response (anti-EBV IgG being produced), the absence of IL-4 might reflect that this cytokine is no longer required for maintaining the ongoing antibody response (32). Instead, a strong polarization to IL-21 production was evidenced. Indeed, the large areas of tissue injury observed in the patient's brain and liver were strongly infiltrated by IL-21-producing lymphocytes, supporting their participation in tissue destruction. Given the cytotoxicity defect of the NK cells and specific cytolytic T lymphocytes, our observations raise the question of the mechanism leading to massive cell death and organ failure in SAP-deficient patients. A possible explanation suggesting that the defect of CD8$^+$ T cell cytotoxicity from SAP^{neg} individuals is restricted to antigens presented by B cells but not by other cell types has recently been offered (33). However, this hypothesis neither explains the role of HLA-I restriction of the EBV viral response nor provides the reason for the inability of NK cells to kill virus-infected cells (34). Our findings of IL-21-producing T lymphocytes within the affected organs suggest instead that once the EBV has gained access to organs containing non-B professional antigen-presenting cells (i.e., Kupffer cells, microglia), the EBV-specific T cells are attracted to them and organ tissue destruction is triggered.

The fact that CD8$^+$ T cells from the XLP-1 patient also expressed and secreted IL-21 is in sharp contrast with the composition of the IL-21-producing population from SAP^{pos} IMN patients and controls, which consisted mainly of CD4$^+$ T cells. Interestingly, when EBV-infected cells were used *in vitro* as a persistent stimulus for T cells, a higher percentage of CD8$^+$ cells expressed IL-21, pointing to sustained viral exposure as being responsible for the massive IL-21 production by SAP^{neg} T lymphocytes. Notably, the population producing IL-21 consisted of either CD4$^+$ or CD8$^+$ cells, raising some points related to the fine-tuning of T cells involved in antiviral responses, among them, the mechanisms responsible for the regulation of IL-21 production in CD4$^+$ and CD8$^+$ cells and their requirements for antigen presentation and costimulation.

Previous studies have shown the importance of IL-21 in the generation, maintenance, and survival of virus-specific CD8$^+$ T cells. It is known that IL-21 augments the proliferation of resting CD8$^+$ T cells *in vitro* and promotes antigen-specific CD8$^+$ T cell
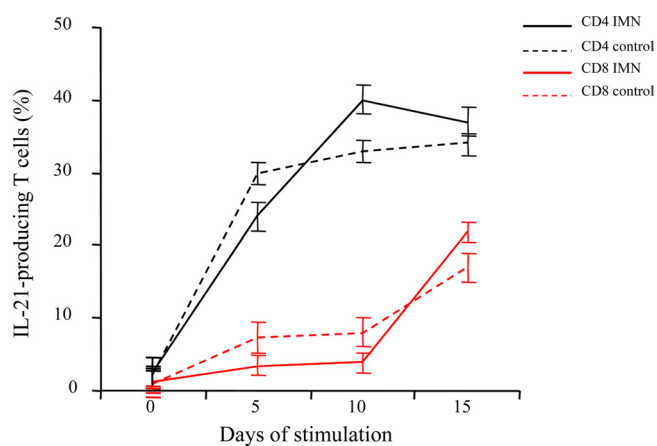
FIG 5 Peripheral blood lymphocytes from SAP^{pos} IMN patients and healthy controls were stimulated *in vitro* with an EBV-transformed cell line. Numbers of T cells expressing IL-21 were determined at day 0 of culture and every 5 days afterwards. CD4$^+$ T cells from IMN patients (solid black line) and controls (dashed black line) produced IL-21 in response to the EBV cell line. The percentages of IL-21$^+$ cells within the CD8$^+$ populations from patients (solid red line) and controls (dashed red line) were found to be increasing at day 15 of culture.

expansion and the response of CD8$^+$ T cells to some viral antigens *in vivo* (35, 36). Therefore, direct IL-21 signaling on CD8$^+$ T cells seems critical for the proliferation of antivirus-specific CD8$^+$ T cells *in vivo*. On this basis, we investigated the role of autocrine IL-21 in the XLP-1 patient's T cell-proliferative activity. Our results showed a significant inhibition of T cell proliferation when the cells were cultured in the presence of anti-IL-21-neutralizing MAb. Importantly, the observation that the IL-21 serum levels were elevated in the XLP-1 patient is of interest, as it may, in a clinical setting, help to provide a simple and rapid diagnosis of a condition that must be considered a medical emergency, especially when a fatal evolution occurs, as in the case of the patient reported here. Consequently, studies on the suitability of anti-IL-21 strategies that might open new paths to finding tools for an earlier diagnosis, as well as be a more adequate therapeutic resource for XLP-1 patients, are on course. Nevertheless, we are aware of the limitations derived from the fact that data were obtained from a single XLP-1 patient and, therefore, should be cautiously interpreted. In this context, it is of importance that the patient did not receive any therapeutic agent known to increase IL-21 production by T cells. However, whether the patient in this study was idiosyncratic and did not represent a typical patient with XLP-1 should also be kept in mind. Indeed, organ failure secondary to a persistent, noncleared EBV infection may contribute to IL-21 serum elevation and IL-21-producing T cell bias in peripheral blood. However, the uniqueness of the clinical setting from which the data were obtained reinforces the interest of these results, which may help in expanding our understanding of the immunopathogenic mechanisms underlying XLP-1.

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LETTER TO THE EDITOR
A Novel IL2RG Mutation Presenting With Atypical T−B+NK+ Phenotype: Rapid Elucidation of NK Cell Origin

To the Editor: IL-2R γ chain is the signaling component of IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, cytokine receptors that are essential in the ontogeny and function of NK and T cells. Mutations in the gene encoding for the γ chain of the interleukin-2 receptor (IL-2RG), mapped to X chromosome, accounts for 50% of diagnosed SCID. X-Linked SCID is characterized by early-onset of severe infections, facilitated by the absence of T and NK lymphocytes (T−B+NK− phenotype) [1,2]. However, different mutations in the IL-2RG gene have been reported associated with a phenotype variant T−B+NK+ [3]. Here we describe a novel mutation of the IL-2RG gene consisting of a triple insertion (ACC) at exon 5, in which NK cells were found in peripheral blood, representing a burden for initial patient diagnosis. The patient was an 8-month-old male born full term to a non-consanguineous couple. Patient's grand-grand mother was of gypsy origin. There was no family history consistent with immunodeficiency. Since 5 months of age the patient suffered several episodes of diarrhea and failure to thrive. At 8 months of age, he was admitted to the hospital due to a severe diarrhea and dehydration. *Staphylococcus auricularis*, *Staphylococcus epidermidis*, and *Candida parasilopsis* were identified in blood cultures. *Candida albicans* and *Pseudomona aeruginosa* were detected in stool's cultures. Immunological findings showed hypogammaglobulinemia IgG: 41 mg/dl (reference range 165–590 mg/dl); IgA: 5 mg/dl (15–50 mg/dl); IgM: 25 mg/dl (30–135 mg/dl). Lymphocyte counting showed CD3+: 8 cells/μl (reference range 2,000–5,900 cells/μl); CD19+: 1,664 cells/μl (610–2600 cells/μl); CD16+CD56+: 353 cells/μl (160–950 cells/μl). Despite the observed T−B+NK+ phenotype, IL-2Rγc chain deficiency was investigated, as it is the most frequent cause of SCID. DNA sequence analysis of the IL-2RG gene revealed a triple insertion (ACC) at exon 5, resulting in the inclusion of a histidine in the γc chain sequence. This pathological variant p. His242_Pro243 insHis has not been previously described. Results from the female-carrier studies demonstrated that this mutation appeared *de novo* in the patient's mother, as she was the only carrier female within the family. All other family females including five sisters, the grand mother as well as the grand-grand mother of the patient, were found free of the mutation.

In spite of the presence of NK cells in the peripheral blood of our patient, no signs of maternal lymphoid engraftment were evident. Maternal T cell engraftment has been reported in up to 50% of diagnosed SCID. However, as it is occurs in our patient, some of them never develop graft versus host disease [4]. Nevertheless, the assessment of the origin of the circulating NK cells was approached by means of a flow cytometric strategy. We reasoned that if NK cells detected were from maternal origin, they should constitutively express at the cell surface, as NK cells from healthy donors, IL-2Rγc chain (CD132) and IL-21R chain (CD360). Conversely, both markers should be absent from patient's resting B cells. Multicolor flow cytometry assay confirmed co-expression of CD132 and CD360 on the NK cell population, showing the maternal origin of NK cells.

The patient received hematopoietic stem cell transplantation from an HLA-identical, non-related donor, which outcome was the immunological reconstitution and successful clinical recovery.

In conclusion, we presented here a novel IL-2Rγc mutation, appearing *de novo* in the patient's mother and presenting NK cells in the peripheral blood. An original and informative approach allowed us to rapidly elucidate the maternal origin of NK avoiding other laborious molecular analysis and facilitating the achievement of an accurate diagnose and appropriate therapy for this patient.

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d.Conclusiones

1.-"A Novel Phenotype Variant Of Severe Congenital Neutropenia Caused by G6PC3 Deficiency". Pediatr Blood Cancer. 2013 60:29-31.

- El tratamiento con factor estimulante de colonia granulocítica recupera cuantitativamente los neutrófilos en sangre periférica. Así, tanto la activación mediante antagonistas de receptores Toll-like y la capacidad de estallido respiratorio dependiente de NADPH se mantienen conservadas.

- La mutación p.Gly260Arg del gen G6PC3 causante del síndrome SCN4 en nuestro paciente no parece relacionarse con la embriogénesis cardíaca

2.-"Interleukin-21 Overxpression Dominates T Cell Response to Epstein-Barr Virus in a Fatal Case of X-Linked Lymphoproliferative Syndrome Type 1". Clin Vaccine Immunol. 2013 20:765-71.

- Las células T CD4$^+$ así como las CD8$^+$ (hallazgo propio de este trabajo y confirmatorio de otras publicaciones de nuestro grupo. Ver anexo Otras Publicaciones) que circulan por la sangre periférica durante la fase aguda de infección en un paciente con síndrome linfoproliferativo tipo 1, producen IL-21 (100% de las células de ambas subpoblaciones).

- Las células T CD4 y CD8 presentes en las áreas dañadas de los órganos infiltrados (cerebro e hígado) producen IL-21.

- La capacidad proliferativa, *ex vivo,* de las células T de dicho paciente se inhibe de manera muy significativa en presencia de anticuerpos neutralizantes anti-IL-21.

- Se detectan niveles séricos elevados de IL-21 a partir del momento en que el numero de linfocitos totales en sangre periférica supera los 5 x 10^4/μL.

3.- A novel IL2RG mutation presenting with atypical T(-)B(+)NK+ phenotype: rapid elucidation of NK cell origin. Pediatr Blood Cancer. 2014 61:178-9.

- La expresión simultanea de CD132 (IL2RG) y CD360 (IL-21R) permite una rápida y univoca forma de establecer el origen de las poblaciones T y NK cuando aparecen en individuos con mutaciones patogénicas del gen que codifica CD132.

- Incidentalmente, la variante p.His242_Pro243insHis en el gen codificante de la cadena gamma común de IL-2 es patogénica, *de novo* en la madre del paciente y no descrita previamente.

e. Resumen

RESUMEN.

La Neutropenia Congénita Severa tipo 4 (SCN4 por sus siglas en ingles) está asociada a mutaciones en el gen que codifica la tercera sub-unidad catalítica de la glucose-6-fosfatasa (G6PC3). En la actualidad, todos los pacientes descritos portadores de la variante p.Gly260Arg en el gen G6PC3 muestran defectos cardíacos. En este trabajo presentamos el caso de una variante p.Gly260Arg en un paciente sin alteraciones funcionales ni estructurales en corazón. La terapia con factor estimulante de colonia granulocítica (G-CSF) elevó el numero absoluto de neutrófilos funcionalmente competentes. Interleuquina-21 (IL-21) es una citoquina cuyas acciones están estrechamente relacionadas con la diferenciación de las células B hacia células plasmáticas, así como en el desarrollo de células CD8$^+$ efectoras y la generación de memoria, con gran influencia en la respuesta celular T frente a virus. El síndrome linfoproliferativo ligado a X tipo 1 (XLP-1) es una inmunodeficiencia primaria caracterizada por una elevada susceptibilidad al virus de Epstein-Barr. En un paciente pediátrico con XLP-1 observamos que IL-21 estaba expresada en casi la totalidad de células T CD4$^+$ y CD8$^+$ de sangre periférica. Sin embargo, no se detectó expresión de IL-21 en ganglios linfáticos, lo que sugiere una movilización celular masiva de esta citoquina hacia los órganos diana afectados, donde fueron identificadas células productoras de IL-21, especialmente en las áreas de daño tisular. La cadena gamma del receptor de IL-2 es el componente de señalización no solo de IL-2 sino también de IL-4, IL-7, IL-9, IL-15 y de IL-21, citoquinas esenciales en la ontogenia y función de las células T y NK. Su mutación es la causa más frecuente de inmunodeficiencia severa combinada ligada al sexo. El fenotipo linfoide en estos pacientes es B+ T- NK- habitualmente. Aquí describimos un paciente con una mutación patogénica y que presentó inicialmente un fenotipo B+ T+ NK+, cuestionando de manera evidente el diagnostico molecular y en consecuencia el tratamiento a recibir. Aquí describimos un estudio fenotípico en el que combinamos la expresión de CD132 y CD360 que nos ha permitido dilucidar en un solo ensayo y mediante citometría de flujo multiparametrica el origen materno de las poblaciones T y NK del paciente.

Summary

Severe congenital neutropenia type 4 (SCN4) is associated with mutations in the gene encoding glucose-6-phosphatase catalytic subunit 3 (G6PC3). To date, all patients bearing the p.Gly260Arg variant of the G6PC3 gene show heart defects. Here, we present a case of the p.Gly260Arg variant in a patient who did not have structural or functional heart anomalies. Treatment with granulocyte colony-stimulating factor recovered the absolute neutrophil count and neutrophil functional competence. Interleukin-21 (IL-21) is a cytokine whose actions are closely related to B cell differentiation into plasma cells as well as to CD8^{+} cytolytic T cell effector and memory generation, influencing the T lymphocyte response to different virus. X-linked lymphoproliferative syndrome type 1 (XLP-1) is a primary immunodeficiency syndrome that is characterized by a high susceptibility to Epstein-Barr virus. We observed in a paediatric patient with XLP-1 that IL-21 was expressed in nearly all peripheral blood CD4^{+} and CD8^{+} T cells. However, IL-21 could not be found in the lymph nodes, suggesting massive mobilization of activated cells toward the infection`s target organs, where IL-21 producing cells were detected, resulting in large areas of tissue damage. IL-2R common gamma chain is the signalling component of IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, cytokine receptors that are essential in the ontogeny and function of NK and T cells. We presented here a novel IL-2RG mutation, appearing the novo in the patient's mother and presenting NK cells in the peripheral blood. We have demonstrated that the use of anti-CD132 (IL-2RG) combined with anti-CD360 (IL-21R) allowed us to rapidly elucidate the maternal origin of NK cells, avoiding other laborious molecular analysis and facilitating the achievement of an accurate diagnose and appropriate therapy for this disease.

f. Otras aportaciones científicas

COLON/SMALL BOWEL

Characterization of Gliadin-Specific Th17 Cells from the Mucosa of Celiac Disease Patients

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OBJECTIVES: Celiac disease (CD) is a disorder characterized by a deregulated immune response to ingested wheat gluten and related cereal proteins in susceptible individuals. It has been considered that the onset of CD is mediated by a skewed Th1 response. However, the participation of Th17 cells in the pathogenesis of the disease, a key cell population in other autoimmune disorders, has not been studied in detail. We have investigated the presence of Th17 cells in the mucosa of active CD patients and their functional implications in the pathogenesis of the disease.

METHODS: T cells obtained from duodenum biopsies from 15 untreated patients and 11 control individuals were characterized by flow cytometry, immunoassays, and real-time PCR.

RESULTS: We found gliadin-specific CD4^{+} interleukin (IL)-17A-producing T cells in the mucosa of CD patients with a phenotype consisting of TCR (T-cell receptor)$\alpha\beta^+$ CD45RO^{+} CD161^{+} CCR6^{+} (C–C chemokine receptor type 6) and IL-23R^{+}. Functional analysis showed that Th17 cells from CD patients are different from those of control individuals in terms of cytokines production. Th17 cells from CD patients, but not from controls, simultaneously express transforming growth factor-β (TGFβ). Th17 CD cells also produce interferon-γ (IFNγ), IL-21, and IL-22. The analysis of the transcription factors revealed a high expression of interferon regulatory factor-4 as a feature of gliadin-specific cells from CD patients with respect to controls.

CONCLUSIONS: Gliadin-specific Th17 cells are present in the mucosa of CD patients having a dual role in the pathogenesis of the disease as they produce proinflammatory cytokines (such as IL-17, IFNγ, IL-21), mucosa-protective IL-22, and regulatory TGFβ, which actively modulates IL-17A production by T cells in the celiac mucosa.

INTRODUCTION

Celiac disease (CD) is a disorder characterized by a deregulated immune response to ingested wheat gluten and related cereal proteins in susceptible individuals (1,2). It has been considered that the onset of CD is mediated by a skewed Th1 response (3) because of the cytokine secretion profile detected in the intestinal mucosa. This is further supported by the transcription factor pattern found in peripheral T lymphocytes from

patients with CD (4) and the effector mechanisms leading to microvillus atrophy of the mucosa (5).

However, a more complex picture of the ongoing T cell-produced cytokine interplay in the CD mucosa is emerging. This is supported by the coexpression of proinflammatory and down-regulatory cytokines by intestinal T cells from CD patients and the presence in the CD mucosa of antigen-specific T-regulatory (Treg) cells (6–8). Indeed, recent evidence suggests that some cell

populations other than Th1 lymphocytes have a significant influence on the pathogenesis of autoimmune responses, as is the case of Th17 cell sub-population (9). The participation of Th17 cells in experimental and human autoimmune disorders has widened the actors to be considered in the immunopathogenesis of CD (9,10). It is known that Th17 cells belong to a separate lineage of T lymphocytes displaying differential features from Th1 or Th2 subtypes of T lymphocytes. These differences include the transcription factors required for interleukin (IL)-17A production and the profile of cytokines produced by these cells, the expression of IL-23 receptor and C–C chemokine receptor type 6 (CCR6), a molecule that has also recently been found to be present on the surface of Treg cells (11). The production of IL-17A in humans is mainly dependent on the expression of the orphan nuclear receptor transcription factor, RORC (12). The cytokine profile of Th17 cells comprises various factors such as IL-21, which is also produced by Th2 cells (13), and IL-22, a cytokine involved in early defense mechanisms against some bacterial pathogens in epithelial tissues (14,15).

The differentiation of naive CD4+ into Th17 cells is controlled by proinflammatory cytokines, such as IL-1β and IL-6, as well as transforming growth factor-β (TGFβ) (16). TGFβ is a pleiotropic cytokine abundantly present in the intestinal mucosa (17) where it is secreted by CD4+ T cells (6) and by some intraepithelial CD8+ lymphocytes (18). The essential role of TGFβ in the regulation of the intestinal immune response and in the maintenance of regional homeostasis was demonstrated by facts that TGFβ-deficient mice have severe autoimmunity (19) and that blockade of TGFβ abrogates the protective activity of Treg cells against colitis observed in a cellular transfer model (20).

Despite the fact that IL-17A-producing T cells (CD4+ and CD8+) have a prominent role in the pathogenesis of autoimmune diseases (21,22), their presence in the small intestine mucosa of CD patients remains largely unexplored. Recent reports have identified cytokine transcripts in duodenal biopsies from CD that follow a pattern consistent with the presence of Th1 and Th17 cells (23,24). However, this indirect evidence has not clarified the relative participation of those cell populations in the pathogenesis of the disease. Therefore, we decided to investigate the presence and functional features of Th17 cells obtained from intestinal explants of untreated celiac patients.

Our results demonstrate the presence of gliadin-specific IL-17A-producing T cells in the duodenum of CD patients. These cells express CCR6, CD161, and IL-23R, show upregulated expression of RORC and interferon regulatory factor-4 (IRF4) transcription factors, and secrete IL-21 and very high levels of IL-22. Remarkably, Th17 cells from CD patients produce TGFβ, which regulates IL-17A production through an RORC- and IRF4-dependent mechanism.

METHODS

Patients and controls

Distal duodenum biopsies were obtained during upper gastrointestinal endoscopy from 15 CD patients undergoing diagnostic protocols (age range 4–13 years); 7 patients were females and 8 males. We obtained control samples from 11 non-CD individuals under-

going biopsies for screening procedures for abdominal symptoms (age range 4–15 years); 4 non-CD individuals were females and 7 males. The CD diagnosis was established based on clinical symptoms, the presence of anti-transglutaminase IgA antibodies, and the histological evidence of total or partial villous atrophy. Human leukocyte antigen (HLA)-class I (PCR-SSP, Invitrogen, Brown Deer, WI) and HLA-class II (PCR-SSO, Innogenetics, Gent, Belgium) high-resolution typing was studied in all patients and in non-CD individuals and their results are shown in **Table 1**. All samples were obtained after informed consent in accordance with the regulations and previous approval of the Institutional Review Board of the Reina Sofia University Hospital. All patients participating in our study were untreated and had never received any specific treatment.

Immunoselection and culture of cells

Biopsies typically consisted of three to four mucosal explants obtained from each patient and non-CD individuals. Tissue explants were independently digested with 1 mg/ml collagenase A for 1 h at 37 °C. Cells obtained from the explants of each individual were pooled and either used for intracellular cytokine staining assays or stimulated with 100 μg/ml transglutaminase-treated peptic–tryptic–gliadin, hereafter gliadin (Sigma, St Louis, MO), in the presence of 1×10^6 irradiated (3,500 rads) autologous peripheral blood mononuclear cells for an additional 7 days. Cells were cultured in RPMI 1640 medium containing 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 100 Units/ml penicillin, and 100 mg/ml streptomycin (all from Life Technologies, Chagrin Falls, OH) supplemented with 5% human serum (complete medium) in the absence of exogenous cytokines. Expanded cells were depleted of the CD8+ population by positive CD8 immunoselection with appropriate immunobeads (Miltenyi, Bergisch Gladbach, Germany), and the negative fraction, consisting of >93% CD4+ cells, was further separated using CCR6 immunobeads (Miltenyi). A total of 1.5×10^4 cells from both positive and negative fractions (CD4+CCR6+ or CD4+CCR6−) were used to determine their intracellular IL-17A expression (CD4+CCR6+ >93% IL-17A; CD4+CCR6− <2% IL-17A+). The remaining cells from each fraction were further expanded in the presence of gliadin (CD patient cells) or anti-CD3/CD28-coated beads (cells from non-CD individuals) (human T-Expander CD3/CD28; Invitrogen Dynal Biotech ASA, Oslo, Norway) at 3 beads/cell in the presence of 20 ng/ml of IL-23 (eBioscience, San Diego, CA). Exogenous IL-23 was removed from cultures 72 h before experiments.

Flow cytometry

For direct immunofluorescence staining, cells were incubated with fluorochrome-conjugated antibodies on ice for 30 min in the dark and washed twice before analysis. fluorescein isothiocyanate-, Alexa-Fluor 647-, phycoerythrin-, antigen presenting cell (APC)-, or phycoerythrin-Cy7-conjugated monoclonal antibodies (mAbs) against CD3, CD4, CD8, CD25, CD45RA, CD45RO, anti-interferon-γ (anti-IFNγ), anti-TGFβ, IL-4, IL-10, and isotype-matched irrelevant control mAbs were purchased from BD Biosciences (San Jose, CA). Antibodies against TCR (T-cell

COLON/SMALL BOWEL

Table 1. High-resolution HLA genotype of CD patients and non-CD control individuals participating in the study

| Individual	HLA-A*	HLA-C*	HLA-B*	HLA-DRB1*	HLA-DQB1*
CD-1	0201, 2501	0303, 0701	0801, 1501	0301, 1102	0201, 0301
CD-2	0301, 3002	0501, 0702	0702, 1801	0301, 1501	0201, 0602
CD-3	2301, 2601	0401, 1203	3801, 4403	0301, 1301	0201, 0603
CD-4	0201, 2402	0304, 1502	1501, 5101	0301, 0401	0201, 0302
CD-5	0201, 1101	0401, 0701	1801, 3501	0401, 1104	0301, 0302
CD-6	3002, 2902	0501, 1601	1801, 4403	0301, 0404	0201, 0301
CD-7	2301, 2902	0701, 1601	4403, 4901	0701, 1101	0202, 0301
CD-8	0301, 3101	0401, 0501	3501, 4402	0101, 0301	0201, 0501
CD-9	2402, 3002	0202, 0702	0702, 1503	0301, 1301	0201, 0603
CD-10	2301, 3301	0401, 0602	4403, 4501	0301, 0701	0201, 0202
CD-11	0101, 0201	0602, 0701	0801, 5701	0301, 0701	0201, 0303
CD-12	3001, 3201	0401, 0602	1302, 3503	0301, 0701	0201, 0202
CD-13	0101, 0201	0701, 0202	0801, 2705	0301, 1001	0201, 0501
CD-14	2902, 6802	1601, 0602	4403, 5001	0701, 1104	0202, 0301
CD-15	0201, —	0501, 0701	0801, 1801	0301, —	0201, —
Non-CD-1	0201, 6802	0701, 1203	3801, 4901	0405, 1301	0302, 0603
Non-CD-2	0201, 2902	0602, 1601	4403, 5701	1302, 1501	0602, 0604
Non-CD-3	0301, 2402	0702, —	0702, —	0402, 1104	0301, 0302
Non-CD-4	0201, 0301	0202, 0702	0702, 5101	0701, 1501	0202, 0602
Non-CD-5	0102, 3201	0802, 1505	0702, 1402	0102, —	0501, —
Non-CD-6	0201, 3002	0701, 1203	3801, 4101	0301, 1301	0201, 0603
Non-CD-7	2402, —	0701, 1601	1801, 4403	1102, 1501	0301, 0602
Non-CD-8	0301, 1101	0302, 0701	4901, 5801	0405, 1401	0302, 0503
Non-CD-9	0301, 2902	0102, 1601	4403, 5101	0701, 1101	0202, 0301
Non-CD-10	0201, 3002	0701, 1402	0801, 5101	0301, 0701	0201, 0202
Non-CD-11	2902, 3301	0802, 1601	1402, 4403	0102, 0701	0202, 0501

CD, celiac disease; HLA, human leukocyte antigen.

receptor)αβ and TCRγδ were obtained from BD Pharmingen (San Diego, CA). Antibodies to CCR6 and IL-23 receptors were purchased from R&D Systems (Minneapolis, MN), and the anti-Foxp3 staining set was purchased from eBioscience. The following anti-human antisera and mAbs were also used: anti-RORC, anti-IRF4 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-IL-22 (R&D Systems), and anti-IL-17A and anti-IL-21 (eBioscience). Mouse IgG1, IgG2, and rat IgG1 (BD Biosciences) were used as isotype-matched controls. For intracellular staining, cells were activated with 40 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin (Sigma) for 6 h at 37 °C in the presence of brefeldin A before being stained, fixed, and permeabilized using the BD Golgi plus cytofix/cytoperm kit according to the manufacturer's protocol (BD Pharmingen); non-activated controls, treated only with brefeldin A, were included in the assays. Flow cytometry was performed on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed using the FACS Diva software.

Cytokine analysis

T cells were stimulated with gliadin (50 μg/ml) or anti-CD3/CD28-coated microbeads (3 beads/cell) (Invitrogen Dynal Biotech ASA). After culture for 24 h at 37 °C, supernatants were collected and stored at −80 °C until used. Secreted IL-17A and IFNγ were determined using a human Cytokine/Chemokine Multiplex Immunoassay (Linco Research, St Charles, MO), and IL-21 and IL-22 using ELISA (enzyme-linked immunosorbent assay) (Bender Medsystems, Vienna, Austria) following the manufacturer's instructions. TGFβ levels in culture supernatants were detected using a Quantikine Human TGFβ ELISA (R&D Systems) according to the manufacturer's protocol.

Gliadin specificity assays

Gliadin specificity of IL-17A-producing T cells (5×10⁴ cells/well) from CD patients was assayed by challenging cells with gliadin (25, 50, or 100 μg/ml) for 5 days. Native transglutaminase (Sigma)
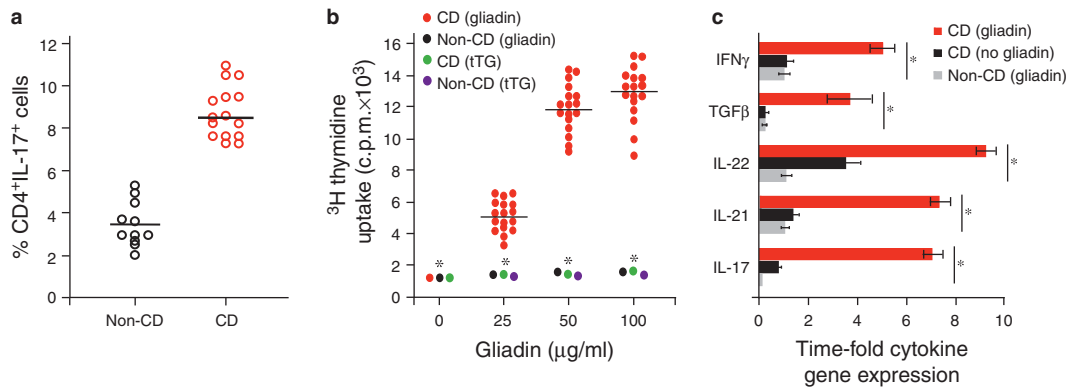
Figure 1. Gliadin-specific CD4$^+$IL-17A$^+$ lymphocytes are present in the duodenal mucosa of CD patients. (**a**) Individual percentages of CD4$^+$IL-17A$^+$ cells obtained upon digestion of duodenal biopsies from CD patients (red empty circles; $n=15$) and non-CD control subjects (black empty circles; $n=11$). Mean±s.d. values were 8.7±2.4% for CD patients and 3.6±1.6% for non-CD control individuals ($P<0.05$). (**b**) Biopsies-derived fresh T cells from CD patients (red solid circles; $n=12$) and non-CD control individuals (black solid circles; $n=7$) were tested for gliadin specificity by challenging the cells for 5 days with indicated amounts of gliadin and pulsed with ^{3}H-thymidine to determine cell proliferation. Circles represent values obtained from each individual. T cells from CD patients (green circles) and controls (purple circles) were also tested in the presence of transglutaminase alone (tTG). Circles represent the mean proliferation value obtained in the experiments. (**c**) Cytokine signature of mucosal T cells from CD patients measured by qPCR in the presence (red bars) or absence (black bars) of gliadin (50 μg/ml) and from non-CD control individuals in the presence of gliadin (gray bars) in the same conditions as indicated in panel **b**. Results were normalized against values of the *HPRT1* gene and expressed as time-fold induction (mean±s.d.; $n=10$); *$P<0.01$. CD, celiac disease; IL, interleukin; qPCR, quantitative PCR.

at the concentrations indicated above was used in the experiments as control. Cells were cultured in complete media, pulsed for the final 16 h of culture with 0.5 μCi/well of ^{3}H-thymidine (ICN Pharmaceuticals, Costa Mesa, CA), harvested, and ^{3}H-thymidine uptake measured by liquid scintillation counting (Betaplate 1205, Perkin-Elmer, Wellesley, MA).

Real-time PCR assays

Cytoplasmic RNA from T cells was extracted using the RNeasy Kit (Qiagen GmbH, Hilden, Germany) and reverse transcribed using the AMV-reverse transcription system (Promega, Madison, WI). For quantitative PCR experiments, equal amounts of cDNA templates were amplified. The specific primers pairs used were as follows: RORC: 5′-AGTCGGAAGGCAAGATC AGA-3′ (forward) and 5′-CAAGAGAGGGTTCTGGGCAAG-3′ (reverse); T-bet: 5′-CCGTGACTGCCTACCAGAAT-3′ (forward) and 5′-ATCTCCCCCAAGGAATTGAC-3′ (reverse); IRF4: 5′-GT CCTGAGCGAAAACAGGAG-3′ (forward) and 5′-ACCCAA GACTCCCACAGTTG-3′ (reverse); IFNγ: 5′-TTCAGCTCT GCATCGTTTTG-3′ (forward) and 5′-TCTTTTGGATGCTC TGGTCA-3′ (reverse); TGFβ: 5′-AAGTGGACATCAACGGGT TC-3′ (forward) and 5′-GTCCTTGCGGAAGTCAATGT-3′ (reverse); IL-17A: 5′-CCCCAGTTGATTGGAAGAAA-3′ (forward) and 5′-GAGGACCTTTTGGGATTGGT-3′ (reverse); IL-21: 5′-GGCAACATGGAGAGGATTGT-3′ (forward) and 5′-AAGC AGGAAAAAGCTGACCA-3′ (reverse); IL-22: 5′-CTCCTTC TCTTGGCCCTCTT-3′ (forward) and 5′-GTTCAGCACCTGC TTCATCA-3′ (reverse); HPRT1: 5′-ACCCCACGAAGTGTTGG ATA-3′ (forward) and 5′-AAGCAGATGGCCACAGAACT-3′ (reverse). The reactions were performed in a LightCycler 480 system (Roche, Indianapolis, IN). An initial denaturation step at 95 °C for 15 min was carried out followed by 35 cycles of 94 °C

for 20 s, 60 °C for 30 s, and 72 °C for 30 s. The expression of target genes was calculated using the comparative method for total quantity upon normalization to HPRT gene expression.

Statistics

Comparisons between groups were performed by means of the Wilcoxon test or the Mann–Whitney test, as appropriate. Differences were considered as statistically significant for $P<0.05$.

RESULTS

Isolation and identification of duodenal Th17 lymphocytes from CD patients

To determine whether Th17 cells are present in duodenum explants of CD patients, freshly obtained T cells were double stained for CD4 and IL-17A and their expression was analyzed by flow cytometry. Our results show that CD patients have an increased population of IL-17A-containing CD4$^+$ T cells (8.7±2.4%) than do controls (3.6±1.6%) (**Figure 1a**). It is well known that pathogenic T cells from the mucosa of CD patients recognize gliadin in the context of HLA-DQ2 or DQ8 (25). Therefore, we stimulated cells with transglutaminase-treated peptic–tryptic–gliadin by overnight loading of the peptide onto irradiated peripheral blood mononuclear cells pooled from four different HLA-DQ2$^+$ healthy donors. For this purpose, peripheral blood mononuclear cells from donors were pooled and gliadin added at the indicated concentrations overnight before co-culture for 5 days. We found that T cells from CD patients vigorously proliferated in response to gliadin in a dose-response manner (**Figure 1b**, red circles), whereas proliferation of cells obtained from non-CD individuals was confined to background levels (**Figure 1b**, black circles). By contrast, presentation of gliadin by HLA-DQ2$^-$ cells did not elicit
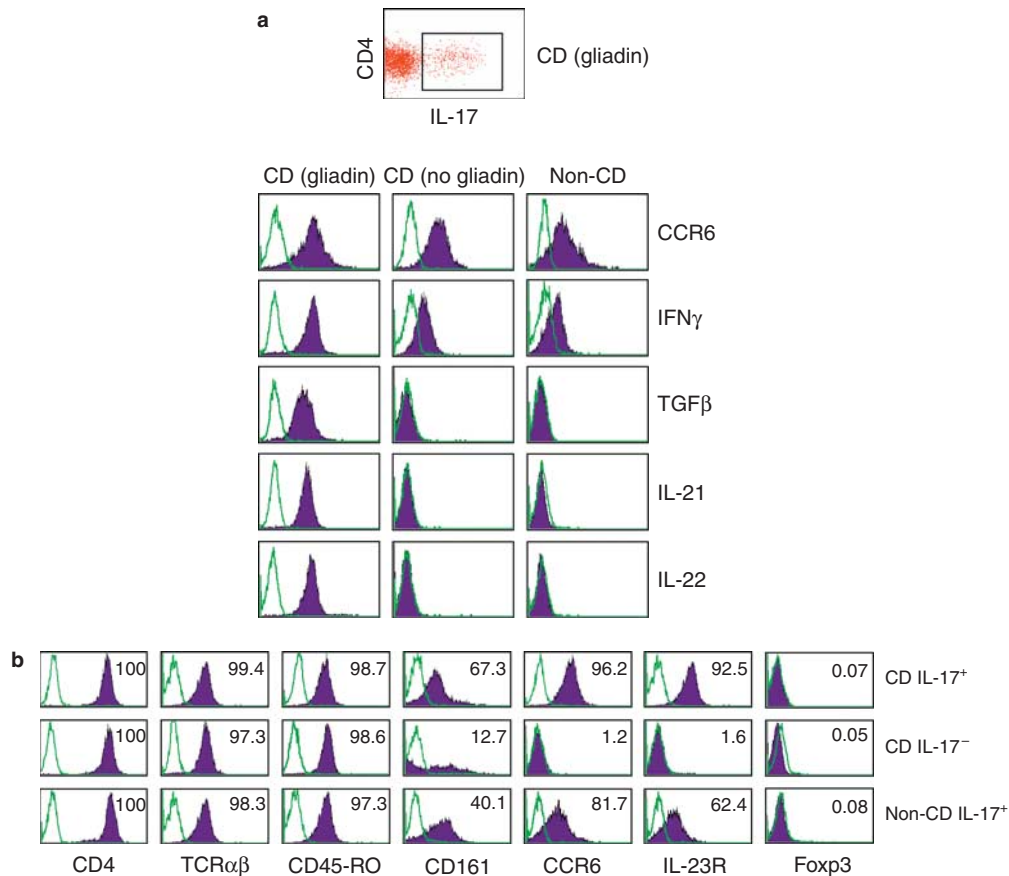
Figure 2. Phenotype of Th17 CD lymphocytes. (**a**) Representative flow-cytometric analysis on gated CD4+IL-17A+ duodenal cells from CD patients and control individuals of CCR6, IFNγ, IL-21, IL-22, and TGFβ expression, in the presence or absence of gliadin (50 μg/ml). (**b**) Representative phenotype of IL-17A+ cells from CD patients (upper panel), IL-17A− cells from CD patients (middle panel), and IL-17A+ cells from control individuals (lower panel). CD, celiac disease; CCR6, C–C chemokine receptor type 6; IFNγ, interferon-γ; IL, interleukin; TGFβ, transforming growth factor-β.

any proliferation of cells from either CD patients or normal individuals. *In vitro* stimulation with tissue transglutaminase did not induce proliferation of T cells derived from either non-CD or CD duodenal biopsies when presented by HLA-DQ2+ cells (**Figure 1b**, green and purple circles), thereby supporting the specificity of the response to gliadin by T cells from CD patients.

The cytokine signature of the responding T-cell population was also analyzed by quantitative PCR. We found a signature profile in CD patients' T cells mainly consisting of IFNγ, IL-17, IL-21, IL-22, and TGFβ, whereas the major cytokines detected in non-CD individuals were IFNγ, IL-21, and IL-22 (**Figure 1c**). These results were confirmed by analysis of the protein expression of these cytokines in fresh populations upon gliadin stimulation by means of intracellular staining and flow cytometry. Our results showed that, when gating on the IL-17A-producing cell sub-population (**Figure 2a**), all the aforementioned cytokines were found in gliadin-stimulated T cells, which also expressed CCR6 (**Figure 2b**). Non-CD T cells showed a different pattern of cytokine expression upon stimulation with anti-CD3/CD28-coupled beads.

The cell isolates were stimulated *in vitro* for 7 days with gliadin in the presence of HLA-DQ2+ feeder cells, and the

expanded cells were sorted to obtain a CD4+ population based on negative immunoselection, followed by a fractioning based on the CCR6 expression. The resulting population (> 93% CD4+CCR6+) was further cultured for 7 days with either gliadin (CD) or anti-CD3/CD28-coupled beads (non-CD individuals) and their phenotypical and functional characteristics were analyzed. Th17+ expanded cells from CD patients showed the widespread surface expression of TCRαβ, CD45RO, CD161, CCR6, and IL-23R (**Figure 2b**, upper panels). By contrast, the comparison between IL-17A+ and IL-17A− populations evidenced that CCR6 and IL-23R expressions were only found in IL-17A-producing cells, whereas CD161 was found in both IL-17A+ (52–76% of cells), and to a lower extent in, IL-17A− (12–23%) duodenum-derived T cells (**Figure 2b**, middle panels). No phenotypical differences appeared between IL-17A+ T cells from CD and non-CD control individuals (**Figure 2b**, lower panels). The expression of Foxp3 was analyzed in both CD- and non-CD-derived cells to explore the possibility that the cells could be in a transition state between Tregs and Th17. We found that this molecule is absent in all cells studied (**Figure 2b**).
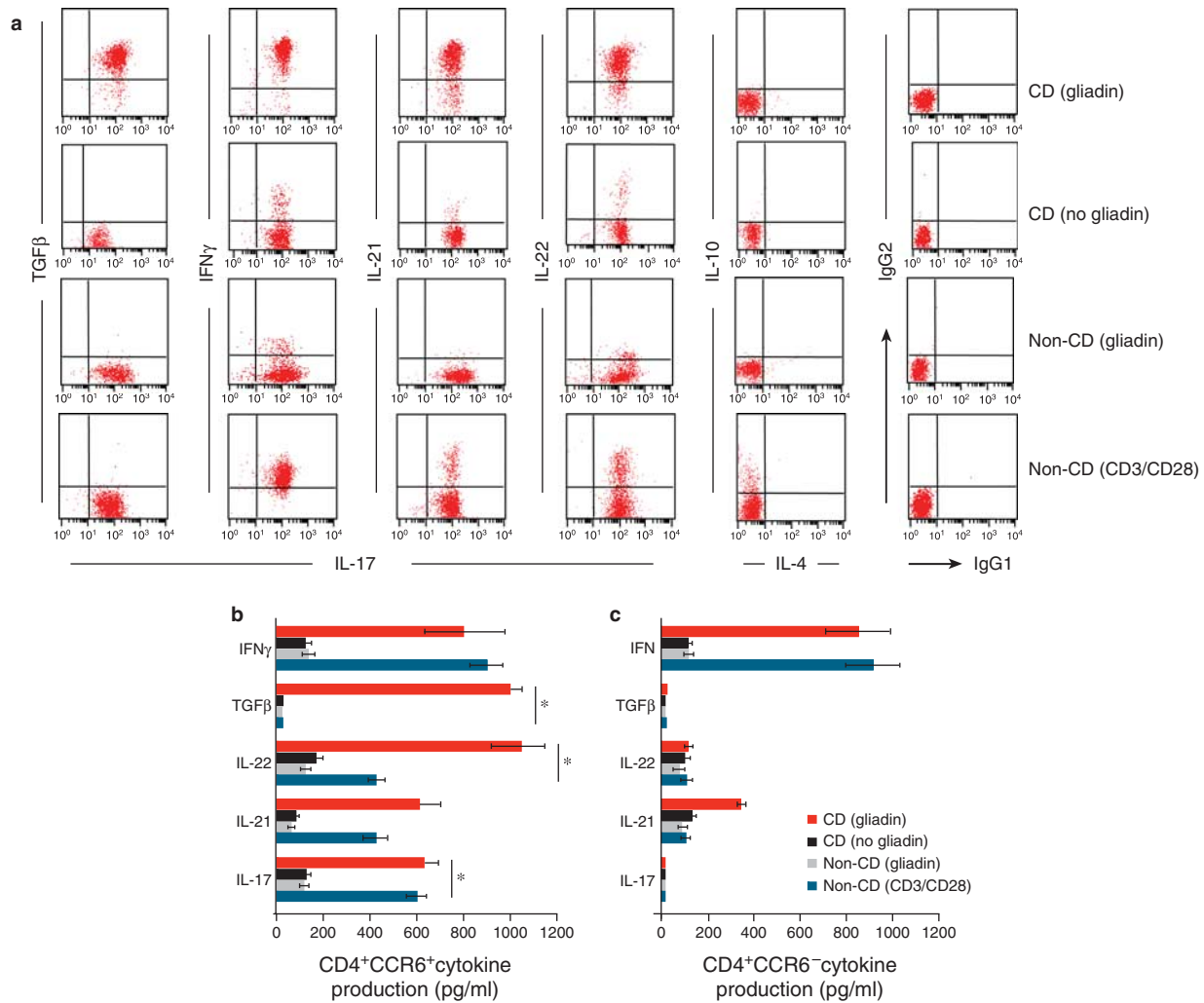
Figure 3. Cytokine production of CD4⁺CCR6⁺ cells from CD patients in response to gliadin stimulation. (**a**) Intracellular cytokines analysis of CD4⁺ CCR6⁺-sorted cells either resting or gliadin stimulated (50 µg/ml) from CD patients (upper panels) and control subjects treated with gliadin or anti-CD3/CD28 beads (lower panels) (one representative experiment out of five is shown). Cells were cultured in 96-well plates, 20×10^3 cells/well. (**b**) Cytokines released to the supernatant by CD4⁺CCR6⁺ cells cultured and analyzed as described above by flow cytometry, either in the presence (red bars) or absence (black bars) of gliadin from CD patients and non-CD control individuals either stimulated with gliadin (gray bars) or anti-CD3/CD28-coated beads (blue bars). (**c**) Cytokines released by CD4⁺CCR6⁻ cells treated as described in panel **b**. Results are expressed as mean±s.d. of 10 samples. *$P<0.01$. CD, celiac disease; CCR6, C–C chemokine receptor type 6; IL, interleukin; TGFβ, transforming growth factor-β.

Functional analysis of gliadin-specific Th17 cells from CD patients

The expression of IL-17A, TGFβ, IFNγ, IL-21, and IL-22 in response to gliadin by the indicated populations from three patients and three control individuals was measured by quantitative PCR in parallel experiments. As IL-17A⁺ cells from non-CD individuals did not respond to gliadin, anti-CD3/CD28-stimulated Th17⁺ cells from non-CD individuals were also included in the experiments as controls. We found that all mentioned cytokines were highly produced by the gliadin-specific Th17 CD population. Remarkably, a vigorous TGFβ expression was detected in gliadin-stimulated cells from CD patients but not in the IL-17A⁺ counterpart population from non-CD individuals (data not shown).

These molecular observations were further studied by assessing intracellular cytokines produced by Th17 cells, at the protein level. We observed that gliadin-stimulated CD4⁺IL-17A⁺ cells from CD patients, but not anti-CD3/CD28-stimulated CD4⁺IL-17A⁺ cells from control subjects, simultaneously produce IFNγ, IL-21, IL-22, and, strikingly, TGFβ, whereas neither IL-4 nor IL-10 production was found within Th17 CD cells (**Figure 3a**). These results were confirmed after analyzing the presence of secreted cytokines in the culture supernatant. Thus, we found that gliadin-specific Th17 CD cells produce significantly higher amounts of IL-21 and IL-22 ($P<0,05$) than do the same cells in the absence of gliadin and also than do their counterparts obtained from control individuals and stimulated with anti-CD3/CD28-coated beads. Remarkably, the vigorous production of TGFβ is confined to Th17 CD cells (**Figure 3b**). The cytokine production pattern of CD Th17⁻ cells and control individuals showed no differences, as IFNγ was the main cytokine produced by both CD and non-CD Th17⁻ cells in response to stimulation, whereas IL-21 and IL-22 were produced in lower amounts (**Figure 3c**).
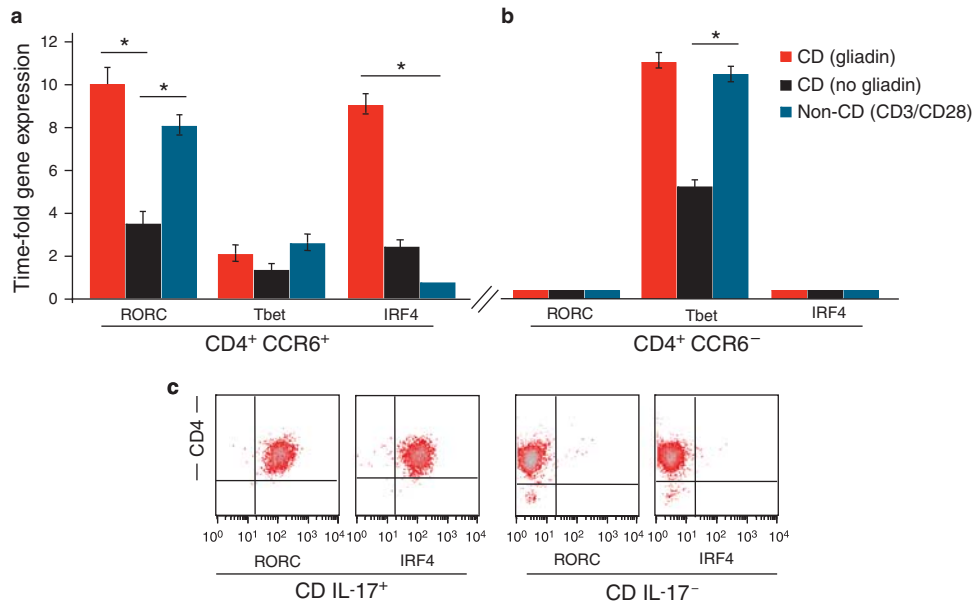
Figure 4. Analysis of transcription factors of stimulated T cells from the duodenal lamina propria. (**a**) Expression of RORC, T-bet, and IRF4 transcription factors in sorted CD4⁺CCR6⁺ populations, in the presence (red bars) or absence (black bars) of gliadin, from CD patients and non-CD control individuals stimulated with anti-CD3/CD28-coupled beads (blue bars). Results were normalized against *HPRT1* gene values and represented as time-fold induction. (**b**) Analysis of CD4⁺CCR6⁻ cells from CD patients and control individuals treated as indicated in panel **a**; values represent mean±s.d. obtained in seven independent experiments. *$P<0.05$. (**c**) Representative flow-cytometric analysis of RORC and IRF4 intracellular protein expression in IL-17A⁺ (left panels) and IL-17A⁻ cells from CD patients challenged with gliadin (50 µg/ml). CD, celiac disease; CCR6, C–C chemokine receptor type 6; IL, interleukin; IRF4, interferon regulatory factor-4.

Transcription factors analysis

The characterization of gliadin-expanded IL-17A⁺ cells from CD patients was extended to the analysis of the pattern of their transcriptional factors. Quantitation of mRNA from gliadin-stimulated cells showed a robust upregulation of the *RORC* gene in Th17 cells of CD patients and stimulated control cells compared with the steady-state mRNA of the *HPRT* gene. CD Th17 cells in the absence of gliadin also expressed RORC mRNA, but to a significantly lower extent. Interestingly, we also found a strong expression of the IRF4 transcription factor in the gliadin-stimulated Th17 CD population (**Figure 5a**). This factor was upregulated neither in IL-17A⁺ cells from non-CD control individuals stimulated with anti-CD3/CD28-coated beads (**Figure 4a**) nor in the IL-17A⁻ fraction of duodenal cells from CD patients (**Figure 4b**). Equivalent low levels of T-bet mRNA were found in IL-17A⁺ cells from CD patients and controls (**Figure 4a**). However, the T-bet factor was highly expressed within the IL-17A⁻-stimulated population of CD patients and non-CD individuals (**Figure 4b**). We confirmed by intracellular staining, followed by flow cytometry, the expression of RORC and IRF4 transcription factors in IL-17A⁺ cells from CD patients and their absence in the IL-17A⁻ cell fraction (**Figure 4c**).

TGFβ produced by CD IL-17A⁺ T cells is biologically active and regulates IL-17A production

As we found that Th17⁺ cells isolated from CD patients also produce TGFβ, we addressed the functional role of this pleiotropic factor on this cell population. Therefore, Th17⁺ cells were cultured for 16 h in the absence or presence of minute amounts of TGFβ (5 ng/ml). We found that addition of TGFβ to the Th17⁺ cell culture resulted in a vigorous stimulation of the production of IL-17A, as determined by a shift in the mean fluorescence intensity channel of the stained cells at the end of the culture, from a mean fluorescence channel of 23 in untreated cells to 246 mean fluorescence channel in TGFβ-treated cells (**Figure 5a**). Finally, we studied the impact of TGFβ on the RORC and IRF4 expression of stimulated Th17⁺ cells. We found that addition of 5 ng/ml TGFβ to Th17⁺ cells significantly increased the expression of RORC and IRF4 transcription factors (**Figure 5b**, solid bars). The specificity of the effect was demonstrated by blocking experiments adding 10 µg/ml anti-TGFβ mAb to the cultures. We found that blockade of TGFβ results in a significant reduction in the mean fluorescence channel (down to mean fluorescence channel 5), number of IL-17A-stained cells (**Figure 5a**), as well as in the expression of RORC and IRF4, which in both cases fell below the levels of stimulated cells alone (**Figure 5b**, empty columns). Finally, IL-17A secreted to culture supernatants was also inhibited in the presence of anti-TGFβ mAb, demonstrating that TGFβ produced by CD IL-17A⁺ T cells is biologically active, thereby supporting the observation that autocrine secretion of TGFβ has a positive regulatory role in the production of IL-17A by Th17 cells derived from the intestinal mucosa of CD patients.

DISCUSSION

Mounting evidence suggests the participation of Th17 cells in the pathogenesis of autoimmune diseases. However, the role of these cells in CD has not been so far extensively studied. Owing to this lack of information, in this study, we have addressed the characterization of
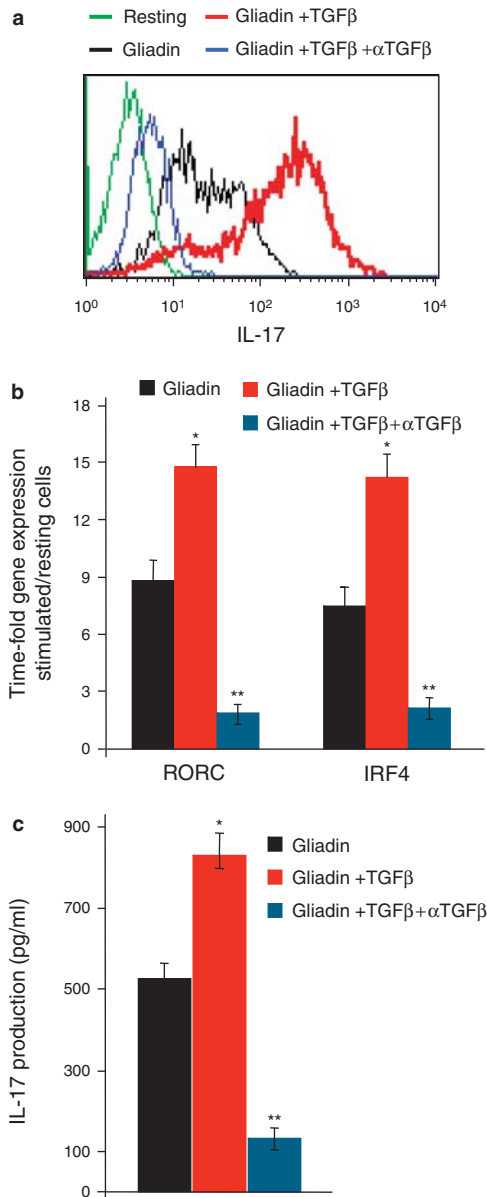
Figure 5. Autocrine TGFβ regulates IL-17A production. (**a**) Flow cytometry overlay of IL-17A⁺ cells from CD patients resting (green line) stimulated with gliadin (black line); gliadin plus 5 ng/ml of TGFβ (red line); or gliadin plus 5 ng/ml of TGFβ in the presence of neutralizing amounts (10 μg/ml) of anti-TGFβ mAb (blue line). (**b**) Expression of RORC and IRF4 transcription factors determined by qPCR in CCR6⁺ cells stimulated as described in panel **a**. Results are expressed as normalized mean±s.d. of time-fold expression over values of non-stimulated cells ($n=5$) (gliadin+TGFβ *$P<0.05$; gliadin+TGFβ+anti-TGFβ **$P<0.01$). P-values are for both RORC and IRF4. (**c**) IL-17A detected in supernatants of cultures treated as described in panels **a** and **b**. Results represent mean±s.d. ($n=5$) (gliadin+TGFβ $P<0.05$; gliadin+TGFβ+anti-TGFβ $P<0.01$). CD, celiac disease; CCR6, C-C chemokine receptor type 6; IL, interleukin; IRF4, interferon regulatory factor-4; mAb, Monoclonal antibody; TGFβ, transforming growth factor-β; qPCR, quantitative PCR.

CD4⁺IL-17A⁺ T lymphocytes present in the duodenal mucosa of CD patients, a tissue where both CD4⁺ and CD8⁺ T cells producing IL-17A have recently been found (24).

Our results indicate that the duodenal mucosa of active CD patients contains CD4⁺ T cells that produce IL-17A in significantly higher amounts than those found in the mucosa of non-CD control individuals. Th17 CD cells share with the "classical" Th17 population phenotypical features such as the expression of CCR6, CD161, and IL-23 receptor, although some studies were unable to find expression of the latter in the active CD mucosa (26). The fact that multiple splice forms of the human interleukin-23 receptor α-chain are found in activated leukocytes (27) and the "*ex vivo*" cell-expansion conditions used may account for the differences observed. CD161, on the other hand, has been proposed as a marker for IL-17A-producing cells (28). However, we found that CD161-positive cells do not include all IL-17A-producing CD cells, or otherwise that not all IL-17A-producing cells are derived from the same cell precursors (29).

We have shown that IL-17A-producing cells from the celiac mucosa, but not those obtained from controls, are gliadin specific. This finding is nevertheless in contradiction with a recent study showing that gluten-reactive T-cell lines from treated patients produce IL-21 but not IL-17 or IL-22 (30). However, there are some important differences in the experimental approaches undertaken by the two studies. First, all participating patients in our study had never received any treatment, whereas Bodd *et al.* used long-term cell lines from treated patients. Second, the cells used in our study were sorted based on their CCR6 surface expression and subsequently expanded by gliadin challenge in culture. Third, our cells were cultured in the presence of exogenous IL-23, a cytokine that is required to sustain the IL-17 phenotype. The combination of these factors apparently results in expanded populations that have different characteristics. Indeed, only a small percentage of cells contained in the cell lines derived from the CD mucosa by Bodd *et al.* showed HLA DQ2-restricted gluten reactivity. Despite these authors' failure to detect gluten-reactive Th17 cells within the cell lines derived from the CD intestinal mucosa, they report, interestingly, a small percentage of CD4⁺IL17⁺ cells in fresh blood peripheral cells of CD patients after an oral gluten challenge.

The reactivity of cells is restricted to gliadin, as we failed to observe any proliferation in response to transglutaminase. This finding is fully consistent with previous data showing no detection of transglutaminase-reactive cells within the CD mucosa (31), although a recent study has found that transglutaminase-specific Th1 cell clones derived from peripheral blood of some, but not all, active CD patients induce *ex vivo* typical lesion changes in the CD intestinal mucosa. The detection of these reactive cells was influenced by the clinical situation of patients and also required 8–10 weeks of repeated stimulation, thus indicating that even a small percentage of transglutaminase-reactive cells that were undetectable in our short-term cultures are nevertheless enough to contribute to intestinal damage (32).

The presence of IL-17⁺ gliadin-specific cells in the CD intestinal mucosa constitutes strong evidence for an active involvement of Th17 cells in the pathogenesis of CD. We found that activation of Th17 cells of CD patients is controlled by the same transcription factors than their counterparts from non-CD control individuals. However, the expression pattern of these factors is not the same. There is a very high expression of IRF4 in CD4⁺IL-17A⁺ cells

from CD patients compared with their non-CD counterparts. This fact suggests that a strong upregulation of IRF4 is relevant for the Th17-specific response to gliadin-derived peptides. IRF4 facilitates RORC expression, a factor that is required for differentiation of Th17 cells, as it was first described in a model of experimental autoimmune encephalomyelitis (33). The detection of the IRF4 protein inside IL-17A$^+$ cells reinforces the idea that IRF4 influences RORC expression in gliadin-specific IL-17A duodenal T cells. It is also noteworthy that, in the aforementioned model of experimental autoimmune encephalomyelitis, a significant IL-17A production was achieved in mice bearing a single copy of the *IRF4* gene that also rescued the IL-22 production ability (34). Thus, it is conceivable that the high amount of IL-22 detected in our Th17 cells from CD patients compared with IL-17A-producing cells from control individuals might be related to the upregulation of IRF4. As expected, equally high levels of RORC are found in Th17 cells from CD and non-CD control individuals. T-bet, the master regulator of Th1 cell differentiation was expressed by Th17 CD cells, although at levels lower than those of RORC and IRF4. This finding leaves open the possibility that CD4$^+$IL-17A$^+$ cells in the CD mucosa are not, *sensu stricto*, classical Th17 cells, or otherwise that classical Th1 and Th17 subtypes are not closed compartments, as a number of groups have recently shown to occur with Th17 cells and Treg cells (35,36). In this regard, the concomitant production of IFNγ, a "Th1 type" cytokine, should be observed. The production of IFNγ by cells committed to the production of IL-17A clearly supports a prominent role of Th17 cells in CD pathogenesis. However, whether both cytokines influence each other's function and/or production requires further investigation.

Our data show that whereas IL17$^-$ cells produce IL-21, the amount of this cytokine produced by Th17 CD cells is significantly higher. The precise role of IL-21 in the pathogenesis of CD remains unresolved. In this regard, the enhanced expression of IL-21 in the mucosa of untreated CD patients related to gluten challenge has been reported (37). These and our data suggest that IL-21 has a role in sustaining T-bet expression and IFNγ production, thereby contributing to the dominant Th1 response observed in the mucosa of CD patients.

We found that Th17 mucosal cells from CD patients express two cytokines with well-known dual opposing effects: IL-22 and TGFβ. Both cytokines have anti-inflammatory and proinflammatory roles (38), depending on the targeted cells (14,39) and the inflamed organ (40–43). As the IL-22 receptor is highly expressed by epithelial cells of the intestine (44), the enhanced IL-22 production by CD IL-17A$^+$ cells, which also produce TGFβ, allows the idea that Th17 cells might have a dual role in CD: a proinflammatory role, by means of Th1 cytokines contributing to mucosal inflammation and disease progression, or an anti-inflammatory, protective role, exerted through TGFβ and IL-22 that would hurdle the Th1 response, as is the case recently observed in an animal model of inflammatory bowel disease (45). Consequently, it is relevant to elucidate more precisely the role of IL-22 in the pathogenesis of CD, as IL-22 might well be considered for future biological therapy because of its selective modulation of tissue response while showing no direct effects on the immune response.

One of the most striking findings of our study is that IL-17A cells from active CD patients also produce TGFβ. This result adds a new

feature to the known homeostatic role of TGFβ in the intestine by modulating the participation of Th17 cells in the gliadin-specific response in an autocrine/paracrine manner. Such a paradoxical effect may be related to the observation that tumors secreting large quantities of TGFβ induce the development of IL-17A$^+$ cells, which in turn promote tumor survival in an IL-17A-dependent manner (46). It has been described in a model in which TGFβ activity is deficient in the CD mucosa because of the action of IL-15, the signals of which activate Jun N-terminal kinase leading to TGFβ inhibition (47). However, the neat dual effects of TGFβ on IL-17A production observed in our study indicate that TGFβ is operative within the context of the CD mucosa and reinforces the significance of TGFβ produced by IL-17A CD cells as biologically active, as it case of CD8$^+$TCRγδ$^+$NKG2A$^+$ intraepithelial lymphocytes from CD patients with regulatory activity mediated by their secreted TGFβ (18). In this regard, our preliminary data point out that Th17 cells that also produce TGFβ do not have the capacity to behave as regulators of effector T cells stimulated with anti-CD3 plus anti-CD28 mAbs (Ortega *et al.*, unpublished observations). We have not found CD4$^+$ cells from CD patients exclusively committed to the production of IL-17A, as most CD4$^+$IL-17A$^+$ cells also produce IFNγ and/or TGFβ. This is a significant difference with canonical Th17 cells, which produce neither IFNγ nor TGFβ and represent another relevant finding of our study. This is apparently in contrast with the results reported in a previous study, which showed the existence of gliadin-specific Treg cells (presumably IL-17A$^-$) in the celiac mucosa (6). In that study, the biological activity of TGFβ was elegantly demonstrated as its *in vitro* neutralization (using an anti-TGFβ mAb) rescued IFNγ production by T cells in response to gliadin when co-cultured in the presence of Treg cells, thereby supporting gliadin-specific Treg cells as the main source for TGFβ in that experimental setting. However, the model by Gianfrani *et al.* differs from that of ours in two relevant aspects: first, Treg cells were obtained from cultures in the presence of high amounts of exogenous IL-10, a cytokine that was neither used by us nor secreted by our IL-17A-producing cells, and second, cells studied by these authors (Treg cells) were grown in culture for a much longer period of time and their ability to produce TGFβ or IL-17A was not formally addressed. However, we cannot exclude the possibility that mucosal CD4$^+$IL-17$^-$ cells from untreated celiac patients and CD4$^+$IL-17$^-$ or CD4$^+$IL-17$^+$ cells from non-CD patients may acquire the ability to secrete TGFβ if cultured for an extended period of time and/or in the presence of other cytokine(s) that could be present at the inflammation site in the small intestine.

Taken together, these differences raise interesting issues, still unraveled, such as: where are IL-17A-producing cells present in the CD mucosa site generated or polarized. In this regard, we have previously shown that IL-17A production by cells from psoriatic skin could be modulated *in vitro* in response to different polarizing conditions (22). It is being accepted that Th17 cells could originate from Treg cells by a conversion mechanism (48). Our observation that Th17 cells concomitantly produce TGFβ may be interpreted in favor of a common origin of Th17 and Treg cells. However, we found that Th17 cells express neither FoxP3 nor IL-10, observations that do not argue against this common origin but rather against the possibility that the Th17 cells shown herein could represent a transitional state

from Treg cells becoming Th17 cells, as this population does not retain any of the characteristics of Treg cells, with the exception of its ability to produce TGFβ (48,49). However, our data do not formally exclude this possibility, which needs to be further explored in detail in CD. Indeed, increasing evidence clearly points to the diversity and functional duality of Th17 cells (50). As an example, Cosmi *et al.* (51), have recently shown the existence of Th17 cells also able to produce IL-4 under appropriate polarizing conditions, thereby adding new data supporting the plasticity of Th17 cells.

In conclusion, our data demonstrate the presence of gluten-specific IL-17A-producing cells in the duodenum of CD patients, which have the ability to secrete some cytokines with known dual opposing effects on the inflammatory process. These findings strongly support the involvement of Th17 cells in the pathogenesis of CD, and suggest that IL-17A-producing cells might not be univocally linked to the promotion of self-tissue damage during autoimmune responses. Thus, the understanding of the activity of Th17 cells in CD is of clinical and therapeutical relevance, as they may open new approaches to the biological treatment of the disease.

CONFLICT OF INTEREST

Study Highlights

WHAT IS CURRENT KOWLEDGE

✓ Th17 cells are a distinct T-lymphocyte sub-population.

✓ Th17 cells participate in the pathogenesis of autoimmune diseases.

✓ Th17 cells secrete interleukin (IL)-17 and IL-21.

✓ Th17 cytokine signature has been described in the celiac mucosa.

WHAT IS NEW HERE

✓ Gliadin-specific Th17 cells are increased in the celiac disease (CD) mucosa.

✓ Th17 cells from CD patients secrete IL-17, interferon-γ (IFNγ), IL-21, IL-22, and transforming growth factor-β (TGFβ) in response to gluten challenge.

✓ In CD patients, but not in control individuals, mucosa-derived Th17 cells require RORC and interferon regulatory factor-4 (IRF4) transcription factor expression.

✓ In CD patients, IL-17 production by Th17 gliadin-specific cells is under TGFβ autocrine regulation.

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ORIGINAL ARTICLE

IL-17 Producing T Cells in Celiac Disease: Angels or Devils?

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Celiac disease (CD) is a very common chronic condition in human beings, affecting approximately one in 100 individuals. It is an autoimmune disease with a defined environmental trigger, the gluten contained in dietary cereals, occurring in genetically susceptible individuals. The disease has a very strong HLA association. More than 90% of CD patients have HLA-DQ2, and almost all of the remaining celiac population possesses HLA-DQ8 molecules. Th17 cells seem to participate in the disease pathogenesis producing and secreting either proinflammatory or anti-inflammatory cytokines.

Keywords celiac disease, IL-17A, IL-21, IL-22, mucosa, Th17

CELIAC DISEASE

Celiac disease (CD) is a very common chronic condition in human beings, affecting approximately one out of 100 individuals. It is an autoimmune disease with a defined environmental trigger, the gluten contained in dietary cereals, occurring in genetically susceptible individuals [1]. The disease has a very strong HLA association. More than 90% of CD patients have HLA-DQ2, and almost all of the remaining celiac population expresses HLA-DQ8 molecules [2, 3]. T cells present in celiac lesions recognize gluten epitopes presented by disease-associated HLA-DQ2 (DQA1∗05:01/DQB1∗02:01) or HLA-DQ8 (DQA1∗03/DQB1∗03:02) molecules [4, 5]. HLA-DQ2 and DQ8 are already used clinically to help exclude disease [6]. However, approximately 40% of the population carry these alleles and the majority never develop CD, indicating that although necessary they are not the only responsible elements for the development of the disease [7]. Also a number of non-MHC genes has been reported as susceptibility factors in celiac disease [8, 9]. Environmental factors also include early life gluten exposure, short duration of breastfeeding, intestinal infections and changes in microbiota [10].

Celiac disease is characterized by small intestinal enteropathy with villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in the epithelium and lamina propria. CD4+ lymphocytes restricted to HLA-DDQ present in the

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lamina propria recognize, preferentially, deamidated gluten peptides, a post-translational modification mediated by the enzymatic action of tissue transglutaminasa 2. In these peptides, some glutamine residues have been converted to glutamate. Such a modification favors this gluten peptides' interaction with HLA-DQ2 and HLA-DQ8 since these alleles show preference to bind negatively charged anchor residues [11–13].

The pathogenic mechanisms leading to villous atrophy in CD remain not completely understood. Not long ago, CD was thought to be mediated by a skewed Th1 response because of the cytokines secretion profile detected in the intestinal mucosa, which induces CD8+ T lymphocytes to kill mucosal cells by a direct cytotoxic mechanism or by Fas-mediated apoptosis [14–17]. In the following years a more complex picture of the ongoing cytokine interplay in CD mucosa has emerged, supported by the findings of proinflammatory and down-regulatory cytokines produced by intestinal T cells from CD patients, as well as the presence of antigen-specific T regulatory cells (Tregs) in CD mucosa. In addition, over the last few years, it has become evident that the involvement of cell populations, other than Th1 lymphocytes, may have significant influence in the pathogenesis of autoimmune responses, as it is the case of the Th17 cell subpopulation.

Th17 CELLS

Th17 cells represent a new arm of the effector CD4+ lymphocytes recognized mainly, though not only, by their ability to produce different forms of IL-17 (i.e. IL-17A, IL-17F) [18]. It seems that Th17 cells originate from a CD161+CD4+ T cell precursor [19]. Th17 cells display differential features from Th1 or Th2 subtypes of T lymphocytes. Although controversial data has been reported, it is generally accepted that these differences include the expression of IL-23 receptor (IL23R) as well as CCR6, a molecule also present on the surface of Tregs [20]. Differences also include the transcription factors required for IL-17A production, RORC and IRF4 [21, 22] and the cytokines profile that comprises a variety of factors such as IL-21, which is also produced by Th2 cells [23], IL-22, a cytokine involved in early defense mechanisms against some bacterial pathogens in epithelial tissues [24, 25], and IL-26 [26]. Th17 lymphocytes also produce TNFα [27], granulocyte-monocyte colony stimulating factor (GM-CSF) [28, 29] and CXCL8 [30]. Th17 cells also produce cytokines classically associated to the Th1 or Th2 subtypes of T lymphocytes such as IFNγ (Th17/Th1) [31] or IL-4 (Th17/Th2) [32], respectively.

Th17 CELLS IN CD

Th17 cells are present in active CD mucosa where they may participate in the onset and/or development of CD. The participation of Th17 cells in experimental and human autoimmune disorders has also widened the actors to be considered on the immune pathogenesis of CD. We shall review and discuss data regarding Th17 cells' contribution to the cellular and cytokine interactions within the CD small bowel context and counter-evidences of Th17 participation in CD. Controversy is served.

The presence of Th17 cell activity in the celiac mucosa was initially identified by detecting cytokine transcripts consistent with the presence of Th1 and Th17 cells in duodenal biopsies from CD patients [33]. In these studies, the expression of IL-21, IFNγ, TGFβ1, IL-6, IL-17A, IL-17F and IL23R was quantified by means of RT-PCR assays. For that purpose, comparison of intestinal samples from active CD patients and biopsies taken after a long-term treatment with gluten free diet (GFD) patients was performed. Results showed up-regulation in the expression of gene characteristic of both Th1 and Th17 cell lineages as the disease progresses. This finding is consistent with the

situation in active inflammation sites of other autoimmune diseases, such as inflammatory bowel disease or psoriasis. The authors found that the most pronounced changes are observed for IFNγ, IL-12 and IL-17A, but there was also an increase, though not statistically significant, in the expression of IL23R and IL-6.

In a single nucleotide polymorphism (SNP) study aimed to identify CD susceptibility factors in a large cohort of CD patients, genes relevant for the Th17 immune response were genotyped. One hundred one SNPs, present in 16 Th17-related genes (IL23R, RORC, IL6R, IL17A, IL17F, CCR6, IL6, JAK2, TNFSF15, IL23R, IL22, STAT3, TBX21, SOCS3, IL12RB1 and IL17RA), were analyzed. Surprisingly no significant results emerged after performing the appropriate statistical corrections, therefore excluding any relevant role of Th17 cells-related factors on CD risk. However, in this study, data corresponding with well-known Th17-related genes such as IL-17F, IL-22 and RORC were eliminated from the study due to deviation from Hardy-Weinberg proportions. In addition, IL23R SNP initially found highly associated to CD risk [34] could not be confirmed in a replication set [35]. These results imply that the initial findings on the presence of Th17 cytokine signature in the small bowel of CD patients may not be related to the individual susceptibility to the disease or to its development. However, a recent study analyzed mRNA levels of the cytokine IL-17A, IFNγ, IL-10, TGFβ1 and the Treg marker FOXP3 in small intestinal biopsies of untreated CD (children on a gluten-containing diet with active disease), treated CD (children with CD on a gluten-free diet and with inactive disease) and controls (pediatric clinical controls with no known food intolerance). Levels of IL-17A and IFNγ were significantly higher in biopsies from patients with untreated CD compared to both treated CD and controls. Foxp3, IL-10 and TGFβ all showed a similar pattern with significantly higher levels in untreated than treated CD, but not in controls. Analysis comparing mRNA levels of the cytokines and Foxp3 in biopsies of the three studied groups revealed significant correlation between IL-17A and both IFNγ and Foxp3 in untreated CD and between Foxp3 and TGFβ in treated CD. In controls, there was no correlation between any of the cytokine mRNAs or between these and Foxp3 mRNA [36].

Interestingly, four hours *ex vivo* stimulation of CD small bowel biopsies with gliadin peptides as a surrogate of an acute re-exposure to gliadin showed a generalized increment in the expression (mRNA) of all cytokines except TGFβ in the active phase of the disease. The expression of IL-17F was undetectable in the majority of samples from CD patients at any stage [33].

Increased IL-17A protein levels in response to gliadin challenge were demonstrated one year late, under strictly controlled culture conditions in a more elaborated condition. Biopsy specimens from active CD patients were cultured in an organ culture dish in an atmosphere containing 95% O_2/5% CO_2 at 37°C and 1 bar pressure [37]. Indeed, in elegant *ex vivo* experiments, these authors showed that either single positive CD4+ cells or double positive CD4+CD8+T cells present in the biopsy specimens produced intracellular IL-17A in response to peptic-tryptic digest of gliadin. Additionally, they reported no response in the same experiments by T cells other than CD4+(including CD4+CD8+), indicating that gliadin-reactive CD4+ cells are an important source of IL-17A within the CD small bowel context. More important is the observation that CD3+ cells contained in duodenal explants from inactive CD individuals up-regulates the production of IL-17A upon stimulation with gliadin. Because IL-21 promotes Th17 cell development [38] and is expressed in excess in CD [39], some biopsy specimens were incubated in the same conditions with or without an anti-human IL-21 neutralizing monoclonal antibody. Results clearly demonstrated that blockade of IL-21 activity significantly reduced IL-17A transcript levels in organ culture of active CD duodenal explants [37]. Remarkably neutralizing IL-21 also inhibited IL-17A production by biopsy specimens from inactive CD stimulated with peptic-tryptic gliadin,

showing that gliadin induces IL-17A through an IL-21-dependent mechanism. A different approach was used by Di Sabatino et al. that explored the expression of CD40 and CD40L in the duodenal mucosa of celiac patients, and the effect of CD40L blockade on the production of proinflammatory cytokines such as IFNγ and IL-17 in duodenal explants. Indeed, explants stimulated with gliadin peptides *ex vivo* produced increased IFNγ and IL-17 when compared to the controls [40].

Despite the observations showing the ability of intestinal samples from untreated CD patients cultured under different conditions to respond to gliadin peptides by increasing the production of Th17-related cytokines, the gliadin specificity of such responses remained unclear as those experiments were carried out using organ cultures of whole biopsy specimens, and not purified cell types. Our group offered demonstration of the presence of gliadin-specific Th17 cells [41]. Gliadin-specific Th17 cells were obtained from CD distal duodenum explants after collagenase A digestion. T cells derived from explants (3–4) of each individual were stimulated with gliadin peptides in the presence of irradiated autologous PBMC for seven days in culture medium containing human serum. After CD8+ cells depletion, CCR6+ immunoselected cells were further expanded in the presence of gliadin and exogenous IL-23 added to the system during Th17 cells isolation and expansion procedures. Phenotype features of the IL-17+ cells from CD patients showed an effector/memory phenotype as well as the widespread surface expression of the Th17 subset defining molecules such as CCR6, IL23R and CD161. However, it is to be noted that the latest was found in approximately half of the IL-17+ cells from either CD and non-CD control individuals. This is not totally concordant with data reported by Monteleone's group where they found that within the CD161+ cells from CD patients, 40% expressed IL-17A whereas more than 90% of IL-17A+ cells lacked expression of CD161 [37]. This discrepancy likely indicates a different balance between memory- and effector-IL-17A producing cells within the IL-17A+ cell populations studied. In any instance, both reports differ from other studies supporting that CD161 is expressed by all Th17 memory cells as well as in CD8+ T cells and double negative CD4–CD8– T lymphocytes [19, 42, 43].

We have found that IL-17A producing cells from CD patients express two cytokines with dual, opposite effects: IL-22 and TGFβ. Both cytokines have anti-inflammatory and pro-inflammatory roles [44, 45]. The presence of IL-22 producing cells observed by us but not in IL-17A producing cells from other CD cohorts might suggest a different infectious biography or a direct effect of the microbiota in both cohorts of patients. This is an interesting issue that deserves attention as IL-22 plays a key role in the control of intestinal homeostasis [46] and the production of antimicrobial peptides cooperatively with IL-17A [47].

It is of interest that although IFNγ is not considered a Th17-related cytokine, all reports on IL-17A production by T cells in the celiac mucosa found concomitant production of IL-17 and IFNγ. This raises the question of whether or not IL-17A producing T cells in CD are "canonical" Th17 cells. It has been suggested that in the presence of IL-12, Th17 cells acquire the ability to secrete IFNγ. This, added to the well-known role of IL-21 in up-regulating and sustaining the expression of the T-bet transcription factor, might explain the reason why no canonical Th17 cells has been yet identified in the mucosa of untreated CD patients. Regarding the transcription factors involved in Th17-related cytokine expression, there is not much information available. We have shown that IL-17A-producing T cells displayed up-regulated expression of transcriptional factors RORC and Interferon-Regulatory Factor 4 (IRF4). IRF4 is a hematopoietic cell-specific transcription factor that regulates the maturation and differentiation of immune cells, and RORC transcription factors. Whereas the role of RORC in the differentiation of Th17 cells is well known, IRF4 participation in the development of IL-17A-producing cells is becoming increasingly clear. Either CD4+ or CD8+ (Tc17)

lymphocytes producing IL-17A has been shown to express IRF4, which is required in mucosal tissues to obtain DCs specialized in instructing IL-17 responses [48] in health as well as [49, 50] in disease conditions [51, 52].

In our study, Th17 cells from CD patients but not from control individuals proliferate in response to gliadin challenge. Gliadin stimulation also results in an increased mRNA and protein level of IL-17A, IFNγ, IL-21, IL-22 and, strikingly, TGFβ. Neither IL-10 or IL-4 was detected within the IL-17A population in our patients. This data strongly support that cells were gliadin-specific. This finding is nevertheless in contradiction with the observations that gluten-reactive T-cell lines from treated patients produced IL-21 but not IL-17 or IL-22 [53]. However, all patients in our study had never received any treatment, whereas Bodd et al. used long-term cell lines from treated patients. In addition, only a small percentage of cells contained in the cell lines derived from the CD mucosa by Bodd et al. showed HLA DQ2-restricted gluten reactivity. Remarkably, the same authors detected CD4+ IL-17+ cells in peripheral fresh blood of CD patients after an oral gluten challenge. This finding is strongly suggestive of the existence of an antigen-driven boost of gliadin-specific cells in CD patients following gluten provocation. Unfortunately, whether these cells come from or goes to the gut was not investigated. This points to the importance of the culture techniques used to isolate, purify and expand T cells from intestinal explants, since different experimental approaches may lead to deep discrepancies in the interpretation of the role of any T cell type in the pathogenesis of CD. A recent report, however, also failed to find increased numbers of Th17 cells in pediatric CD patients. Instead, they found elevated number of cells producing IL-21 and IFNγ. These authors, however, reported increased representation of Th17 cells in the mucosa of a subgroup of adult CD patients. In a smart group of histopathological experiments, IL-21 producing cells were clearly established as different from cells producing IL-17. The authors attribute this finding to the expression of TLR2 in lesional tissues, a receptor known to trigger IL-17A secretion. Unfortunately, no data regarding co-expression of IL-21 by IL-17A-producing cells was reported in this subgroup of patients [54].

OTHER CYTOKINES DETECTED IN Th17 CELLS IN CD MUCOSA

Other cytokines of the IL-17 family participate at some stages of the disease. While participation of IL-17A and IL-22 remains controversial, there is a unanimous perception that, among Th17-related cytokines, IL-21 plays a relevant role in the pathogenesis of the CD [41, 55, 56]. This cytokine attracted attention since a first genome-wide study carried out in CD performed by Heel et al. [57] showed a strong association with a close region to the IL21 gene, which is located in human chromosome 4. IL-21 is a cytokine produced by activated CD4+ T cells, NKT cells and neutrophils [58], and also by CD8+ T cells under certain conditions of prolonged T lymphocyte stimulation, such as in some autoimmune or infectious conditions [27, 59]. Indeed, IL-21 is critical for CD8$^+$-T cell survival [60, 61] and memory generation [62] as well as for promoting the activity of CD8-T cell effectors during viral infections enhancing the cytotoxic response to virally infected cells by NK cells and CD8-T lymphocytes [63, 64]. This relation to CD8+ cells is of importance to our debate, as both CD4+ and CD8+ play a role in CD development and tissue damage. Overproduction of IL-21 was observed in the intestinal mucosa of patients with active CD, its origin thought to be lamina propria unique CD4+ cells [39]. The main contribution to CD inflammatory response was shown to be the stimulation of IFNγ production by CD4+ lymphocytes. However, IL-21 also stimulates the release of metalloproteases by enterocytes and fibroblast [65] in the intestine, contributes to recruitment of dendritic cells by inducing enterocyte production of CCL20 and acts synergistically with IL-15 in the modulation of intraepithelial CD8+

T cells upon gliadin challenge in CD mucosa. Studies from two independent groups have shown that IL-21 might be critical to confer resistance of CD4+ T lymphocytes to non-antigen specific Treg cells [27, 66]. It is also relevant regarding the function of IL-21 in CD pathogenesis that this cytokine acts directly on B cells to regulate Bcl-6 expression and germinal centers responses contributing to CD-associated autoantibodies production [67].

EMERGING TREATMENTS FOR CELIAC DISEASE

The recent progress in unraveling the pathogenic mechanisms underlying CD allows us to envision imaginative treatments for the disease. As this is a condition triggered by gluten intake, a gluten-free diet is effective in most patients and the only recommended treatment that has demonstrated long-term benefits [68]. Unfortunately, around 5% of patients become refractory to the treatment and remain symptomatic even after strict adherence to a gluten-free diet [69]. Resistant patients have been classified into type I and type II refractory CD based on clinical and histological differences, but it has been speculated that these two types may represent evolved stages of the disease [70]. Despite a significant improvement in the availability of processed gluten-free foods, some compliant patients considered as type I refractory are not resistant but rather affected by trace contamination [71], a fact that limits but does not exclude intrinsically resistant patients. The need for more effective treatments, therefore, arises not only from the presence of resistant patients but also from the long-term morbidity observed in compliant celiac patients, who show a high rate of gastrointestinal symptoms [72] and nutritional deficiencies [73].

In addition to this, and as we discuss in this work, the development of CD is clearly favored by an abnormal cytokine balance in a way that is common to other autoimmune disorders [6, 74, 75]. The role of high levels of IFNα in promoting excessive Th1 responses in the small intestine of celiac patients was discovered long ago [76]. More recently we became aware of the role of those cytokines in modulating the production of IL-17 such as IL-15 and IL-21, potent proinflammatory cytokines [77]. A transgenic mouse model that overexpresses IL-15 in the gut lamina propria to levels comparable to those found in celiac patients reveals that this cytokine plays a relevant role in the overproduction of IL12p70 and IL-23 [78]. This up-regulation of Th1 responses is a co-adjuvant effect with retinoic acid, a vitamin A metabolite involved in the induction of intestinal responses [79]. Furthermore, IL-15 triggers an anti-apoptotic pathway in human intraepithelial lymphocytes depending from Bcl-2 and/or Bcl-XL [80], which could explain the prolonged survival of this cellular subpopulation in celiac patients. The damage caused to the epithelia by the overproduction of IL-15 may be further enhanced by the fact that this cytokine interferes with the suppressive activity of intestinal Tregs [81]. Therefore, although Tregs are induced *in situ* by gliadin, their suppressive capacity may be impaired *in vivo* and therefore this could represent another important factor contributing to the development of the disease. And finally, IL-15 positively regulates the production of IL-21 in the celiac intestinal mucosa [55], a relevant finding since it is known that this cytokine amplifies Th1 [82] and Th17 responses [38]. The pleiotropic downstream effects of the IL-15/IL-21/lL-17 network make it an attractive candidate for the development of new immunotherapeutic targets in CD. Blockade of any of these cytokines may result in the restoration of homeostasis in celiac intestinal mucosa as Th1 and Th17 responses would be down-modulated and the suppressive capacity of gliadin-specific Tregs restored.

Finally, there is a huge territory to be discovered regarding the role of Th17 cytokines in small bowel inflammatory processes. Like angels and devils, pro- and anti-inflammatory effects of these cytokines play the delicate game of balance in the

mucosa, with projections into the disease geography. The description of distinct subtypes of Th17 cells, such as natural or inducible Th17 cells, memory Th17 cells either central or peripheral, and further division as pathogenic or non-pathogenic according to IL-23 receptor expression, is relatively novel and their activities in disease still undefined [83]. The plasticity of Th17 cells makes them highly unstable during *ex vivo* isolation and experimental procedures. Their ability to change in response to environmental variations enable Th17 cells to modify their functional abilities, such as the cytokine they produce and the phenotype modifications that may be required for an optimal intervention against pathogens or to maintain or recover tissue homeostasis. Th17 cells and their cytokines represent an impressive lesson on the biological and medical implications of T-cell plasticity in health and disease and provide new concepts and tools to look for more adequate targets to hit in the endless ways of translational knowledge into patients' therapy. The magnitude of this task is only comparable to the fascination that this "a-million-faces" cell provokes in most of us.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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IL-17 Producing T Cells in Celiac Disease: Angels or Devils?

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Celiac disease (CD) is a very common chronic condition in human beings, affecting approximately one in 100 individuals. It is an autoimmune disease with a defined environmental trigger, the gluten contained in dietary cereals, occurring in genetically susceptible individuals. The disease has a very strong HLA association. More than 90% of CD patients have HLA-DQ2, and almost all of the remaining celiac population possesses HLA-DQ8 molecules. Th17 cells seem to participate in the disease pathogenesis producing and secreting either proinflammatory or anti-inflammatory cytokines.

Keywords   celiac disease, IL-17A, IL-21, IL-22, mucosa, Th17

CELIAC DISEASE

Celiac disease (CD) is a very common chronic condition in human beings, affecting approximately one out of 100 individuals. It is an autoimmune disease with a defined environmental trigger, the gluten contained in dietary cereals, occurring in genetically susceptible individuals [1]. The disease has a very strong HLA association. More than 90% of CD patients have HLA-DQ2, and almost all of the remaining celiac population expresses HLA-DQ8 molecules [2, 3]. T cells present in celiac lesions recognize gluten epitopes presented by disease-associated HLA-DQ2 (DQA1∗05:01/DQB1∗02:01) or HLA-DQ8 (DQA1∗03/DQB1∗03:02) molecules [4, 5]. HLA-DQ2 and DQ8 are already used clinically to help exclude disease [6]. However, approximately 40% of the population carry these alleles and the majority never develop CD, indicating that although necessary they are not the only responsible elements for the development of the disease [7]. Also a number of non-MHC genes has been reported as susceptibility factors in coeliac disease [8, 9]. Environmental factors also include early life gluten exposure, short duration of breastfeeding, intestinal infections and changes in microbiota [10].

Celiac disease is characterized by small intestinal enteropathy with villous atrophy, crypt hyperplasia and increased numbers of infiltrating lymphocytes in the epithelium and lamina propria. CD4+ lymphocytes restricted to HLA-DDQ present in the

lamina propria recognize, preferentially, deamidated gluten peptides, a post-translational modification mediated by the enzymatic action of tissue transglutaminasa 2. In these peptides, some glutamine residues have been converted to glutamate. Such a modification favors this gluten peptides' interaction with HLA-DQ2 and HLA-DQ8 since these alleles show preference to bind negatively charged anchor residues   40
[11–13].

The pathogenic mechanisms leading to villous atrophy in CD remain not completely understood. Not long ago, CD was thought to be mediated by a skewed Th1 response because of the cytokines secretion profile detected in the intestinal mucosa, which induces CD8+ T lymphocytes to kill mucosal cells by a direct cytotoxic mech-   45
anism or by Fas-mediated apoptosis [14–17]. In the following years a more complex picture of the ongoing cytokine interplay in CD mucosa has emerged, supported by the findings of proinflammatory and down-regulatory cytokines produced by intestinal T cells from CD patients, as well as the presence of antigen-specific T regulatory cells (Tregs) in CD mucosa. In addition, over the last few years, it has become evident   50
that the involvement of cell populations, other than Th1 lymphocytes, may have significant influence in the pathogenesis of autoimmune responses, as it is in the case of the Th17 cell subpopulation.

Th17 CELLS

Th17 cells represent a new arm of the effector CD4+ lymphocytes recognized mainly,   55
though not only, by their ability to produce different forms of IL-17 (i.e. IL-17A, IL-17F) [18]. It seems that Th17 cells originate from a CD161+CD4+ T cell precursor [19]. Th17 cells display differential features from Th1 or Th2 subtypes of T lymphocytes. Although controversial data has been reported, it is generally accepted that these differences include the expression of IL-23 receptor (IL23R) as well as CCR6, a molecule also present   60
on the surface of Tregs [20]. Differences also include the transcription factors required for IL-17A production, RORC and IRF4 [21, 22] and the cytokines profile that comprises a variety of factors such as IL-21, which is also produced by Th2 cells [23], IL-22, a cytokine involved in early defense mechanisms against some bacterial pathogens in epithelial tissues [24, 25], and IL-26 [26]. Th17 lymphocytes also produce TNFα [27],   65
granulocyte-monocyte colony stimulating factor (GM-CSF) [28, 29] and CXCL8 [30]. Th17 cells also produce cytokines classically associated to the Th1 or Th2 subtypes of T lymphocytes such as IFNγ (Th17/Th1) [31] or IL-4 (Th17/Th2) [32], respectively.

Th17 CELLS IN CD

Th17 cells are present in active CD mucosa where they may participate in the on-   70
set and/or development of CD. The participation of Th17 cells in experimental and human autoimmune disorders has also widened the actors to be considered on the immune pathogenesis of CD. We shall review and discuss data regarding Th17 cells' contribution to the cellular and cytokine interactions within the CD small bowel context and counter-evidences of Th17 participation in CD. Controversy is served.   75

The presence of Th17 cell activity in the celiac mucosa was initially identified by detecting cytokine transcripts consistent with the presence of Th1 and Th17 cells in duodenal biopsies from CD patients [33]. In these studies, the expression of IL21, IFNγ, TGFβ1, IL6, IL17A, IL17F and IL23R was quantified by means of RT-PCR assays. For that purpose, comparison of intestinal samples from active CD patients and biopsies   80
taken after a long-term treatment with gluten free diet (GFD) was performed. Results showed up-regulation in the expression of gene characteristic of both Th1 and Th17 cell lineages as the disease progresses. This finding is consistent with the situation in

active inflammation sites of other autoimmune diseases, such as inflammatory bowel
disease or psoriasis. The authors found that the most pronounced changes are observed for IFNγ, IL12 and IL17A, but there was also an increase, though not statistically significant, in the expression of IL23R and IL6.

In a single nucleotide polymorphism (SNP) study aimed to identify CD susceptibility factors in a large cohort of CD patients, genes relevant for the Th17 immune response were genotyped. One hundred one SNPs, present in 16 Th17-related genes (IL23R, RORC, IL6R, IL17A, IL17F, CCR6, IL6, JAK2, TNFSF15, IL23R, IL22, STAT3, TBX21, SOCS3, IL12RB1 and IL17RA), were analyzed. Surprisingly no significant results emerged after performing the appropriate statistical corrections, therefore excluding any relevant role of Th17 cells-related factors on CD risk. However, in this study, data corresponding with well-known Th17-related genes such as IL-17F, IL-22 and RORC were eliminated from the study due to deviation from Hardy-Weinberg proportions. In addition, IL23R SNP initially found highly associated to CD risk [34] could not be confirmed in a replication set [35]. These results imply that the initial findings on the presence of Th17 cytokine signature in the small bowel of CD patients may not be related to the individual susceptibility to the disease or to its development. However, a recent study analyzed mRNA levels of the cytokine IL-17A, IFNγ, IL10 and TGFβ1, and the Treg marker FOXP3 in small intestinal biopsies of untreated CD (children on a gluten-containing diet with active disease), treated CD (children with CD on a gluten-free diet and with inactive disease) and controls (pediatric clinical controls with no known food intolerance). Levels of IL-17A and IFNγ were significantly higher in biopsies from patients with untreated CD compared to both treated CD and controls. Foxp3, IL-10 and TGFβ all showed a similar pattern with significantly higher levels in untreated than treated CD, but not in controls. Analysis comparing mRNA levels of the cytokines and Foxp3 in biopsies of the three studied groups revealed significant correlation between IL-17A and both IFNγ and Foxp3 in untreated CD and between Foxp3 and TGFβ in treated CD. In controls, there was no correlation between any of the cytokine mRNAs or between these and Foxp3 mRNA [36].

Interestingly, four hours *ex vivo* stimulation of CD small bowel biopsies with gliadin peptides as a surrogate of an acute re-exposure to gliadin showed a generalized increment in the expression (mRNA) of all cytokines except TGFβ in the active phase of the disease. The expression of IL-17F was undetectable in the majority of samples from CD patients at any stage [33].

Increased IL-17A protein levels in response to gliadin challenge were demonstrated one year late, under strictly controlled culture conditions in a more elaborated condition. Biopsy specimens from active CD patients were cultured in an organ culture dish in an atmosphere containing 95% O2/5% CO2 at 37°C and 1 bar pressure [37]. Indeed, in elegant *ex vivo* experiments, these authors showed that either single positive CD4+ cells or double positive CD4+CD8+T cells present in the biopsy specimens produced intracellular IL-17A in response to peptic-tryptic digest of gliadin. Additionally, they reported no response in the same experiments by T cells other than CD4+(including CD4+CD8+), indicating that gliadin-reactive CD4+ cells are an important source of IL-17A within the CD small bowel context. More important is the observation that CD3+ cells contained in duodenal explants from inactive CD individuals up-regulates the production of IL-17A upon stimulation with gliadin. Because IL-21 promotes Th17 cell development [38] and is expressed in excess in CD [39], some biopsy specimens were incubated in the same conditions with or without an anti-human IL-21 neutralizing monoclonal antibody. Results clearly demonstrated that blockade of IL-21 activity significantly reduced IL-17A transcript levels in organ culture of active CD duodenal explants [37]. Remarkably neutralizing IL-21 also inhibited IL-17A production by biopsy specimens from inactive CD stimulated with peptic-tryptic gliadin,

showing that gliadin induces IL-17A through an IL-21-dependent mechanism. A different approach was used by Di Sabatino et al. that explored the expression of CD40 and CD40L in the duodenal mucosa of celiac patients, and the effect of CD40L blockade on the production of proinflammatory cytokines such as IFNγ and IL-17 in duodenal explants. Indeed, explants stimulated with gliadin peptides *ex vivo* produced increased IFNγ and IL-17 when compared to the controls [40].

Despite the observations showing the ability of intestinal samples from untreated CD patients cultured under different conditions to respond to gliadin peptides by increasing the production of Th17-related cytokines, the gliadin specificity of such responses remained unclear as those experiments were carried out using organ cultures of whole biopsy specimens, and not purified cell types. Our group offered demonstration of the presence of gliadin-specific Th17 cells [41]. Gliadin-specific Th17 cells were obtained from CD distal duodenum explants after collagenase A digestion. T cells derived from explants (3–4) of each individual were stimulated with gliadin peptides in the presence of irradiated autologous PBMC for seven days in culture medium containing human serum. After CD8+ cells depletion, CCR6+ immunoselected cells were further expanded in the presence of gliadin and exogenous IL-23 added to the system during Th17 cells isolation and expansion procedures. Phenotype features of the IL-17+ cells from CD patients showed an effector/memory phenotype as well as the widespread surface expression of the Th17 subset defining molecules such as CCR6, IL23R and CD161. However, it is to be noted that the latest was found in approximately half of the IL-17+ cells from either CD and non-CD control individuals. This is not totally concordant with data reported by Monteleone's group where they found that within the CD161+ cells from CD patients, 40% expressed IL-17A whereas more than 90% of IL-17A+ cells lacked expression of CD161 [37]. This discrepancy likely indicates a different balance between memory- and effector-IL-17A producing cells within the IL-17A+ cell populations studied. In any instance, both reports differ from other studies supporting that CD161 is expressed by all Th17 memory cells as well as in CD8+ T cells and double negative CD4–CD8– T lymphocytes [19, 42, 43].

We have found that IL-17A producing cells from CD patients express two cytokines with dual, opposite effects: IL-22 and TGFβ. Both cytokines have anti-inflammatory and pro-inflammatory roles [44, 45]. The presence of IL-22 producing cells observed by us but not in IL-17A producing cells from other CD cohorts might suggest a different infectious biography or a direct effect of the microbiota in both cohorts of patients. This is an interesting issue that deserves attention as IL-22 plays a key role in the control of intestinal homeostasis [46] and the production of antimicrobial peptides cooperatively with IL-17A [47].

It is of interest that although IFNγ is not considered a Th17-related cytokine, all reports on IL-17A production by T cells in the celiac mucosa found concomitant production of IL-17 and IFNγ. This raises the question of whether or not IL-17A producing T cells in CD are "canonical" Th17 cells. It has been suggested that in the presence of IL-12, Th17 cells acquire the ability to secrete IFNγ. This, added to the well-known role of IL-21 in up-regulating and sustaining the expression of the T-bet transcription factor, might explain the reason why no canonical Th17 cells has been yet identified in the mucosa of untreated CD patients. Regarding the transcription factors involved in Th17-related cytokine expression, there is not much information available. We have shown that IL-17A-producing T cells displayed up-regulated expression of transcriptional factors RORC and Interferon-Regulatory Factor 4 (IRF4). IRF4 is a hematopoietic cell-specific transcription factor that regulates the maturation and differentiation of immune cells, and RORC transcription factors. Whereas the role of RORC in the differentiation of Th17 cells is well known, IRF4 participation in the development of IL-17A-producing cells is becoming increasingly clear. Either CD4+ or CD8+ (Tc17)

lymphocytes producing IL-17A has been shown to express IRF4, which is required in mucosal tissues to obtain DCs specialized in instructing IL-17 responses [48] in health
190   as well as [49, 50] in disease conditions [51, 52].

In our study, Th17 cells from CD patients but not from control individuals proliferate in response to gliadin challenge. Gliadin stimulation also results in an increased mRNA and protein level of IL-17A, IFNγ, IL-21, IL-22 and, strikingly, TGFβ. Neither IL-10 or IL-4 was detected within the IL-17A population in our patients. This data strongly
195   support that cells were gliadin-specific. This finding is nevertheless in contradiction with the observations that gluten-reactive T-cell lines from treated patients produced IL-21 but not IL-17 or IL-22 [53]. However, all patients in our study had never received any treatment, whereas Bodd et al. used long-term cell lines from treated patients. In addition, only a small percentage of cells contained in the cell lines derived from the
200   CD mucosa by Bodd et al. showed HLA DQ2-restricted gluten reactivity. Remarkably, the same authors detected CD4+ IL-17+ cells in peripheral fresh blood of CD patients after an oral gluten challenge. This finding is strongly suggestive of the existence of an antigen-driven boost of gliadin-specific cells in CD patients following gluten provocation. Unfortunately, whether these cells come from or goes to the gut was not investi-
205   gated. This points to the importance of the culture techniques used to isolate, purify and expand T cells from intestinal explants, since different experimental approaches may lead to deep discrepancies in the interpretation of the role of any T cell type in the pathogenesis of CD. A recent report, however, also failed to find increased numbers of Th17 cells in pediatric CD patients. Instead, they found elevated number of cells
210   producing IL-21 and IFNγ. These authors, however, reported increased representation of Th17 cells in the mucosa of a subgroup of adult CD patients. In a smart group of histopathological experiments, IL-21 producing cells were clearly established as different from cells producing IL-17. The authors attribute this finding to the expression of TLR2 in lesional tissues, a receptor known to trigger IL-17A secretion. Unfortunately,
215   no data regarding co-expression of IL-21 by IL-17A-producing cells was reported in this subgroup of patients [54].

OTHER CYTOKINES DETECTED IN Th17 CELLS IN CD MUCOSA

Other cytokines of the IL-17 family participate at some stages of the disease. While participation of IL-17A and IL-22 remains controversial, there is a unanimous perception
220   that, among Th17-related cytokines, IL-21 plays a relevant role in the pathogenesis of the CD [41, 55, 56]. This cytokine attracted attention since a first genome-wide study carried out in CD performed by Heel et al. [57] showed a strong association with a close region to the IL21 gene, which is located in human chromosome 4. IL-21 is a cytokine produced by activated CD4+ T cells, and NKT cells and neutrophils [58], and also by
225   CD8+ T cells under certain conditions of prolonged T lymphocyte stimulation, such as in some autoimmune or infectious conditions [27, 59]. Indeed, IL-21 is critical for CD8$^+$-T cell survival [60, 61] and memory generation [62] as well as for promoting the activity of CD8-T cell effectors during viral infections enhancing the cytotoxic response to virally infected cells by NK cells and CD8-T lymphocytes [63, 64]. This relation to
230   CD8+ cells is of importance to our debate, as both CD4+ and CD8+ play a role in CD development and tissue damage. Overproduction of IL-21 was observed in the intestinal mucosa of patient with active CD, its origin thought to be lamina propria unique CD4+ cells [39]. The main contribution to CD inflammatory response was shown to be the stimulation of IFNγ production by CD4+ lymphocytes. However, IL-21 also stim-
235   ulates the release of metalloproteases by enterocytes and fibroblast [65] in the intestine, contributes to recruitment of dendritic cells by inducing enterocyte production of CCL20 and acts synergistically with IL-15 in the modulation of intraepithelial CD8+

T cells upon gliadin challenge in CD mucosa. Studies from two independent groups have shown that IL-21 might be critical to confer resistance of CD4+ T lymphocytes to non-antigen specific Treg cells [27, 66]. It is also relevant regarding the function of IL-21 in CD pathogenesis that this cytokine acts directly on B cells to regulate Bcl-6 expression and germinal centers responses contributing to CD-associated autoantibodies production [67].

EMERGING TREATMENTS FOR CELIAC DISEASE

The recent progress in unraveling the pathogenic mechanisms underlying CD allows us to envision imaginative treatments for the disease. As this is a condition triggered by gluten intake, a gluten-free diet is effective in most patients and the only recommended treatment that has demonstrated long-term benefits [68]. Unfortunately, around 5% of patients become refractory to the treatment and remain symptomatic even after strict adherence to a gluten-free diet [69]. Resistant patients have been classified into type I and type II refractory CD based on clinical and histological differences, but it has been speculated that these two types may represent evolved stages of the disease [70]. Despite a significant improvement in the availability of processed gluten-free foods, some compliant patients considered as type I refractory are not resistant but rather affected by trace contamination [71], a fact that limits but does not exclude intrinsically resistant patients. The need for more effective treatments, therefore, arises not only from the presence of resistant patients but also from the long-term morbidity observed in compliant celiac patients, who show a high rate of gastrointestinal symptoms [72] and nutritional deficiencies [73].

In addition to this, and as we discuss in this work, the development of CD is clearly favored by an abnormal cytokine balance in a way that is common to other autoimmune disorders [6, 74, 75]. The role of high levels of IFNα in promoting excessive Th1 responses in the small intestine of celiac patients was discovered long ago [76]. More recently we became aware of the role of those cytokines in modulating the production of IL-17 such as IL-15 and IL-21, potent proinflammatory cytokines [77]. A transgenic mouse model that overexpresses IL-15 in the gut lamina propria to levels comparable to those found in celiac patients reveals that this cytokine plays a relevant role in the overproduction of IL12p70 and IL23 [78]. This up-regulation of Th1 responses is a co-adjuvant effect with retinoic acid, a vitamin A metabolite involved in the induction of intestinal responses [79]. Furthermore, IL-15 triggers an anti-apoptotic pathway in human intraepithelial lymphocytes depending from Bcl-2 and/or Bcl-XL [80], which could explain the prolonged survival of this cellular subpopulation in celiac patients. The damage caused to the epithelia by the overproduction of IL-15 may be further enhanced by the fact that this cytokine interferes with the suppressive activity of intestinal Tregs [81]. Therefore, although Tregs are induced *in situ* by gliadin, their suppressive capacity may be impaired *in vivo* and therefore this could represent another important factor contributing to the development of the disease. And finally, IL-15 positively regulates the production of IL-21 in the celiac intestinal mucosa [55], a relevant finding since it is known that this cytokine amplifies Th1 [82] and Th17 responses [38]. The pleiotropic downstream effects of the IL-15/IL-21/lL-17 network make it an attractive candidate for the development of new immunotherapeutic targets in CD. Blockade of any of these cytokines may result in the restoration of homeostasis in celiac intestinal mucosa as Th1 and Th17 responses would be down-modulated and the suppressive capacity of gliadin-specific Tregs restored.

Finally, there is a huge territory to be discovered regarding the role of Th17 cytokines in small bowel inflammatory processes. Like angels and devils, pro- and anti-inflammatory effects of these cytokines play the delicate game of balance in the

mucosa, with projections into the territories of disease. The description of distinct sub-
types of Th17 cells, such as natural or inducible Th17 cells, memory Th17 cells either
290   central or peripheral, and further division as pathogenic or non-pathogenic accord-
ing to IL-23 receptor expression, is relatively novel and their activities in disease still
undefined [83]. The plasticity of Th17 cells makes them highly unstable during *ex vivo*
isolation and experimental procedures. Their ability to change in response to envi-
ronmental variations enable Th17 cells to modify their functional abilities, such as the
295   cytokine they produce and the phenotype modifications that may be required for an
optimal intervention against pathogens or to maintain or recover tissue homeostasis.
Th17 cells and their cytokines represent an impressive lesson on the biological and
medical implications of T-cell plasticity in health and disease and provide new con-
cepts and tools to look for more adequate targets to hit in the endless ways of transla-
300   tional knowledge into patients' therapy. The magnitude of this task is only comparable
to the fascination that this "a-million-faces" cell provokes in most of us.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the
content and writing of the article.   **AQ2**

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g. Informe Bibliográfico

1.- *"A Novel Phenotype Variant of Severe Congenital Neutropenia   Caused by G6PC3 Deficiency"*

En este articulo se describe el primer caso de una forma de neutropenia congénita severa (SCN), designada como Tipo 4, que cursa SIN la concurrencia de malformaciones cardiacas. Hasta el momento, el numero de pacientes detectados en todo el mundo con esta enfermedad, debida a mutaciones en el gen que codifica la tercera unidad (C3) de la glucosa 6 fosfatasa en los neutrófilos, no llega al centenar y en todos ellos se ha detectado la presencia de defectos interventriculares cardíacos que requieren cirugía correctora. La consecuencia de mayor alcance científico derivada de nuestro artículo es el hecho de que no parece existir relación entre el gen mutado y la morfogénesis cardiaca humana, como podría inferirse de la asociación, hasta ahora unívoca, de las malformaciones cardiacas y la mutación en dicho gen en el resto de pacientes diagnosticados de déficit de G6PC3, aportando así nueva información a la patogenia de esta forma de neutropenia congénita severa. La información bibliométrica se describe a continuación:

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Revista: *Pediatric Blood and Cancer*
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Categoría	Total de revistas	Lugar	Cuartíl
Pediatría	**117**	**21**	**1er cuartil**

2. "Interleukin-21 Overexpression Dominates T Cell Response to Epstein-Barr Virus in a Fatal Case of X-Linked Lymphoproliferative Syndrome Type 1"

La inmunodeficiencia primaria humana conocida como síndrome linfoproliferativo ligado a X (XLP) representa una enfermedad rara cuyas consecuencias en más del 93% de los casos es el exitus del paciente en un plazo que no supera los 30 días desde el inicio de las manifestaciones clínicas. Se sabe en la actualidad que el defecto causante del XLP tipo 1 (XLP-1) es la mutación del gen que codifica la denominada proteína asociada (SAP) a la molécula de señalización de activación linfocítica (SLAM). La consecuencia de la perdida de esta proteína o su función es la incapacidad de los linfocitos T citotóxicos y de las células NK para destruir células infectadas por virus mediante mecanismos de citotoxicidad. Sin embargo, se desconocía hasta ahora el mediador responsable de la proliferación linfoide que tiene lugar durante la destrucción aguda de los órganos diana, que son habitualmente hígado y cerebro. Nosotros aportamos en este papel la primera evidencia de que es la IL-21 el mediador responsable de la linfoproliferación. Este hecho abre nuevas perspectivas terapéuticas en esta enfermedad en la que solo un diagnóstico e intervención muy rápidos puede ayudar al paciente. Las referencias bibliométricas se indican a continuación.

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Microbiología	**119**	**63**	**3er cuartil**

3. "A Novel IL2RG Mutation Presenting With Atypical T-B+NK+ Phenotype: Rapid Elucidation of NK Cell Origin"

En este trabajo presentamos datos originales en las que una nueva mutación en el gen que codifica la cadena gamma común del receptor de IL-2 (IL-2RG) que además aparece *de novo* en la madre del paciente que presenta células NK en sangre periférica, lo que constituye una forma paradójica de presentación de esta inmunodeficiencia humana (XSCID). Un abordaje original e informativo nos ha permitido dilucidar con suma rapidez el origen materno de dichas células, permitiéndonos así evitar laboriosos análisis moleculares y facilitando el diagnostico de certeza, lo que a su vez nos ha permitido establecer la terapia apropiada para este paciente. La información bibliométrica se muestra a continuación:

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Categoría	Total de revistas	Lugar	Cuartíl
Pediatría	117	21	1er cuartil