El microambiente y la autoinmunidad en la leucemia linfática crónica

Gerardo Ferrer Aguilar

Tesis Doctoral

Facultad de Medicina

Programa de Doctorado

Medicina

El microambiente y la autoinmunidad en la leucemia linfática crónica

Directores de Tesis

Dr. Emili Montserrat Costa

Dra. Carol Moreno Atanasio

Gerardo Ferrer Aguilar

Barcelona, Julio 2012

A mis padres, mis hermanos

y Mari Carmen

Agradecimientos

Quiero mostrar mi más sincero agradecimiento a todas las personas que me han acompañado y orientado en la elaboración de esta tesis:

En primer lugar a mis dos directores de tesis, el Profesor Emili Montserrat y la Dra. Carol Moreno que me han transmitido su entusiasmo por el estudio de una enfermedad tan compleja clínica y biológicamente como la LLC. De ellos he aprendido la importancia de los detalles, que siempre se puede mejorar, que los datos no son en sí mismos resultados, que la investigación debe partir siempre de una pregunta clara y pertinente ("si la pregunta no es buena, la respuesta es irrelevante"). Siempre han estado a mi lado. Para la Dra Carol Moreno siempre ha existido un experimento más a hacer, algo que perfeccionar, algún detalle más a pulir. El Profesor Emili Montserrat me ha enseñado a "leer" e "interpretar" artículos ajenos y hallazgos propios, en ocasiones de forma sorprendentemente "nueva", y jamás podré olvidar su ejemplo y enseñanzas por lo que hace a la claridad y precisión en identificar los problemas, analizar los resultados de los trabajos y exponerlos con claridad.

A Kate Hodgson y Alfons Navarro con los que he pasado muchísimas horas en el laboratorio. Kate es una persona maravillosa que siempre ha estado dispuesta a ayudarme, explicarme lo que fuese necesario, cariñosamente crítica y de la que he aprendido mucho. Por supuesto, Kate, nunca olvidaré ni tu risa ni tus postres. Alfons, ha sido genial trabajar con alguien con tantas inquietudes y ganas de probar cosas nuevas. Además de haber encontrado un buen amigo.

Al profesor Marià Monzó quiero agradecerle el haberme permitido trabajar en su laboratorio, su generosidad incondicional y el haberme hecho sentir como uno más de

su grupo. De él he aprendido muchas cosas, sobre todo que la modestia y el trato llano no son incompatibles, antes al contrario, con una investigación seria y rigurosa.

Durante los mis primeros meses en el laboratorio Sonia, Aina y Silvia me enseñasteis y me ayudasteis en todo. Con el tiempo hemos forjado una bonita amistad y colaborar sin reservas en nuestros respectivos trabajos.

Estos años no habrían pasado tan rápido sin la compañía de Tania, Rut y Marina, que me han cuidado, echado broncas por mi desorden (tu no Marina, tu mesa esta peor que la mía!), trabajado codo con codo y reído mucho. Con vosotras, mis dos compañeras de mesa primero, Tania y ahora Rut he compartido también cientos de canciones.

En el laboratorio también he trabajado con investigadores y personas maravillosas como Berni, Rosa, Carme y Renée, de las que he aprendido mucho. Gracias también a los recién llegados, a los estudiantes Marc y Anna que me han ayudado a ver con otra perspectiva la evolución que he hecho desde que empecé hasta este momento.

A Carme Torres, siempre amable y disponible, por sus magníficas dotes de gestión.

A otros grupos de investigación y servicios del IDIBAPS. El grupo de inmunoreceptores, malaria, citometría (mi segunda casa), entre otros varios.

Esta investigación no habría sido posible sin muestras biológicas y sin la información con respecto a los pacientes. Por ello quiero agradecer especialmente a Tycho Baumman, Dolors Colomer, Marta Aymerich, Montse Elena y Gael Roué, así como a todas las personas que trabajan con ellos, toda su ayuda. Y también, de forma

muy especial, al Dr Arturo Pereira por su apoyo incondicional, consejo, y tener siempre la puerta abierta para discutir nuestras dudas estadísticas.

Finalmente a todas aquellas que han estado junto a mí: mis amigos, Bet y especialmente mis padres que siempre me han apoyado en mis estudios y proyectos.

GRACIAS

Financiación: Becas y ayudas

Los trabajos de investigación que configuran esta tesis han sido en parte financiados gracias a diversas becas y ayudas:

- Red Temàtica de Investigación Cooperativa en Cáncer beca RT 06/0020/002051 del Ministerio de Educación y Ciencia.
- Instituto Carlos III FISS PI080304 y PI040698.
- Generalitat de Catalunya 2009SGR1008.
- CLL Global Foundation (USA).

Durante este periodo también he percibido becas personales:

- Beca IDIBAPS.
- Beca PFIS del Instituto de Salud Carlos III.

Índice

Página

Introducción

Leucemia linfática crónica

Definición

La leucemia linfática crónica (LLC) es un síndrome linfoproliferativo que se caracteriza por la acumulación progresiva de células linfoides B monoclonales con un inmunofenotipo característico (CD5+, CD19+, CD20 ^{débil}, CD23+, SmIg^{débil}) en sangre periférica, medula ósea y tejidos linfáticos [1, 2]. En la clasificación de la Organización Mundial de la Salud, la LLC y el linfoma linfocítico de células pequeñas se consideran la misma enfermedad, que se diferencian únicamente por el lugar donde este trastorno se manifiesta de forma fundamental: sangre (LLC) o ganglios linfáticos (linfoma linfocítico de células pequeñas) [2].

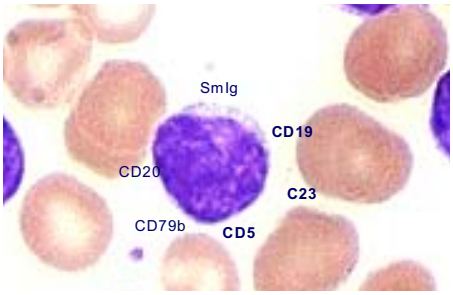


Figura 1. Marcadores característicos del linfocito de la LLC

Epidemiología

La LLC es la leucemia más frecuente en adultos en los países occidentales. La incidencia global es de 2-6 casos/100.000 habitantes y año, aumentando de forma considerable con la edad. Así, la incidencia en mayores de 70 años es de casi 40/100.000 habitantes y año. La mediana de edad al diagnóstico es de 72 años, pero el porcentaje de pacientes diagnosticados cuando todavía son jóvenes ha aumento en la última década, de forma que un tercio de ellos tiene menos de 55 años. La distribución por sexos es desigual (hombres 1,5-2/mujeres 1)[2].

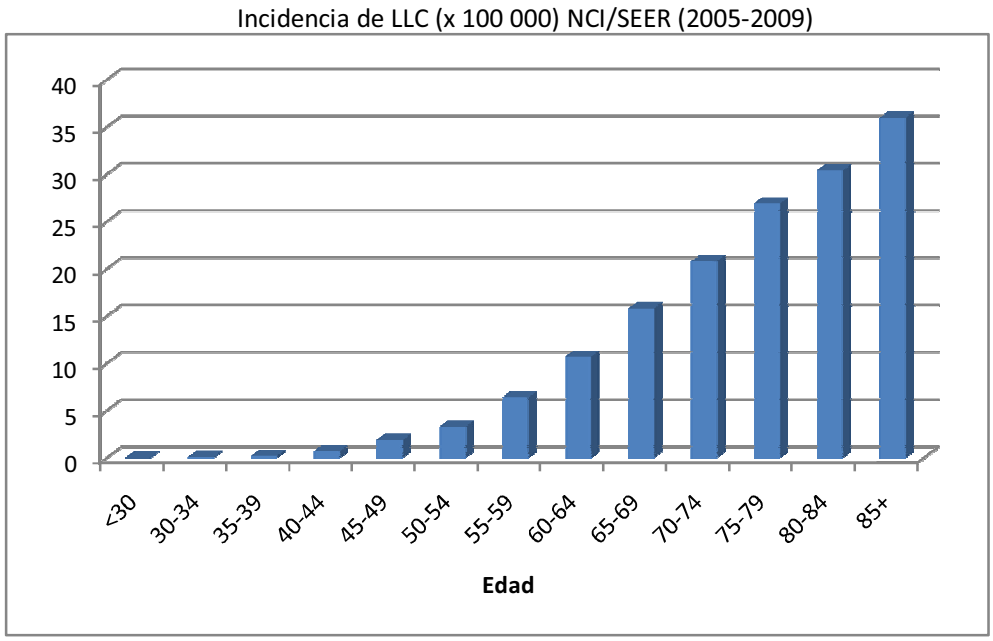


Figura 2. Distribución de la incidencia de la LLC en los distintos grupos de edad

La frecuencia de la LLC varía según los países y raza. Así, en los países occidentales representa el 20-40% de todas las leucemias, mientras que en los orientales la LLC representa únicamente el 5-10% de todas las leucemias. Cabe destacar que en personas de origen oriental emigradas a países occidentales y sus familiares la incidencia de LLC no se ha visto incrementada sino que se ha mantenido baja [3, 4].

Por otra parte, existen familias en las que varios miembros se hallan afectos de LLC. Los parientes de primer grado de pacientes con esta forma de leucemia presentan un riesgo relativo 8.5 veces superior de padecer LLC que la población general [5]. En los casos de LLC familiar, en los miembros de la segunda generación la enfermedad suele aparecer de 10 a 20 años antes (fenómeno de "anticipación") [6].

Un 13-18% de los familiares en primer grado de pacientes con LLC presentan un cuadro conocido como linfocitosis B monoclonal (LBM) de características fenotípicas semejantes a la LLC pero en el que la cifra de linfocitos monoclonales en sangre periférica es inferior a 5.000 por microlitro (ver Diagnóstico; página 21) [7, 8]. La LBM también puede encontrarse en un 3-5% de la población general, sobre todo en personas de edad avanzada [9,

10]. La transición de LBM a LLC es sumamente rara de tal forma que sólo ocurre en un 1-2% de sujetos con LBM al año [11, 12].

Etiología

La causa o causas que originan la LLC son desconocidas. A diferencia de lo que ocurre con otras leucemias, no se ha podido demostrar una relación convincente entre la exposición a radiaciones ionizantes u otros agentes citotóxicos y esta enfermedad. Los casos familiares, la dispar incidencia de la LLC según los países y etnias, así como el hecho de que la incidencia de LLC en personas origen asiático que han emigrado a países occidentales, como por ejemplo USA, se mantenga baja hablan a favor de una base genética en la LLC [4, 5, 9, 13-15].

La célula que origina la LLC es controvertida. El inmunofenotipo de las células neoplásicas se asemeja al de las células B de la zona marginal de los folículos linfoides y es por ello que se cree que esta enfermedad podría tener su origen en células de dicha zona. Sin embargo, se ha sugerido que en el origen de la LLC podría jugar un papel fundamental las células madre hematopoyéticas de la LLC (CMH-LLC) [16] (Figura 3). La CMH-LLC produciría, en primer lugar, un gran número de células B policlonales; seguidamente, los clones de células B se seleccionarían y expandirían dando lugar a un tipo de LBM. A continuación, diversas alteraciones genéticas –posiblemente la deleción del miR-15 y miR-16 en el cromosoma 13q14 – darían lugar a la transformación de algunos clones de LBM en LLC. [17-19].

En el caso de las LLC familiares se ha identificado por medio de SNP *arrays* en algunos locus (p. ej. 16q24.1 and 6q21.3) que podrían estar asociados con un mayor riesgo de padecer LLC [9, 15]. Además, esta personas presentan con mayor frecuencia un polimorfismo en la región promotora del gen B *cell activating factor of the TNF family* (BAFF), que promueve un aumento de los niveles de esta proteína antiapoptótica y que juega un importante papel en el

desarrollo de LLC [20-22]. En modelos murinos, el aumento de esta molécula se ha visto asociado con la aparición de un síndrome linfoproliferativo similar a la LLC [23, 24].

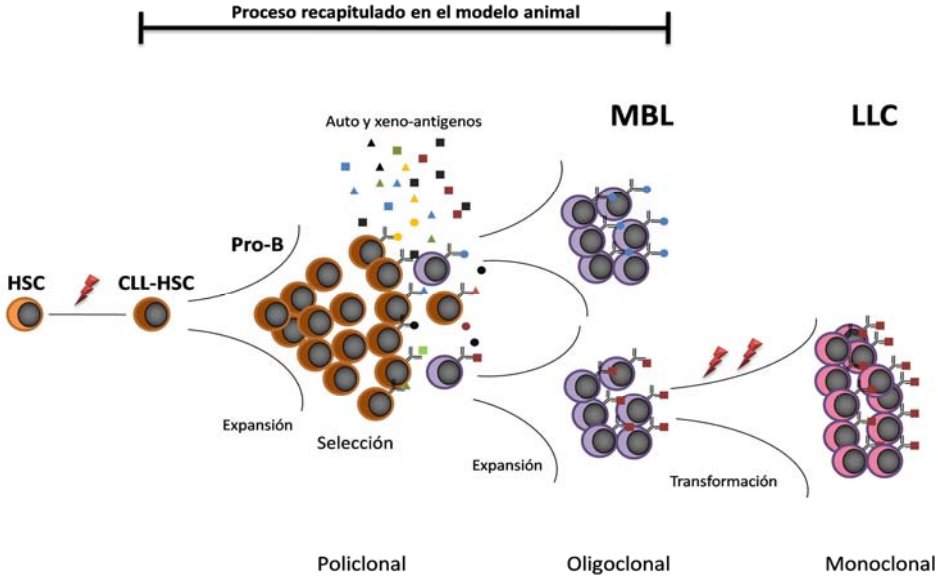


Figura 3 Representación esquemática del desarrollo de la LLC humana a partir del modelo animal. Modificado de Kikushige, Y. Cancer Cell 20(2): 246-59; 2011.

Aunque la mayor parte de las células de LLC se encuentran en las fases G0-G1 del ciclo celular, una fracción de las mismas se multiplica activamente [25]. Las células neoplásicas de la LLC se caracterizan por presentar niveles elevados de proteínas antiapoptóticas como BCL2 y MCL1, mientras que la proteína pro-apoptótica BCLX está disminuida. El predominio de las proteínas anti-apoptóticas sobre las apoptóticas, así como la interacción de las células neoplásicas con las células del microambiente (p. ej. células T, *nurse like*, estromales) (ver Biología; página 24) promueven la acumulación de las células leucémicas en el organismo. Finalmente, los genes *IGHV* pueden estar mutados o no [26, 27] (ver Biología; página 24). La mutaciones somáticas de estos genes tienen lugar en los centros germinales, por lo que se considera que la LLC puede ser una enfermedad de origen pre- o post germinal.

Diagnóstico

En más de la mitad de los pacientes, la enfermedad se descubre de forma casual al realizar una analítica por cualquier motivo, sin que exista sospecha alguna de LLC. En los casos sintomáticos, el cansancio, la aparición de adenopatías o las infecciones de repetición son los síntomas que más a menudo conducen al diagnóstico [28].

El diagnóstico de certeza de LLC requiere la presencia de más de 5.000 linfocitos B monoclonales por microlitro con un inmunofenotipo característico (CD5+, CD19+, CD20-/+, CD23-) y que persista al menos 3 meses. La clonalidad debe ser confirmada por citometría de flujo [2, 29].

En las extensiones de sangre periférica se observan linfocitos de tamaño pequeño, con escaso citoplasma y un núcleo con cromatina condensada y un nucléolo apenas visible. En las extensiones de sangre periférica es frecuente observar sombras nucleares de Gumprecht (o células "fantasma"), que no son más que células linfoides rotas, debido a su fragilidad, al preparar las extensiones de sangre periférica.

Inmunofenotípicamente, las células de LLC coexpresan el marcador CD5 y marcadores de células B (CD19, CD20, y CD23). Los niveles de las inmunoglobulinas (Ig) y el CD20 son bajos en comparación con las células B normales, y cada clon expresa una sola cadena ligera de las inmunoglobulinas, kappa o lambda.

El aspirado medular y la biopsia no son necesarios para establecer el diagnóstico. Sin embargo, pueden ser necesarios para averiguar el origen de las citopenias (fallo medular *vs.* inmune o mecanismos periféricos). En la biopsia de médula ósea se pueden observar cuatro tipos de infiltración: nodular, intersticial, mixto y difuso. (Figura 4) [1].

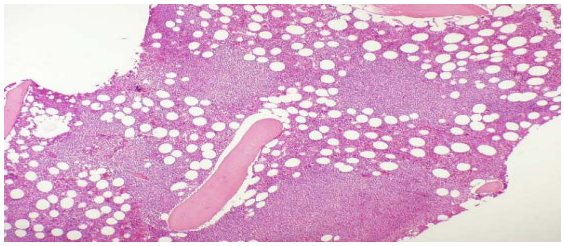


Figura 4. Patrón de infiltración de médula ósea por LLC. Esta imagen muestra un patrón de infiltración mixto con afectación nodular e intersticial de la médula ósea, característico de LLC.

Aunque normalmente el diagnóstico no presenta dificultades, a veces puede plantearse el diagnóstico diferencial con linfomas no hodgkinianos con expresión leucémica, particularmente el linfoma de células del manto, el linfoma linfoplasmocitoide y el linfoma de zona marginal. En la sangre periférica de los pacientes de LLC se puede encontrar un porcentaje bajo (10-20%) de linfocitos atípicos (p. ej. prolinfocitos, células linfoplasmocitoides o centrocitos), pero el diagnóstico de LLC "atípica" no puede hacerse sin descartar antes un linfoma con expresión leucémica. Una proporción igual o superior al 55% de prolinfocitos en sangre es propia de la leucemia prolinfocítica, enfermedad que en la mayoría de casos corresponde a un linfoma del manto. El inmunofenotipado de las células leucémicas es imprescindible para el diagnóstico. El estudio de la medula ósea, ganglios linfáticos, así como diversas pruebas genéticas y moleculares pueden ser de gran ayuda en los casos de diagnóstico difícil. (Tabla 1)

Tabla 1. LLC y síndromes linfoproliferativos B crónicos: Características inmunofenotípicas, genéticas y moleculares

	IG	CD20	CD5	CD10	CD23	CD11C	CD25	CD103	OTROS
Leucemia linfática crónica	-/+	-/+	+	-	+	-/+	-	-	CD19(+), FMC7(–)
Leucemia prolinfocítica	+	+	-/+	-/+	-/+	-	+	-	FMC7(+)
Tricoleucemia	+	+	-	-	-	+	+	+	Anexina A1 (+), DBA44(+), TRAP, BRAFF (V600E) mutado
Linfoma linfoplasmocitoide	+	+	-	-	-		+/-	-	Ig citoplasmática (+)
Linfoma de la zona marginal esplénico	+	+	-/+	-	-/+	-/+	-/+	-	Bcl-2(+); del(7q32)
Linfoma de células del manto	+	+	+	-	-	-	-/+	-	t(11;14)(q13;q32); ciclina D1(+); SOX11 (+)
Linfoma folicular	+	+	-	+/-	-/+	-	+	-	t(14;18); Bcl-2 +

Complicaciones de la LLC

Entre las complicaciones que los pacientes con LLC pueden presentar, son de destacar las infecciones, las alteraciones autoinmunes, la transformación a linfoma agresivo y las segundas neoplasias. Al ser la autoinmunidad uno de los puntos clave de esta tesis, los aspectos autoinmunes de la LLC se discuten por separado en el apartado "Autoinmunidad en la LLC" (página 35)

Con respecto a las infecciones, es preciso destacar que la hipogammaglobulinemia, alteraciones de la inmunidad celular [30] y el propio tratamiento de la enfermedad, facilitan la aparición de infecciones que son la principal causa de mortalidad [31, 32]. Los agentes terapéuticos con efecto inmunosupresor aumentan el riesgo de infecciones oportunistas (p. ej., CMV, pneumocystis, toxoplasma, micobacterias).

La LLC puede transformarse en otros síndromes linfoproliferativos de carácter agresivo [33-35]. Así, en un 3-10% de casos la enfermedad se asiste a la aparición de un linfoma de células grandes (síndrome de Richter), que suele ponerse de relieve en forma de empeoramiento del estado general, fiebre, aumento del tamaño de los ganglios linfáticos o del bazo, incremento del lactato deshidrogenasa (LDH) sérica o, menos frecuentemente, hipercalcemia o aparición de un componente monoclonal. También pueden observarse transformaciones a linfoma de Hodgkin, en cuyo caso suele estar presente el virus de Epstein-Barr (VEB), y a leucemia prolinfocítica. La transformación linfomatosa puede tener su origen en células de la propia LLC o en células no relacionadas con la leucemia y su pronóstico es ominoso.

Alrededor de un 10-15% de pacientes con LLC presentan segundas neoplasias, una incidencia más alta que la observada en la población general [36-38]. Su aparición se puede producir de forma previa, simultánea o después del diagnóstico. Los tipos más frecuentes son melanoma, cáncer de pulmón, linfoma, sarcoma de Kaposi, tumores del sistema nervioso central y tubo digestivo. Los pacientes con LLC tienen un riesgo superior de desarrollar un

tumor cutáneo poco frecuente conocido por el nombre de carcinoma de células de Merkel, y viceversa [39].

Biología

Hace tan solo unas décadas lo único que se sabía sobre la LLC era lo que se podía observar por medio del microscopio, es decir que la enfermedad se caracterizaba por la presencia en sangre periférica de una gran cantidad de células linfoides pequeñas, con una morfología característica. Aparte de ello y de que en medula ósea y tejidos linfoides se observaban células de características similares a las presentes en sangre, poca cosa más se sabía de esta enfermedad. En los últimos años, gracias sobre todo a los progresos en la tecnología disponible para estudiar la biología del cáncer, se han realizado importantes avances en el conocimiento de la LLC.

Receptor de células B

Los genes de la región variable (V), diversidad (D) y de unión (J) codifican para la región variable de la cadena pesada de las Ig (*IGHV*) (Figura 5) que constituye, junto con la porción variable de la cadena ligera de las Ig, el sitio de unión al antígeno en el receptor de células B (RCB). En aproximadamente un 10% de casos se observa el uso de determinados genes V y combinaciones preferentes con genes específicos D y J. En estos casos, la estructura de los RCBs y, por tanto, el sitio de unión al antígeno son prácticamente idénticos [40, 41]. Este hecho es llamativo puesto que, dada la diversidad y el gran número de combinaciones posibles de los genes V, no cabría esperar encontrar dos casos con una estructura del RCB similar en más de un millón de pacientes de LLC. A este fenómeno se le conoce como estereotipo del RCB [42].

Las mutaciones de los genes *IGVH* se detectan por secuenciación, de forma que una secuencia que difiera en un 2% o más de la línea germinal se considera como mutada. De acuerdo con este criterio, la LLC se divide en dos grupos: aquellos casos cuyas células leucémicas presentan un 2% o más de mutaciones en los genes *IGVH* (LLC “mutada”) y los que

no presentan mutaciones o en los que éstas son muy escasas (< 2%) (LLC "no mutada"). Estos pacientes presentan una evolución de la enfermedad totalmente distinta [26] (ver Pronóstico página 29).

Las señales producidas por la unión del antígeno al RCB son distintas en los diferentes subgrupos de LLC. Así, en la LLC "mutada" las células B tienen menor capacidad de apoptosis, supervivencia o proliferación. Ello parece deberse a la incapacidad de unión del antígeno debido a cambios en la conformación del receptor causados por las mutaciones de los genes IGHV o a defectos en las señales de transducción mediadas por el RCB. La expresión de la proteína ZAP-70 suele asociarse a los casos no mutados y facilita una transducción de señales a través del RCB más potente, con la consiguiente mayor actividad celular, proliferación y adquisición de lesiones genéticas. [43-46].

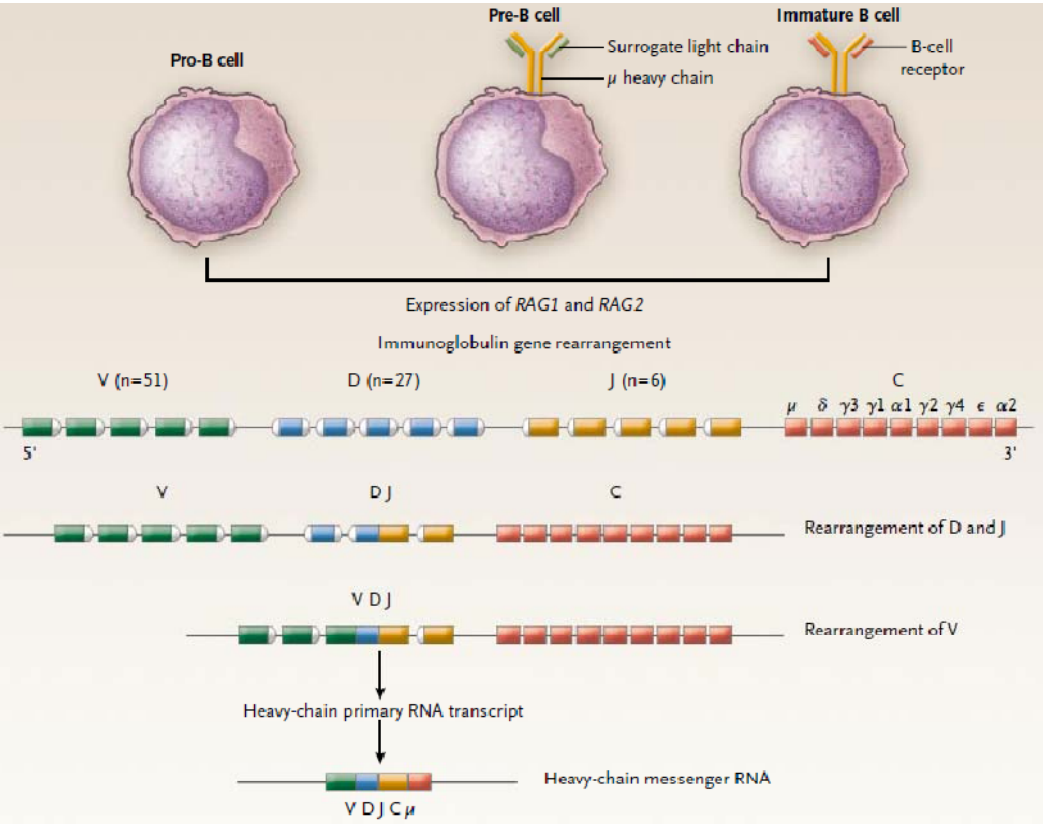


Figura 5. Esquema del reordenamiento de los genes de las *IGHV* (Chiorazzi, N. The New England Journal of Medicine 352 (8): 804-15; 2005).

Alteraciones genéticas

Citogenética y mutaciones

Un 80% de pacientes de LLC presentan alteraciones citogenéticas detectables por la técnica FISH. Las alteraciones más comunes son las deleciones en 13q (14-60% de los casos), 11q (10-32%), 17p (3-27%), 6q (2-9%) y trisomía del cromosoma 12 (11-18%). La deleción 13q14 se considera un fenómeno primario en la patogenia de la LLC [47, 48]. La deleción del 17p provoca la disfunción del gen *TP53*, pero este gen también esta mutado en un 1-4% de los pacientes con ausencia de la deleción. Así mismo, la deleción y mutaciones en la región 11q promueven la perdida de función del *ATM.* En ocasiones se puede observar la translocación (14;19)(q32;q13) donde se encuentran el gen de las *Igs* y el locus del BCL3.[49-53].

Aplicando técnicas de resecuenciación del genoma, se han identificado mutaciones de genes como *NOTCH1, SF3B1, BIRC3* y *MYD88* que parecen correlacionarse con progresión de la enfermedad y resistencia al tratamiento [54-57]. Estas observaciones, sin embargo, requieren ser confirmadas en series estudiadas de forma prospectiva y mediante análisis estadísticos rigurosos (p. ej., análisis multivariados basados en la información individualizada de cada estudio). Otra cuestión de gran importancia radica en la posibilidad de que determinadas alteraciones moleculares afecten a diferentes subclonas celulares.

Estado de los telómeros

Los telómeros son los extremos de los cromosomas, y están formados por DNA repetitivo y su función es mantener la estabilidad cromosómica. Los telómeros se erosionan con la proliferación celular lo que provoca la senescencia de las células [58]. En células normales dicha erosión está compensada con una transcriptasa conocida como telomerasa [59, 60]. Aunque en la LLC el índice proliferativo de las células es muy bajo, los telómeros son anormalmente cortos [61].

Epigenética

Las modificaciones epigenéticas son alteraciones cromosómicas no hereditarias que afectan a la transcripción de los genes. En la LLC entre el 2 y el 8% de las islas CpG están metiladas de forma aberrante, lo que se asocia a una expresión génica peculiar [62]. Un ejemplo de ello es la *death-associate protein Kinase 1* cuya expresión está silenciada por la metilación de la región promotora de las células de LLC [63]. Las metilaciones pueden tener un papel clave en el mal pronóstico que comportan diversos biomarcadores (p. ej., mutaciones de genes IGHV o TP53) [64].

Expresión génica

microRNAs

Los microRNAs son RNAs no codificantes que actúan como represores de la traducción de RNA a proteína. La relación de los microRNAs con el cáncer se describió en primer lugar en LLC donde en la región 13q14.3 delecionada se halló un gen no transcrito y dos genes microRNAs (miR-15a y miR-16-1) (Figura 6) [17] Así mismo se conoce que miR-16-1, miR-29, miR-181 y miR-34a regulan la expresión de genes tan importantes como BCL-2, MCL-1, TCL-1, CDK-2 y p53 [65-69].

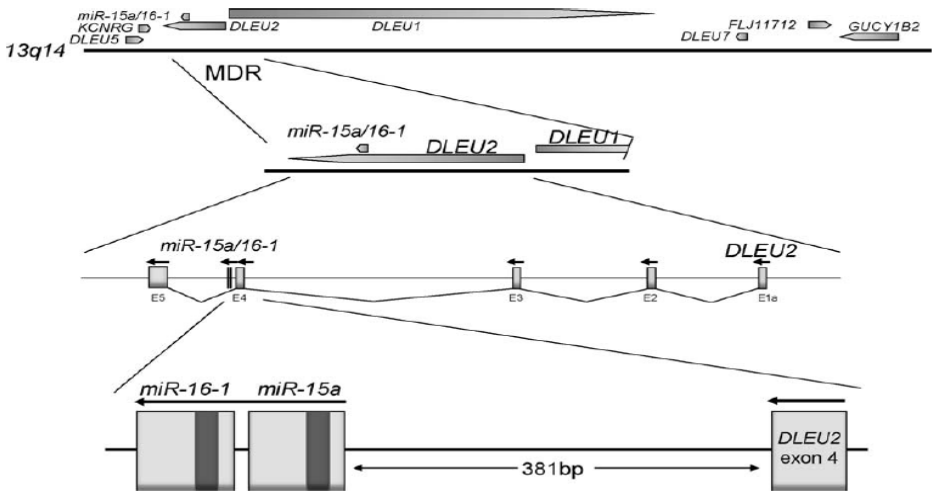


Figura 6. Representación esquemática de la región 13q14, comúnmente delecionada en las células de LLC que contiene dos mircroRNAs: miR-16-1 y miR-15a (Klein, U; Seminars in Cancer Biology. 20 (6): 377-83; 2010)

Cinética de las células de LLC

La visión convencional es que la LLC es una enfermedad que se caracteriza por defectos en la muerte celular programada, o apoptosis, y la consiguiente acumulación de células neoplásicas en el organismo. Este concepto se basa en que la gran mayoría (>98%) de las células de LLC circulantes se encuentran en estado G0 o G1 del ciclo celular. Sin embargo, un gran número de células de LLC expresan marcadores de activación, tales como el CD38, CD69 y CD49d. En una serie de importantes estudios se demostró, mediante el empleo de deuterio, que tasa de células neoplásicas nuevas que se producen cada día equivale al 0,1-1% de toda la clona de LLC. Por tanto, en la LLC además del componente "acumulativo" existe también un importante factor "proliferativo" [25].

Microambiente

A lo largo de la última década, se ha puesto de relieve el importante papel del microambiente tumoral en la supervivencia y proliferación de las células neoplásicas. El microambiente tumoral de la LLC se halla en la medula ósea y ganglios linfáticos y consiste en una mezcla de células inmunitarias, estromales y vasculares que crean un nicho en el que se evita que las células neoplásicas entren en apoptosis y, por el contario, proliferen (Figura 7).

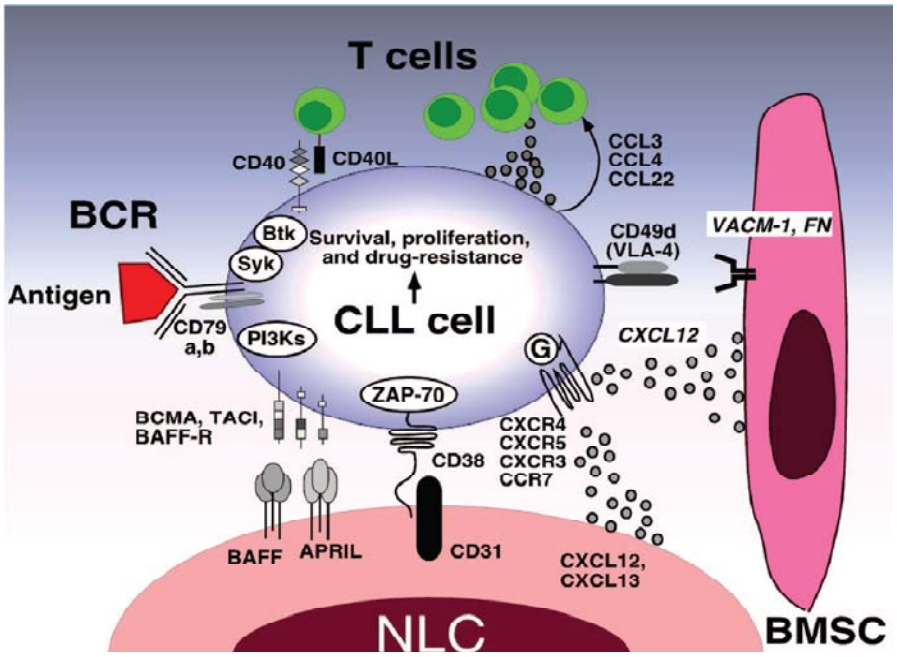


Figura 7. Interacciones moleculares entre las células de LLC y células estromales y del microambiente en la médula ósea y/o tejidos linfáticos, que se consideran relevantes para la supervivencia y proliferación de las células de LLC. Las células *nurse like* expresan la quimoquinas CXCL12 y CXCL13, mientras que las de la médulas ósea expresan principalmente el CXCL12. Las integrinas (CD49d), cooperan con los receptores de quimioquinas estableciendo la adhesión célula-célula. Además, las células *nurse like* también expresan miembros de la familia TNF: BAFF y APRIL, promoviendo señales de supervivencia a través de sus receptores (BAFF-r, TACI y BCMA) a las células de LLC. El receptor CD38 interactúa con el CD31 de las células estromales y *nurse like*, lo que promueve la activación de vías de supervivencia. Determinados antígenos propios o del microambiente son factores clave en la activación y expansión de la clona de LLC a través de la activación del RCB. Finalmente, la estimulación vía el RCB o el cocultivo con células *nurse like* promueve que las células de LLC expresen CCL3, CCL4 y CCL22 que induce el reclutamiento de células inmunológicas. En la LLC las células T se encuentran preferentemente en los centros proliferativos y pueden interaccionar vía CD40. (Burger, JA; Hematology Am Soc Hematol Educ Program.;2011: 96-103; 2011).

Las células *nurse like* (NLCs) son células mononucleares $CD14^{+}$ que abundan en los órganos linfáticos secundarios [70]. Del mismo modo que las células estromales, estas células expresan quimioquinas como el CXCL12 y moléculas antiapoptóticas como BAFF y APRIL [71]. El "diálogo" que establecen las células T-CD4 + con las células leucémicas a través del sistema CD40-CD40L (CD154) contribuye también, de forma muy importante, a la proliferación y supervivencia de las células tumorales de la LLC.

Pronóstico

El curso de la enfermedad es extremadamente variable. La supervivencia de los pacientes con LLC va desde unos 2-3 años hasta más de 10 años según el estadio clínico y las

características de la enfermedad. Una serie de parámetros clínicos permiten predecir con bastante exactitud la esperanza de vida de los pacientes.

Marcadores clásicos

La introducción de estadios clínicos significó un gran avance en el pronóstico de los pacientes con LLC. Los sistemas más utilizados son el de Rai y el de Binet [72, 73]. Ambas clasificaciones se basan en el número de áreas "linfoides" afectas, valoradas clínicamente, y la existencia o no de anemia y trombocitopenia (tabla 2).

Además de los estadios clínicos, otros factores pronósticos clásicos son el grado de infiltración de la médula ósea, cifra de leucocitos, tiempo de duplicación linfocitario en sangre y niveles séricos de Beta2 microglogulina (B2M)[74-76] (Tabla 3).

Tabla 2. Criterios de clasificación para los estadios de Binet y Rai.

z		ESTADIOS DE BINET		ESTADIOS DE RAI
Bajo riesgo	A	Hb ≥ 10g/dL, plaquetas ≥ $100x10^9$/L y ≤ 2 áreas linfoides* afectas	0	Linfocitosis en sangre periférica y médula ósea
Riesgo intermedio	B	Hb ≥ 10g/dL, plaquetas ≥ $100x10^9$/L y ≥ 3 áreas linfoides* afectas	I	Linfocitosis + adenopatías
			II	Linfocitosis + esplenomegalia y/o hepatomegalia
Alto riesgo	C	Hb < 10g/dL o plaquetas < $100x10^9$/L	III	Linfocitosis + Hb ≤ 11,0 g/dL
			IV	Linfocitosis + plaquetas ≤ $100x10^9$/L

(*) En los estadios de Binet, se toman en cuenta cinco áreas linfoides para asignar el estadio: adenopatías (tanto uni- como bilaterales) en las siguientes zonas: 1) cervicales; 2) axilares y 3) inguinales, así como 4) bazo y 5) hígado.

A pesar de su importancia, los estadios clínicos no dejan de tener limitaciones [77]. Por ejemplo, no predicen si la enfermedad se mantendrá estable o, por el contario, será progresiva. Además, la mayoría de pacientes se diagnostican en fase asintomática de la enfermedad o, en otras palabras, en estadios clínicos precoces, lo que limita el valor pronóstico de los estadios en su conjunto [78, 79]. Por último, los estadios clínicos no anticipan la respuesta al tratamiento.

Tabla 3. Marcadores pronóstico de la LLC.

MARCADORES CLÁSICOS	MARCADORES BIOLÓGICOS
Estadio clínico	Mutaciones de los genes IGHV
Recuento linfocitario en sangre	Alteraciones citogenéticas
Morfología linfocitaria en sangre	CD38
Tiempo de duplicación de linfocitos en sangre	ZAP-70
Grado de infiltración medular	Marcadores séricos: B2-microglobulina, timidín-cinasa, CD23 soluble

Marcadores biológicos

La técnica FISH permite detectar anomalías citogenéticas hasta en un 80% de los pacientes. Algunas de estas alteraciones, a pesar de no ser específicas de la LLC, se correlacionan con la evolución clínica de los enfermos. Así, los pacientes con alteraciones de los cromosomas 11 (11q-) o 17 (17p-) tienen mal pronóstico ya que no suelen responder al tratamiento, mientras que aquellos con un cariotipo normal o deleción del cromosoma 13q como única anomalía tienen mejor pronóstico. Por otro lado, la trisomía del cromosoma 12 se asocia a un pronóstico intermedio [49, 50, 80] (Tabla 4).

Tabla 4 Correlaciones clínico-biológicas con alteraciones citogenéticas y mutaciones.

Alteración	Correlación clínico-biológica
Deleción de 13q (de forma aislada)	Fases iniciales y no progresivas de la enfermedad, buen pronóstico
Deleción de 11q	Varones jóvenes, enfermedad agresiva. Asociación con la mutación de *SF3B1.* Mala respuesta al tratamiento y supervivencia libre de enfermedad corta
Deleción de 17p/ mutación *TP53*	Enfermedad progresiva y resistente a la terapia convencional
Trisomía 12	Morfología e inmunofenotipo atípicos. Asociada con la mutación de *NOTCH1*
Deleción 6q	Linfocitos con aspecto linfoplamocitoide
Mutación NOTCH1	Transformación de la enfermedad, mal pronóstico
Mutación SF3B1	Mal pronóstico
Mutación BIRC3	Resistencia al tratamiento, mal pronóstico

Las mutaciones de los genes de las *IGHV* distinguen dos formas de la enfermedad. [26, 27]. Los pacientes sin mutaciones de las *IGHV* tienen mal pronóstico y presentan un curso clínico agresivo y no responden al tratamiento. En cambio, aquellos con mutaciones de las *IGHV* suelen presentar un curso clínico estable, carecen de otros signos de mal pronóstico y su pronóstico es, por lo general, bueno [26, 27]. Asimismo, la expresión del antígeno de superficie CD38 en las células neoplásicas se relaciona con las mutaciones de *IGHV* aunque no de forma absoluta y, además, su expresión puede variar a lo largo del tiempo [81]. De forma paralela, la expresión de la proteína ZAP-70 en los linfocitos neoplásicos de la LLC se correlaciona muy estrechamente con la presencia o ausencia de mutaciones de *IGHV* [82-84]. Por último, la expresión de lipoproteín lipasa, así como la de determinados microRNA y la actividad de la telomerasa y longitud de los telómeros también se han correlacionado con el estado mutacional de las *IGHV* y la expresión de ZAP-70 [85-87].

Marcadores de respuesta al tratamiento

La respuesta al tratamiento es el parámetro pronóstico más importante [88]. Debido a ello, identificar qué pacientes van a responder a una determinada terapia es importante para elegir el tratamiento más adecuado en cada caso y evitar la exposición a tratamientos tóxicos y poco útiles. En este sentido, la deleción 17p y las mutaciones de *TP53* se asocian a una respuesta pobre al tratamiento con análogos de las purinas [51, 89]. A su vez, los pacientes con deleción 11q (gen *ATM*) presentan respuestas de corta duración a no ser que se traten con quimioinmunoterapia. [90, 91].

Tratamiento

Únicamente deben tratarse los pacientes con enfermedad sintomática o que muestra signos de progresión.

Las dos últimas décadas han contemplado una importante mejoría en el tratamiento de la LLC. La tabla 5 resume los resultados de los principales ensayos clínicos que han permitido identificar a la quimioinmunoterapia (la combinación de un anticuerpo monoclonal contra el CD20 juntamente con quimioterapia basada en los análogos de las purinas) como el tratamiento de elección para la LLC [92-94] (Tabla 5 y Figura 8).

Tabla 5. Resultados de ensayos clínicos con diferentes agentes.

	Tratamiento	Mediana edad, años	N	TRG%	RC %	SLE,m	SG%
Rai et al, 2000	Fludarabina	64	170	63	20	20	mediana, 55 m
	Clorambucil	62	181	37	4	14	mediana, 56 m
Catovsky et al, 2007	C	65	387	72	7	20	
	F	64	194	80	15	23	
	FC	65	196	94	38	43	
Hallek et al, 2010	FCR	61	409	95	44	52	87 (3-años)*
	FC	61	408	85	22	33	82.5 (3-años)

C: clorambucil; F: fludarabina; R: rituximab

En 1999, el grupo del MD Anderson Cancer Center desarrolló la combinación de fludarabina, ciclofosfamida y rituximab (FCR) con unos resultados nunca vistos hasta entonces en el tratamiento de la LLC [95, 96]. Cabe resaltar que la combinación FCR y tratamientos similares han hecho posible alcanzar no solo una tasa de respuestas completa (RC) elevada sino conseguir la RC molecular (sin enfermedad residual detectable). Por desgracia, las personas mayores de 70 años, aquellas con enfermedades asociadas graves, mal estado general, función renal deteriorada, o infección activa por VHB o VHC no pueden tratarse con FCR o tratamientos similares.

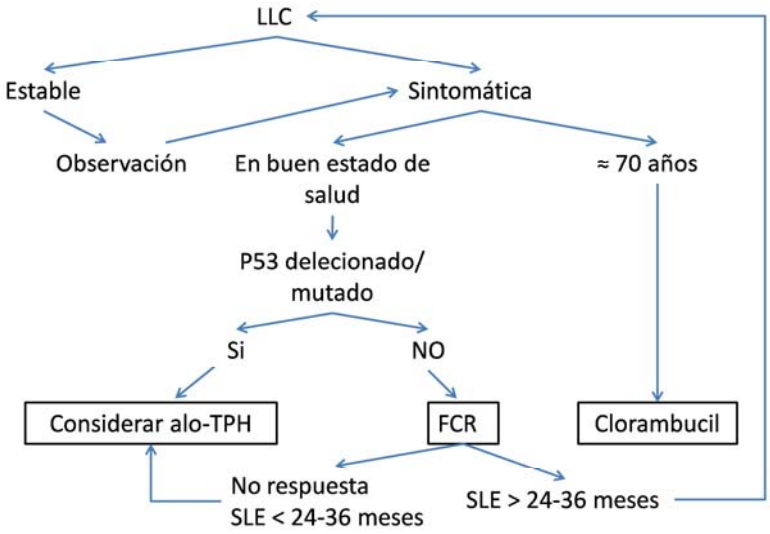


Figura 8. Algoritmo de tratamiento de los pacientes de LLC no incluidos en ensayos clínicos

Una fracción de pacientes (20-40%) no responden al tratamiento (enfermedad "refractaria"). Normalmente se trata de enfermos que presentan una deleción de 17p o mutaciones de *TP53.* Estos pacientes tienen muy mal pronóstico (mediana de supervivencia <24 meses) y en ellos el trasplante alogénico de progenitores hematopoiéticos (alo-TPH) es el único tratamiento capaz de controlar la enfermedad [88, 97, 98].

Los recientes avances en la compresión de la biología de la LLC han permitido el desarrollo de nuevos agentes terapéuticos capaces de actuar sobre vías metabólicas especialmente involucradas en esta leucemia [99-101]. Algunos de los agentes cuya eficacia ya

se ha comprobado y están en uso clínico son el ofatumumab, la bendamustina y la lenalidomida. Otros medicamentos que están en un estado avanzado de desarrollo son el GA-101 (un anticuerpo monoclonal anti-CD20), los inhibidores de quinasas (p. ej. flavopiridol), los inhibidores de la transducción de señal el RCB (p. ej., CAL-101, PCI 32765), las moléculas anti-BCL2 (p. ej. ABT-26 o Navitoclax), así como linfocitos T modificados (p. ej. linfocitos T con antígenos quiméricos o CART-cells).

Autoinmunidad en la LLC

Epidemiología

La asociación de la LLC con citopenias autoinmunes fue descrita a finales de los años 60 [102-105]. La incidencia se encuentra entre <5-10% [106]. La complicación más común es la anemia hemolítica autoinmune (AHAI) (aproximadamente 7%) mientras que la trombocitopenia inmune (PTI), la neutropenia autoinmune y la aplasia pura de serie roja son menos frecuentes (<1-2%). No existen pruebas de una correlación entre la LLC y enfermedades autoinmunes de la coagulación, tales como la hemofilia o la enfermedad de von Willebrand adquiridas.

Aunque en estudios antiguos la incidencia de enfermedades autoinmunes en la LLC se consideró muy alta, llegando a cifrarse en el 41% de casos si se incluían los enfermos con tan sólo "marcadores séricos" de autoinmunidad [102], en estudios recientes se estima, de forma mucho más plausible, que la frecuencia es del 2-12% (Tabla 6).

Respecto a los síndromes autoinmunes no hemáticos, en la década de los 80 se publicaron diversos estudios en los que se sugería que los familiares de pacientes con LLC presentaban con cierta frecuencia enfermedades autoinmunes [107]. Uno de los objetivos de esta tesis ha sido, precisamente, discernir mediante un análisis metódico de la literatura si existe o no una relación entre la LLC y las enfermedades autoinmunes no hemáticas.

Tabla 6. Estudios de grandes series de citopenias autoinmunes en LLC

AUTOR Y FECHA	POBLACIÓN	NÚMERO DE PACIENTES Y TIEMPO	PRONÓSTICO	CORRELACIÓNES CLINICO-BIOLÓGICAS
Hamblin 1986	195 pacientes de una institución	19 de 195 (9.7%), 1972-1985; 15 AHAI y 4 PTI	No descrito	No descritas
Kyasa 2000	132 pacientes de un único sistema de atención primaria	12 de 132 (9.1%), 1989-2001; 6 AHAI, 5 PTI y 1PRCA	La SG no es diferente de aquellos pacientes de LLC sin autoinmunidad	No descritas
Mauro 2000	1203 pacientes de una institución	52 de 1203 (4.3%), 1986-1996	La SG no difiere de aquellos pacientes Coombs + y anémicos de aquellos Coombs- y no anémicos	Leucocitosis, edad avanzada, hombre y LLC activa
Barcellini 2006	3150 pacientes del grupo GIEMA, 17 instituciones	194 de 3150 (6.2%); 129 AHAI y 35 PTI	No descrito	Estadio avanzado, enfermedad activa, edad avanzada.
Duek 2006	Registro nacional de LLC de 964 pacientes	63 de 964 (6.55%), 1971-2006; 55 Coombs + al diagnóstico, 9 PTI o Evans	No descrito	B2M elevada y CD38 elevado.
Visco 2008	1278 pacientes de 3 instituciones	64 de 1278 (5%) PTI, 1996-2004 47AHAI y 28 únicamente Coombs +	SG inferior en aquellos pacientes con PTI. La SG de los pacientes que al diagnóstico presentaban trombocitopenia es significativamente inferior a la de aquellos que no la presentaban independientemente de su origen	Leucocitosis, el gen *IGHV* no mutado y ZAP-70 elevada
Zent 2008,2009	1750 pacientes de una institución	75 de 1750(4.5%), 1995-2004; 41 AHAI, 35 PTI, 9APCR y 3AIG	SG no diferente de aquellos que no desarrollan citopenias. SG superior en pacientes con citopenia de origen autoinmune comparado con aquellos por fallo medular	Varones, gen *IGHV* no mutado, ZAP-70 elevada y citogenética de alto riesgo
Moreno 2010	961 pacientes de una institución	70 de 960 (7%), 1980-2008; 49 AHAI, 20 PTI y 1 Evans	SG no diferente de aquellos pacientes que no desarrollan autoinmunidad. SG superior en pacientes con citopenia de origen autoinmune en comparación con pacientes en estadío C al diagnóstico	Leucocitosis, tiempo de duplicación linfocitario corto, B2M elevada y CD38 elevado

SG: supervivencia global; B2M: Beta 2 microglobulina; APCR: Aplasia pura de células rojas

Se considera que las personas que padecen AHAI tienen un mayor riesgo de padecer LLC (riesgo relativo, RR: 108.4), también se ha observado, aunque en un solo estudio, un incremento de la LLC en la anemia perniciosa (RR: 1.94) [108]. La asociación entre la AHAI y el riesgo a desarrollar LLC es difícil de interpretar ya que bastantes de estos casos podrían presentar una LBM detectable tan sólo mediante citometría de flujo. Tanto en la LLC [109, 110] como en la autoinmunidad [111] se ha descrito un componente genético. Sin embargo hasta el momento no se ha encontrado ninguna asociación genética entre estos dos tipos de enfermedades [112].

Aspectos Biológicos

La LLC se caracteriza por defectos en los linfocitos T: incremento del número de linfocitos T, inversión del ratio entre las CD4+ y CD8+ y la producción de citoquinas inhibitorias (p. ej. IL-6, IL-10, TNF y TGF-β) [113-118]. Finalmente cabe destacar que la LLC se ha visto asociada con alteración de la inmunidad innata [119-122].

La explicación biológica para las citopenias autoinmunes en la LLC es compleja y en gran parte desconocida, aunque se sabe que en su origen intervienen células neoplásicas, linfocitos T y el microambiente [123-125] (Figura 9).

En los modelos murinos de LLC, las células B CD5+ (células B1a) son capaces de producir anticuerpos polireactivos que se unen al DNA y actúan como factor reumatoide [126, 127]. Sin embargo las células B CD5+ en humanos raramente producen auto-anticuerpos, hecho que está en consonancia con el hecho de que las células B CD5+ humanas y de ratón no son equivalentes [125, 127].

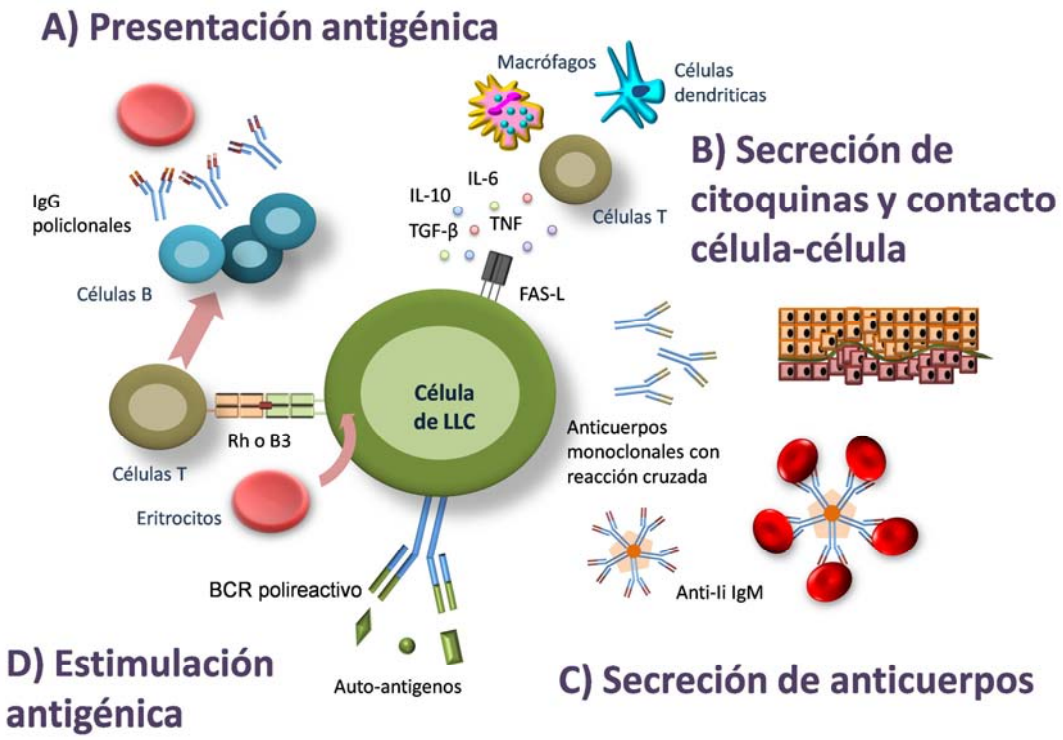


Figura 9. Mecanismos de la autoinmunidad en la LLC
A) Las células de LLC podrían procesar los antígenos de las células rojas y actuar como células presentadoras de antígenos, induciendo la respuesta de los linfocitos T y la formación de anticuerpos policlonales por parte de células B normales, induciendo de forma indirecta AHAI. B) Las células de LLC producen citoquinas inhibitorias que alteran la tolerancia, lo que provoque el "escape" de células autoreactiva. C) Raramente las células de LLC actúan como efectoras y secretan autoanticuerpos monoclonales patológicos. Dos de estas enfermedades son el pénfigo paraneoplásico, done la inmunoglobulinas presentan reacción cruzado con epitopos localizados en la unión dermo-epidérmica, y la crioglobulinemia donde las IgMs se unen a los eritrocitos. D) A su vez, las células de LLC pueden ser estimuladas a través de su BCR polireactivo que reconoce autoantígenos. Modificación de Hodgson K. Haematologica 96 (5): 752-61; 2011.

En las células B la respuesta antigénica está mediada por el BCR. En las células de LLC, particularmente en aquellos casos con *IGHV* no mutado pueden presentar un RCB altamente polireactivo que reconoce auto-antígenos [128-131]. Estos mismos antígenos son reconocidos por los anticuerpos "naturales", los cuales son patológicos en algunas enfermedades autoinmunes [132]. Sin embargo, la señalización del BCR en las células de LLC puede ser defectuosa, hecho que se relaciona con el bajo número de inmunoglobulinas presentes en la superficie de las células [133], ensamblajes no funcionales del RCB [134, 135] y mutaciones en las proteínas accesorias [136]. No obstante, se ha demostrado que las células de LLC son

capaces de producir anticuerpos autoreactivos *in vitro*, aunque es un hecho raro que las células de LLC produzcan anticuerpos *in vivo* en suficiente cantidad como para que ello tenga traducción clínica, aunque existen excepciones (p. ej. crioglobulinemia).

Los anticuerpos causantes de las citopenias son policlonales [137]. La capacidad de las células de LLC de actuar como células presentadoras de antígenos es casi nula, como se observa en los estudios *in vitro*, a excepción de los antígenos de los eritrocitos Rh y B3, los cuales son procesados y presentados a los linfocitos T de forma efectiva [138, 139]. También se ha descrito que la frecuencia de AHAI es mayor en aquellos casos con LLC avanzada, donde el bazo está muy infiltrado por células neoplásicas [140], lo que hace que las células de LLC se encuentren muy cerca de eritrocitos dañados. Además en el bazo también se encuentran linfocitos T que expresan el CD40 ligando, una molécula que es capaz de inducir un incremento de la capacidad presentadora de las células de LLC [141].

Citopenias autoinmunes en la LLC

Correlaciones clínico–biológicas

Diversas características clínicas y biológicas de la LLC se han visto asociadas con la aparición de citopenias autoinmunes (Tabla 7). Así, en la mayoría de estudios se ha observado una correlación entre AHAI y el estadio avanzado de la enfermedad [102, 142], LLC activa [140] y pacientes de mayor edad. [140, 142, 143].

Debido a que la mayoría de los estudios son retrospectivos, la relación entre citopenias autoinmunes con los nuevos marcadores pronósticos de la LLC no se ha podido analizar de forma adecuada. Sin embargo, tanto la AHAI y la PTI se han relacionado con marcadores de mal pronóstico como la ausencia de mutaciones en los genes *IGHV*, la expresión alta de ZAP-70 y los niveles elevados de la B2M [144, 145].

Las citopenias autoinmunes son más frecuentes en las fases avanzadas de la enfermedad, sin embargo las mismas pueden preceder a la LLC o diagnosticarse conjuntamente [144-146]. La asociación de la AHAI y la PTI previa a la LLC hay que considerarla con cuidado, ya que para el diagnóstico de la AHAI no se utiliza la citometría de flujo de forma rutinaria de tal forma que la posible existencia de una LBM, que no deja de ser en muchos casos más que una forma preleucémica de la LLC, no puede descartarse [147]. Este hecho remarca la importancia de descartar la LLC y otras enfermedades linfoproliferativas en el diagnóstico de la AHAI [148].

Tabla 7. Factores pronósticos correlacionados con citopenias autoinmunes en LLC

MARCADORES CLÍNICOS	MARCADORES BIOLÓGICOS
Estadio avanzado	Beta 2 microglobulina
Edad avanzada	CD38 elevado
Género masculino	ZAP-70 elevada
Recuento elevado de leucocitos	Genes *IGHV* no mutado
Tiempo de duplicación linfocitario corto	Citogenética de mal pronóstico (11q-, 17p-)

Las descripciones iniciales de la enfermedad asociaban la terapia contra la LLC como elemento que favorecía la aparición de citopenias autoinmunes [105, 149]. Especialmente los análogos de las purinas (p. ej. fludarabina) [150-152], por una supresión prolongada de las células T CD4+, un descenso en la población de células T reguladoras (CD4+CD25+FOXP3+). La afectación de estas células se ha visto asociada al desarrollo de enfermedades autoinmunes [153].

Actualmente, sin embargo, existen pruebas de que el riesgo de desarrollar citopenias autoinmunes después del tratamiento con análogos de purinas no es superior al que existe con otros tratamientos [143, 154, 155].

Diagnóstico

Las múltiples causas de la citopenia en la LLC (autoinmunidad, fallo medular, hiperesplenismo, quimioterapia, sepsis) hacen necesario una cuidadosa valoración clínica y de laboratorio. El examen morfológico de la sangre periférica a la búsqueda de esferocitos o plaquetas de gran tamaño, la prueba de Coombs, los niveles de LDH, bilirrubina, haptoglobinas y el recuento de reticulocitos, además del examen de la médula ósea por medio de aspirado o biopsia son obligadas ante la sospecha de una citopenia autoinmune [106].

La mayoría de los pacientes con LLC y AHAI presentan una anemia con un test de Coombs positivo en el contexto de reticulocitosis y un incremento de la bilirrubina. Los niveles de LDH son menos fiables ya que puede estar elevados en los casos de LLC activa. Además, en la AHAI se han descrito casos con test de Coombs negativo, particularmente asociados con la terapia [154]. Se debe tener en cuenta que la reticulocitosis puede estar ausente o ser muy poco marcada debido a la infiltración de células leucémicas en la médula ósea o por el tratamiento quimioterápico. El examen de la médula ósea es esencial para distinguir la citopenias relacionadas con el tratamiento [1]. El diagnóstico de la PTI es particularmente difícil, puesto que no existe una prueba realmente específica para el mismo. Sin embargo, la trombopenia puede considerarse de origen inmune si se observa un repentino descenso de la cifra de plaquetas, en ausencia de esplenomegalia, infección o quimioterapia, así como un número correcto de megacariocitos en la médula ósea [143]. El descenso gradual en lugar de repentino del recuento plaquetario es más frecuente en la PTI de los adultos que en niños, hecho que puede dificultar el diagnóstico. La respuesta a corticosteroides también se puede considerar una prueba diagnóstico. El diagnostico de eritroblastopenia se basa en la existencia de una anemia con cifra muy baja o reticulocitos y escasos precursores de la serie roja en la medula ósea. La interpretación morfológica de la medula ósea puede ser difícil si se halla rellena de linfocitos. En estos casos la tinción para la glicoforina es de ayuda para detectar los

precursores de la serie roja. En los casos de eritroblastopenia es preciso descartar un origen vírico (por ej., parvovirus, CMV). Los pocos casos de neutropenias autoinmunes descritos en la LLC corresponden con toda probabilidad no tanto a casos de neutropenia asociada a LLC sino de neutropenia en el curso de leucocitosis con linfocitos grandes granulares [156].

Pronóstico

La influencia de las citopenias autoinmunes en el pronóstico de los pacientes con LLC es motivo de debate.

En dos estudios de más de mil pacientes se observó que la AHAI se asociaba con enfermedad activa, pero sin un efecto negativo en la supervivencia. Sin embargo, en otro estudio los pacientes con un test de Coombs positivo y AHAI presentaban una supervivencia inferior a aquellos sin estos datos, pero cabe destacar que este estudio se realizó en pacientes que requirieron tratamiento y, por consiguiente, con mal pronóstico [140, 143, 144].

En un estudio donde se evaluó la PTI en más de 1200 pacientes, se observó que los pacientes que presentaron PTI al diagnóstico o a lo largo de la enfermedad presentaron un peor pronóstico independiente de otros marcadores pronósticos [157], hecho probablemente relacionado con la asociación de la PTI con casos no mutados de los genes *IGHV* [145]. Respecto a otras citopenias autoinmunes no se conoce el impacto pronóstico por su baja frecuencia.

BAFF y APRIL

Estructura, expresión y regulación

BAFF es una proteína de membrana homotrimérica de tipo II que pertenece a la familia de las proteínas del *tumor necrosis factor* (TNF), aunque también se puede encontrar de forma soluble como resultado de una escisión entre la región extracelular y transmembra por parte de una proteína denominada convertasa. BAFF comparte el 20-30% de la secuencia con otros miembros de la familia TNF (LT-alfa, CD40L, TRAIL) y hasta 50% con APRIL [158, 159]. Sin embargo, APRIL sólo existe de forma secretan y su escisión se produce en el compartimiento de Golgi [160]. Aunque la estructura cristalina de las proteínas de la familia de las TNF ha revelado una organización trimérica, BAFF posee un *loop* exterior que permite la interacción entre trímeros, pudiendo formar estructuras más complejas [161, 162].

BAFF y APRIL se expresan en monocitos, macrófagos, células dendríticas y neutrófilos, aunque también se pueden observar en células no mieloides [163-167]. Citoquinas conocidas en la regulación del sistema inmune tales como Interferon-γ (INF-γ) y interleuquina-10 (IL-10), se ha demostrado in vitro, que estimulan la expresión de BAFF en células mieloides [168].

Receptores

BAFF y APRIL son capaces de unirse a dos receptores de la familia TNF, *B-cell maturation antigen* (BCMA) y *Transmembrane activator and calcium-modulator and cyclophilin ligand (CAML) interactor* (TACI). Existe un tercer receptor específico para BAFF conocido como BAFF-r o BR3 [169, 170]. Se ha observado que APRIL interacciona con los proteoglicanos heparán sulfato pero su función es totalmente desconocida.

BCMA, TACI y BAFF-r son proteínas de membrana tipo III con un dominio extracelular donde se une BAFF o APRIL, en esta región de unión los miembros de esta familia suelen presentar cuatro dominios ricos en cisteína (CDRs) estabilizados por puentes disolfuro, en la

región intracelualar interaccionan con las proteínas *TNF-R associated factor* (TRAF) que son los responsables de la transmisión de la señal a núcleo mediada por el factor nuclear Kappa B (NF-kB) [169]. Los tres receptores son expresados por los linfocitos B aunque su expresión depende del estado de maduración en el que se encuentra la célula [171, 172].

BCMA fue el primero en ser descrito al estar involucrado en una translocación cromosómica en un linfoma T humano [173]. Su expresión parece estar restringida a las células B maduras. Aunque la función de BCMA en la maduración de células B humanas está poco estudiada, trabajos recientes apoyan la hipótesis que BCMA participa en los últimos eslabones de la diferenciación de los linfocitos B, además de promover la supervivencia de células plasmáticas en la médula ósea [174, 175].

TACI se expresa sobre todo en células B memoria y de la zona marginal, aunque también se puede encontrar en células B y T activadas [171, 176]. A diferencia de lo que ocurre con BCMA y BAFF-r, TACI inhibe la proliferación y la producción de inmunoglobulinas. Sin embargo, TACI juega un importante papel en el cambio de isotipo de las Ig, de forma dependiente e independiente de las células T al interaccionar con BAFF y APRIL [177-180]. Asimismo, las mutaciones del gen TACI se han visto asociadas con inmunodeficiencias, como por ejemplo déficits en la producción de IgA tanto en casos familiares como esporádicos [181].

El receptor BAFF-r, que es específico de BAFF, esta expresado en la mayoría de linfocitos B y es crucial en el desarrollo y mantenimiento de las células B. Por otra parte, y de igual forma que TACI, también se ha visto asociado con el cambio de isotipo de las Igs [174].

Señalización

Los miembros del TNF y sus ligandos son unos reguladores muy importantes de la respuesta inmune. En particular, BAFF y APRIL participan en la supervivencia, proliferación y diferenciación de los linfocitos B. Ambas proteínas inducen la activación del NF-κB por la

interacción de su dominio intracellular con varios TRAF [182]. Se han identificados dos vías de activación de NF-κB: la vía canónica y la alternativa. La vía canónica de NF-κB se inicia con la activación del complejo IkB Quinasa (IKK) compuesto por IKKβ y IKKγ subunidad reguladora. Esto induce la degradación del inhibidor de NF-κBα (IκBα), y promueve la activación y la translocación al núcleo del heterodímero NF-κB, compuesto por p50, p65, y RelA. La vía alternativa se inicia por la fosforilación de κB2/p100 por IKKα (la vía alternativa es independiente de IKKγ), y permite la translocación al núcleo de p52 con RelB. La activación de la vía canónica y la alternativa inducen una expresión génica diferente [183].

BCMA y TACI se unen a TRAF2, 5, 6 induciendo la activación de la ruta canónica de NF-κB. Sin embargo BAFF-r induce la ruta alternativa por medio de TRAF3 pero también es capaz de activar la vía canónica. De forma remarcable, TRAF3 actúa como un regulador negativo de la activación de la ruta y es la retención de TRAF3 por parte de BAFF-r que permite la activación [184-187].

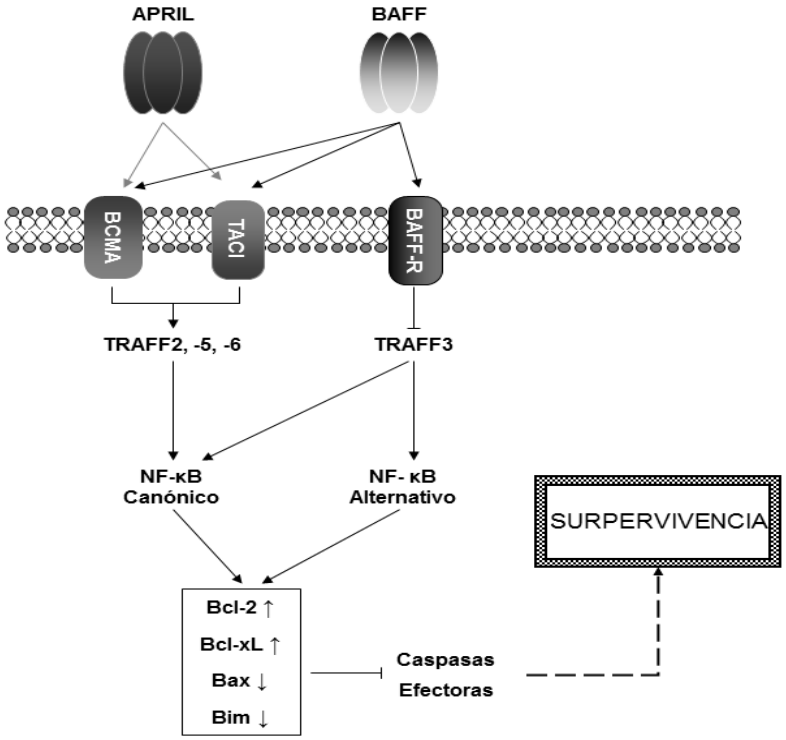


Figura 10. Señalización de BAFF y ARIL inducida por la interacción con sus receptores, promoviendo la supervivencia por la vía de NF- κB.

BAFF, APRIL y enfermedades

La expresión y concentración de BAFF y APRIL se hallan alteradas en enfermedades autoinmunes y trastornos de las células linfoides (Tabla 8).

Autoinmunidad

BAFF tiene un papel importante en el proceso de la tolerancia antigénica. En condiciones fisiológicas las células B autorreactivas son eliminadas para evitar la generación de anticuerpos patológicos. En este proceso la expresión normal de BAFF es insuficiente para rescatar las células B autorreactivas y se mantienen los mecanismos de tolerancia. Diversos estudios con ratones transgénicos para BAFF han demostrado un incremento de la población B autorreactica cuando BAFF se encuentra sobrexpresado [188].

Asimismo, la sobreexpresión de BAFF se ha visto asociada con un conjunto de enfermedades autoinmunes como lupus eritematoso sistémico, artritis reumatoide y síndrome de Sjögren. En algunas de ellas los niveles de BAFF en suero y algunos órganos o tejidos (p. ej., articulaciones y ganglios linfáticos) se han visto asociados con la gravedad de la enfermedad y en general con títulos positivos de autoanticuerpos. De forma interesante algunos polimorfismos en la región promotora de *BAFF* y *APRIL* se han asociado lupus eritematoso sistémico y PTI [189-194]. Aunque en la patogenia de los trastornos autoinmunes suele ponerse mucho énfasis, y con razón, en el papel que juegan los linfocitos T, estudios recientes en modelos murinos de lupus eritematoso sistémico sugieren que la sobreexpresión de BAFF puede iniciar la enfermedad de forma independiente de células T [195].

Por último, la relativa eficacia de antagonistas o anticuerpos contra BAFF y APRIL en algunas enfermedades autoinmunes apoyan la importancia de dichas moléculas en estas enfermedades [163, 196-199]. En estos momentos, uno de estos anticuerpos ha sido aprobado por la FDA y otros están en diversas fases de desarrollo clínico [200].

Tabla 8. Enfermedades autoinmunes con niveles elevados de BAFF o APRIL

NOMBRE	
Lupus eritematoso sistémico	Enfermedad celíaca
Síndrome de Sjögren	Hepatitis autoinmune
Artritis reumatoide	Pénfigo
Trombocitopenia autoinmune	Granulomatosis de Wegener
Miastenia gravis	Cirrosis biliar primaria
Dermatitis atópica	Diabetes tipo 1 en adultos
Esclerosis múltiple	Endometriosis
Anemia hemolítica autoinmune	

Neoplasias linfoides de células B

Por su importancia en los linfocitos B, BAFF y APRIL está siendo estudiadas las neoplasias B tales como linfomas no hodgkinianos (LNH), LLC, mieloma múltiple (MM) y macroglobulinemia de Waldenström (MW) [201-205].

En todas las neoplasias de las células B maduras se ha observado la capacidad de unión a BAFF y en algunos casos a APRIL. De forma interesante las diferentes neoplasias presentan patrones de la expresión de los receptores característicos; TACI y BAFF-r están expresados por células de LLC y LNH, BCMA y TACI se encuentran principalmente en MM y linfoma de Hodgkin. Además la expresión de BAFF-r se ha visto que varía dentro de los LNH. Así, en el linfoma folicular y LLC es inferior que en otros tipos de linfomas como el difuso, de manto y de zona marginal [206, 207]. BAFF y APRIL son capaces de rescatar de la apoptosis a las células neoplásicas *in vitro* [21, 208, 209].

Al mismo tiempo diversos grupos han encontrado que la concentración en suero de BAFF y APRIL en pacientes con LNH y MW es superior a los de las personas sanas. Este hecho afirma la idea que BAFF y APRIL son importantes en la biología de estas neoplasias [210]. Por otra parte, en algunos estudios se ha correlacionado el incremento de BAFF con la transformación de la LLC en un linfoma agresivo y la respuesta al tratamiento. Un elemento a destacar es el aumento de producción de BAFF y APRIL por las células infectadas por el VEB, promoviendo su supervivencia [211]. En estos momentos se están realizando estudios para introducir elementos que bloqueen BAFF y APRIL [212].

En la LLC

BAFF se ha visto implicado en procesos que se dan con frecuencia en la LLC, así como la resistencia a la apoptosis de las células B, la sobreexpresión del BCL-2 y también el desarrollo de fenómenos autoinmunes.

Los niveles de BAFF en suero de pacientes con LLC son inferiores a los de las personas sanas [170, 213]. Así mismo, las LLC familiares presentan con mayor frecuencia un polimorfismo en la región promotora del gen de *BAFF,* induciendo una mayor expresión y unos niveles en suero superiores. Además, en diferentes estudios se ha correlacionado los niveles de BAFF y APIRL con diferentes marcadores pronósticos [20, 21, 213, 214].

Cabe destacar que estas dos moléculas se expresan por las células neoplásicas y por el microambiente que las rodea, y en particular las NLC (Figura 11) [70, 71, 201, 205, 215]. En cultivos *in vitro* se ha observado que al introducir BAFF y APRIL en el medio las células se protegen de los fenómenos de apoptosis [71]. Como hemos mencionado anteriormente, BAFF activa la vía canónica y alternativa del NF-kB al interaccionar con los receptores BAFF-r, TACI y BCMA, aunque en la LLC la vía canónica parece ser la más importante para el mantenimiento de la supervivencia celular [216].

La expresión génica de las células de LLC está influenciada por el contexto donde se encuentran, existiendo diferencias entre sangre periférica, medula ósea y ganglios linfáticos [217, 218]. Este hecho también se ha observado al estudiar la expresión de BAFF. Así, las células de LLC que se encuentran en los ganglios linfáticos –donde se hallan los seudofoliculos o centros proliferativos- expresan en mayor cantidad BAFF que aquellas que se encuentran en medula ósea o sangre periférica [219].

La sobreexpresión de BAFF en modelos murinos de LLC, promueve una aceleración el desarrollo de la enfermedad en los ratones comparado con aquellos en que no se modifican los niveles de BAFF [23, 24]. Así mismo, los ratones transgénicos para APRIL desarrollan una neoplasia B1 similar a la LLC [220]. En estos momentos se están estudiando ratones con varias alteraciones combinadas con aumento de expresión de BAFF o APRIL [221, 222].

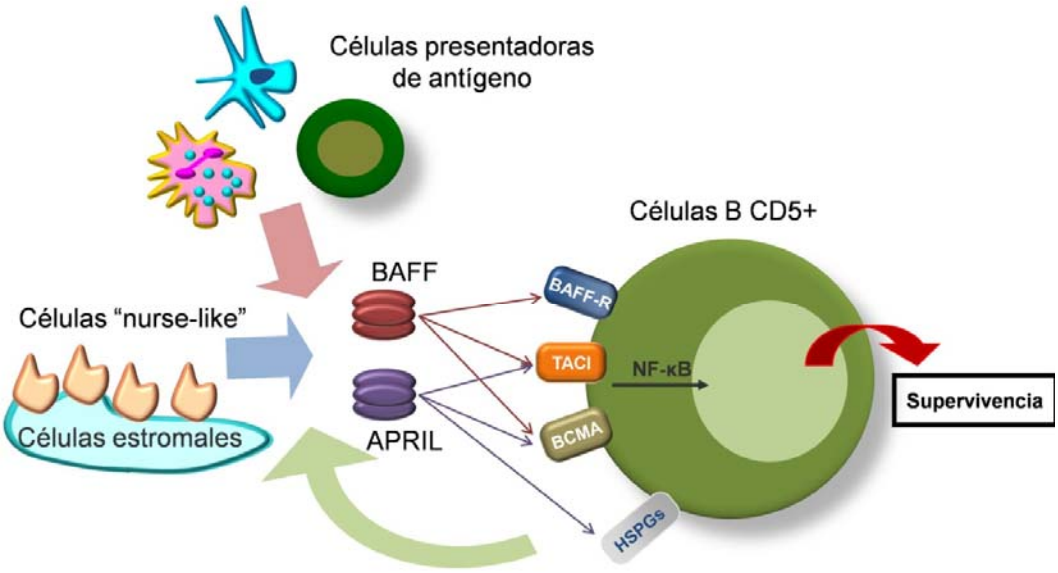


Figura 11. Representación esquemática de BAFF y APRIL en la LLC

Un porcentaje elevado de los pacientes con LLC desarrollan algún síndrome autoinmune. En un sistema biológico tan complejo como el de esta forma de leucemia es muy probable que no exista un único elemento que inicie este tipo de trastornos, pero todo indica que BAFF y/o APRIL tienen un papel importante; por ejemplo, en el mantenimiento de las células B autoreactivas [223].

Finalmente, también se están llevando acaba estudios para evaluar la eficacia de los bloqueantes de estas dos moléculas en el tratamiento de la LLC. De forma interesante se ha comprobado que BAFF conjugado con una toxina induce la muerte de las células de LLC *in vitro* [222, 224].

Hipótesis y objetivos

Hipótesis de trabajo

Esta tesis doctoral se basa en una serie de trabajos que analizan la relación entre la LLC y la autoinmunidad tanto desde el punto de vista clínico (incidencia, características y repercusiones pronósticas de las citopenias autoinmunes en pacientes con LLC) como biológico (estudios sobre las moléculas BAFF y APRIL de la familia TNF como vínculo entre la LLC y la autoinmunidad). Las hipótesis de trabajo fueron las siguientes.

1. Los sistemas pronósticos de Rai y Binet no consideran el origen de la citopenia para asignar el estadio clínico de un determinado paciente. Sin embargo, el origen de la citopenia (inmune o no inmune) podría ser importante para el pronóstico y mejorar el tratamiento de los pacientes considerados en estadio avanzado de la enfermedad.

2. Las proteínas BAFF y APRIL están implicadas en la patogénesis de la LLC y en la autoinmunidad por lo que pueden constituir un vínculo fisiopatológico entre los componentes neoplásico e inmune de la LLC.

Objetivos

Objetivos fundamentales

1. Determinar la prevalencia, características y pronóstico de la anemia hemolítica autoinmune y trombopenia autoinmune en una amplia serie de pacientes con LLC.

2. Correlacionar las citopenias autoinmunes con marcadores clínicos y biológicos: mutaciones de los genes IGHV, expresión de ZAP-70 y de CD38, anomalías citogenéticas y expresión de miRNAs.

3. Correlacionar los niveles de BAFF y APRIL en suero con otros marcadores biológicos tales como las mutaciones de los genes IGHV, expresión de ZAP-70 y de CD38, así como las anomalías citogenéticas, con el pronóstico de la LLC. Comprobar, con respecto a este último aspecto, si la utilización conjunta de BAFF y APRIL aporta más información pronóstica que la de cada una de estas proteínas por separado.

Objetivos secundarios

1. Llevar a cabo un análisis metódico de la literatura para que sirviese de base de los distintos trabajos que configuran esta tesis y determinar el estado actual sobre el conocimiento de la relación entre LLC y fenómenos autoinmunes en general.

2. Analizar los pacientes con LLC en estadio avanzado por causas autoinmunes y no autoinmunes como grupos pronósticos independientes.

3. Cuantificar la concentración de BAFF y APRIL en suero así como su expresión por parte de las células neoplásicas de pacientes con LLC y compararlos en distintos grupos de sujetos, particularmente aquellos con y sin citopenias autoinmunes.

Resultados

Artículo 1: Autoimmune cytopenia in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance.

Carol Moreno*, Kate Hodgson*, Gerardo Ferrer*, Montse Elena, Xavier Filella, Arturo Pereira, Tycho Baumann, Emili Montserrat. Blood. 2010; 116(23):4771-6.

(*) Equal contribution.

Este artículo mereció ser publicado junto con una editorial que glosaba diferentes aspectos del mismo: Dearden, C. Stage C or not stage C...?. Blood. 2010 ; 116(23):4735-6.

Resumen

En este estudio se analizó la prevalencia, características, correlaciones clínicas y la importancia pronóstica de las citopenias autoinmunes en pacientes con LLC. Setenta de 960 pacientes (7%) estudiados en el Hospital Clínic de Barcelona en los últimos 28 años han presentado citopenias autoinmunes, de las cuales 19 fueron detectadas al diagnóstico, 3 antes del diagnóstico y 48 durante la enfermedad. Cuarenta y nueve pacientes presentaron anemia hemolítica autoinmune, 20 trombocitopenia inmune y uno ambos cuadros (Síndrome de Evans-Fisher). Se observó una clara asociación entre las citopenias autoinmunes y variables de mal pronóstico (recuento linfocitario elevado, tiempo de duplicación linfocitario rápido, niveles altos de B2M sérica y expresión de la proteína ZAP-70). El pronóstico de los pacientes con citopenias autoinmunes como grupo no difirió del de aquellos que no presentaron dicha complicación. El hallazgo más importante de este estudio fue que los pacientes con enfermedad avanzada (estadio de Binet C) de origen autoinmune presentan mejor pronóstico que aquellos que el estadio avanzado se debe a la infiltración medular por parte del tumor (mediana de supervivencia: 7.4 años *vs.* 3.7 años, p=0.02). Estos resultados ponen de relieve lo importante que es el averiguar el origen de la citopenia en pacientes con leucemia linfática crónica y aconsejan separar a los pacientes con enfermedad avanzada en dos grupos de acuerdo con el origen de la citopenia: estadio C “inmune” y estadio C “infiltrativo”.

Autoimmune cytopenia in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance

*Carol Moreno,[1] *Kate Hodgson,[1] *Gerardo Ferrer,[1] Montse Elena,[2] Xavier Filella,[2] Arturo Pereira,[3] Tycho Baumann,[1] and Emili Montserrat[1]

[1]Institute of Hematology and Oncology, Hematology Department, [2]Clinical Biochemistry and Molecular Genetics Department, and [3]Hemostasis and Blood Bank Department, Hospital Clínic University of Barcelona, Institut d'Investigacions Biomediques August Pi i Sunyer (IDIBAPS), Barcelona, Spain `

We analyzed prevalence, characteristics, clinical correlates, and prognostic significance of autoimmune cytopenia in patients with chronic lymphocytic leukemia. Seventy of 960 unselected patients (7%) had autoimmune cytopenia, of whom 19 were detected at diagnosis, 3 before diagnosis, and 48 during the course of the disease. Forty-nine patients had autoimmune hemolytic anemia, 20 had immune thrombocytopenic purpura, and 1 had both conditions. A clear association was observed between autoimmune cytopenia and poor prognostic variables (ie, high blood lymphocyte count, rapid blood lymphocyte doubling time, increased serum -2 microglobulin level, and high expression of -associated protein 70 and CD38). Nevertheless, the outcome of patients with autoimmune cytopenia as a whole was not significantly different from that of patients without this complication. Furthermore, no differences were observed according to time at which cytopenia was detected (ie, at diagnosis, during course of disease). Importantly, patients with advanced (Binet stage C) disease because of an autoimmune mechanism had a significantly better survival than patients in advanced stage related to a massive bone marrow infiltration (median survivals: 7.4 years vs 3.7 years; *P* .02). These results emphasize the importance of determining the origin of cytopenia in patients with chronic lymphocytic leukemia for both treatment and prognostic purposes. (*Blood*. 2010; 116(23):4771-4776)

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of B cells with mature appearance and a distinctive immunophenotype (ie, SmIg^{dim}, CD5 CD19 , CD20^{dim}, CD23) in peripheral blood, bone marrow, lymph nodes, and other lymphoid tissues.[1,2] In addition, CLL is frequently associated with autoimmune phenomena, particularly autoimmune hemolytic anemia (AIHA).[3] Thus, between 5% and 25% of patients with CLL develop AIHA, whereas immune thrombocytopenic purpura (ITP) is less frequent with an estimated 1%-5% in most recent studies.[4-7]

Prognosis of patients with CLL is extremely variable. Despite the increasing importance of biomarkers in prognostication, clinical staging systems remain the backbone for assessing prognosis in patients with CLL. Of note, neither Rai et al[8] nor Binet et al[9] staging systems consider the origin of cytopenia when assigning a clinical stage to a given patient. However, to ascertain the outcome of patients with CLL in advanced clinical stage on the basis of the origin of the cytopenia (ie, "immune" vs "infiltrative") can be important because of prognostic and treatment considerations.[10]

Although remarkable advances have been made in our understanding of the pathophysiology of CLL, little is known about the causes and clinical implications of autoimmune cytopenia in patients with this form of leukemia. Furthermore, the effect of autoimmune cytopenias on the clinical outcome of patients with CLL remains controversial.[4,5,7,11] In this regard, there are few reports that analyzed the prevalence, clinical correlates, prognostic factors, and outcome of autoimmune cytopenia in large, unselected series of patients with CLL from single institutions.[4,5]

The aims of this study were 3-fold. First, we wanted to determine the prevalence of autoimmune cytopenia in a large series of unselected patients with CLL. Second, we sought to correlate autoimmune cytopenia with clinical and biologic features. Third, we wished to ascertain the prognosis of autoimmune cytopenia in CLL, particularly in patients with advanced clinical stage (Binet stage C).

Methods

Patients

The study population comprises 960 patients with CLL diagnosed at the Hospital Clínic of Barcelona between January 1980 and December 2008. Patients with a diagnosis of autoimmune cytopenia were identified from this database, and their case records were reviewed. Demographic as well as characteristics at diagnosis and prognostic factors, including blood lymphocyte count and lymphocyte doubling time (LDT), percentage of lymphocytes in bone marrow, -2 microglobulin (B2M) serum levels, cytogenetic abnormalities, *IGHV* mutation status, -associated protein 70 (ZAP-70), and CD38 expression, were registered, and their association with immune cytopenias was analyzed. The study was approved by the Ethics Committee of the Hospital Clínic of Barcelona.

Submitted May 20, 2010; accepted August 15, 2010. Prepublished online as *Blood* First Edition paper, August 24, 2010; DOI 10.1182/blood-2010-05-286500.

C.M., K.H., and G.F. contributed equally to this study.

An Inside *Blood* analysis of this article appears at the front of this issue.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Diagnostic criteria

The diagnosis of CLL was made according to criteria from the National Cancer Institute,[12,13] and whenever possible the diagnosis was confirmed by flow cytometry. The diagnosis of AIHA was based on the presence of an otherwise unexplained hemoglobin level 100 g/L (10 g/dL) or hematocrit 30% and a positive direct antiglobulin test (DAT) for either immunoglobulin G or C3 and the presence of 1 indirect marker of hemolysis: high reticulocyte count, low serum haptoglobin levels, increased serum lactate dehydrogenase or bilirubin levels. For patients in whom DAT was negative, the diagnosis of AIHA was made if 2 of the indirect signs of hemolysis were present.[5] ITP was defined as a sudden and otherwise unexplained fall in platelet count to 100 10^9/L (100 000/mm^3) with 2 of the following: evidence of normal bone marrow function (normal or increased megakaryocytes in bone marrow, or no reticulocytopenia if bone marrow aspirate was not available), no splenomegaly, no chemotherapy within the last month from study entry.[7] Patients were confirmed as having stage C "infiltrative" if they had either hemoglobin level 100 g/L (10 g/dL) or platelet count 100 10^9/L (100 000/mm^3) with no positive DAT and no indirect signs of hemolysis, and further confirmed whenever possible by a significant marrow infiltration as defined by either a diffuse bone marrow histologic pattern or, when this was not available, by the less accurate bone marrow aspirate (80% lymphocytes) or reticulocytopenia (1%).

Study design and statistical end points

Demographic and clinical characteristics were compared with the chi-square and Mann-Whitney tests for categorical and ordinal samples, respectively.[14] Patients in whom the diagnosis of CLL and that of autoimmune cytopenia was made within a month of each other were considered to have C "immune" stage at diagnosis. Survival analyses were performed according to the Kaplan-Meier method and analyzed by the log-rank test.[14] Overall survival was defined as the time interval from the diagnosis of CLL to death from any cause or last follow-up. Statistical analysis was performed with SPSS 15.0 (SPSS Inc) and Stata 10 software (www.stata.com), and a *P* value of .05 was considered significant. Different treatment protocols used over this period of time were approved by the ethics committee of our institution. Briefly, 419 of 960 patients received treatment for their CLL. Fifty-five percent of the patients (231 of 419) were given chlorambucil-based regimens, and 49% of the patients (204 of 419) were given fludarabine-containing regimens at some point during their follow-up; among the latter, 64 patients received fludarabine alone and 140 received fludarabine-containing regimens.

Most patients with autoimmune cytopenia were initially treated with corticosteroids. Details of treatment modalities and response to therapy are shown in Table 1.

Table 1. Treatment and response to therapy of autoimmune cytopenia in patients with CLL

Treatment	AIHA DAT (n 43)*	AIHA DAT (n 7)	ITP (n 20)	All (n 70)
Corticosteroid-based therapy (n 58)				
Steroids alone	16	5	9	30
Steroids other†	20	1	7	28
Chemotherapy‡	6	0	1	7
Other§	0	0	3	3
Unknown	1	1	0	2
Outcome				
Single resolved episode	19	2	10	31
1 episode	8	1	8	17
Steroid dependent	5	1	1	7
Died with active immune disease	4	0	1	5
Unknown	7	3	0	10

CLL indicates chronic lymphocytic leukemia; AIHA, autoimmune hemolytic anemia; DAT, direct antiglobulin test; and ITP, immune thrombocytopenic purpura.

*One patient presented with both AIHA DAT and ITP.

†Refers to patients who in addition to steroids received other treatments: cyclophosphamide (n 19), splenectomy (n 6), rituximab (n 3), intravenous immunoglobulin (n 3), vincristine (n 3), azathioprine (n 1).

‡Chemotherapy regimens were chlorambucil (n 4); cyclophosphamide, hydroxydaunorubicin, Oncovin, prednisone (CHOP; n 2); CHOP BCNU (bis-chloroethyl-nitroso-urea), etoposide, cytosine arabinoside, melphalan autologous stem cell transplantation (n 1).

§Spontaneous resolution (n 2), *Helicobacter pylori* eradication (n 1).

In no case could death be attributed to autoimmune cytopenia solely.

Results

CLL population and prevalence of autoimmune cytopenia

Over the 28-year study period the diagnosis of autoimmune cytopenia was entertained in 93 of 961. However, on review, 23 patients were excluded from the study because they failed to fulfill the strict diagnostic criteria described in "Methods." As a result, the diagnosis of autoimmune cytopenia was confirmed in 70 patients, thus giving a prevalence of 7%. Forty-nine patients had AIHA (42 DAT , 7 DAT), 20 had ITP, and 1 patient presented with both AIHA and ITP (Evans syndrome). Three of the patients who initially had ITP later developed AIHA. The presentation of autoimmune cytopenia was 1 year before the diagnosis of CLL in 3 patients (1 AIHA, 2 ITP), at the time of diagnosis in 19 patients (12 AIHA, 7 ITP), and in 48 patients it appeared during the evolution of the disease (36 AIHA, 11 ITP, 1 Evans syndrome).

Thirty-five of 70 patients developed either ITP or AIHA during (n 11) or after (n 24) therapy with a median time of 6 months (range, 0-120 months) since treatment exposure. Eight patients had received fludarabine-containing regimens (1 fludarabine alone and 7 fludarabine in combination with cyclophosphamide and mitoxantrone) and 18 received chlorambucil. Thus, the prevalence of autoimmune cytopenia after fludarabine and chlorambucil regimens was 8 of 204 patients (4%) and 12 of 231 patients (5%), respectively (*P* NS).

Clinical and biologic characteristics in patients with and without autoimmune cytopenia

The main clinical and biologic characteristics of patients with and without autoimmune cytopenia are listed in Table 2. Clinical characteristics associated with autoimmune cytopenia were a high lymphocyte count, short LDT, and advanced clinical stage. Furthermore, although not significantly different, there was a trend for a higher bone marrow infiltration in patients who developed autoimmune cytopenia.

In patients whose condition was more recently diagnosed and in whom these parameters were available, high serum B2M levels (*P* .02) and increased expressions of ZAP-70 (*P* .02) and CD38 (*P* .07) were significantly associated with autoimmunity. No correlation was observed between autoimmune cytopenia and *IGHV* mutational status or adverse cytogenetics; these variables however were only available in 16% and 30% of the patients, respectively.

Outcome of patients with and without autoimmune cytopenia

Forty-six of the 70 patients with autoimmune cytopenia (66%) have died (5 of them while having active autoimmune disease) and 24 are alive (14 AIHA, 10 ITP) at the last follow-up. Among patients without autoimmune cytopenia, 443 of 867 (51%) have died and 424 are alive.

No significant differences were observed in the overall survival between patients with and without autoimmune cytopenia, the median survival being, respectively, 8 years (95% CI, 7-9) and

Table 2. Clinical and biological characteristics at diagnosis in patients who presented with autoimmune cytopenia versus patients without autoimmune cytopenia

	With autoimmune cytopenia (n 70)	Without autoimmune cytopenia (n 867)	*P*
Median age, y (range)	65.5 (33-89)	66 (24-97)	NS
Male (%)	63	57	NS
WBC count, 10^9/L (range)	28.3 (4.8-461)	20.4 (2.5-454)	NS
Hb level, 10^9/L (range)	12.7 (4.6-18.10)	13.8 (5-18.5)	.05*
Platelet count, 10^9/L (range)	155 (10-347)	188 (10-483)	.05*
ALC, 10^9/L (range)	20.4 (2.8-410)	14.2 (0.95-445)	.004
BM infiltration, % (range)†	60 (15-99)	53 (3-100)	.075
Binet stage, n (%)			
A	37 (53)	671 (79)	
B	9 (13)	131 (15)	.05*
C	24 (34)	54 (6)	
PS 2, n (%)	64 (92)	824 (95)	NS
B2M 2.5 mg/L, n/N (%)	24/44 (55)	185/502 (37)	.02
LDT 12 mo, n/N (%)	17/46 (37)	107/515 (21)	.01
ZAP-70 20%, n/N (%)	18/35 (51)	142/432 (33)	.02
CD38 30%, n/N (%)	16/33 (48)	129/393 (33)	.07
Unmutated *IGHV*, n/N (%)	11/22 (50)	67/131 (51)	NS
Poor risk cytogenetics, n/N (%)‡	3/24 (13)	44/254 (17)	NS
Follow-up, y (range)	6.5 (0-25)	5.5 (0-25)	NS

NS indicates not significant; WBC, white blood cell; Hb, hemoglobin; ALC, absolute lymphocyte count; BM, bone marrow; PS, performance status; B2M, -2 microglobulin; LDT, lymphocyte doubling time; and ZAP-70, -associated protein 70.

*Significant differences are due to immune cytopenias.

†Assessed by BM aspirate or biopsy or both.

‡Poor cytogenetics include del 17p, del 11q, trisomy 12, and the presence of 2 cytogenetic abnormalities as determined by fluorescence in situ hybridization and conventional cytogenetics.

9 years (95%CI, 8-10; *P* NS). Furthermore, median survival was 7, 7.4, and 8 years for patients in whom the autoimmune cytopenia appeared before, at diagnosis, or during the course of the disease. Patients with Binet stage A at diagnosis who later developed autoimmune cytopenia had a median survival from diagnosis of 9 years (95% CI, 7-11 years) versus 10 years (95% CI, 9-11 years) in patients who did not present this complication (*P* NS; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Likewise, median survival was 8.5 years (95% CI, 8-9 years) in patients with B stage who developed an autoimmune cytopenia versus 5.5 years (95% CI, 4-7years) in patients who did not present this complication (*P* NS; supplemental Figure 2).

Comparison between C immune and C infiltrative and restaging after initial therapy

Table 3 shows clinical characteristics of patients who presented with CLL in stage C on the basis of the origin of the cytopenia (ie, immune vs infiltrative). Demographics, clinical characteristics, and median follow-up were comparable in both groups except for the bone marrow infiltration and serum B2M levels that were significantly higher in patients with Binet stage C infiltrative compared with patients with Binet stage C immune.

After initial therapy, significant differences were observed in the response rate according to the origin of the cytopenia. Whereas 16 of the 19 patients with stage C immune disease responded to corticosteroids and, as a result, switched from stage C to stage A, only 9 of 54 patients with stage C infiltrative had a similar response to chemotherapy (*P* .001). Furthermore, within C immune

Table 3. Clinical characteristics in patients with advanced stage at diagnosis according to the type of cytopenia: C immune versus C infiltrative

	C immune (n 19)	C infiltrative (n 54)	*P*
Median age, y (range)	69 (40-86)	70 (28-90)	NS
Male (%)	74	65	NS
WBC count, 10^9/L (range)	25.9 (9.9-334)	30.1 (2.5-454)	NS
Hb, 10^9/L (range)	10.5 (6-16.6)	10 (5-15.4)	NS
Platelet count, 10^9/L (range)	132 (19-347)	89.5 (10-483)	NS
ALC, 10^9/L (range)	15.3 (4.4-257)	23 (1.4-444)	NS
BM infiltration, % (range)*	54 (16-99)	80 (20-100)	04
PS 2%	67	83	NS
B2M 2.5 mg/L, %	50	85	.03
No. of nodal areas			NS
2 LN areas, n	14	40	NS
2 LN areas, n	5	12	NS
Follow-up, y			NS
All patients, median (range)	5 (0-13)	3.5 (0-13)	NS
Alive patients, median (range)	5.6 (0-12.8)	4 (0-7.3)	NS

NS indicates not significant; WBC, white blood cell; Hb, hemoglobin; ALC, absolute lymphocyte count; BM, bone marrow; PS, performance status; LN, lymph node.

*Assessed by BM aspirate or biopsy or both.

patients, 9 of 12 with AIHA and 7 of 7 with ITP were downstaged to A disease. This difference, however, was not statistically significant.

Survival analysis of patients with stage C immune versus stage C infiltrative at diagnosis

Overall 57 of 73 patients with advanced clinical stage at diagnosis have died: 46 of 54 (85%) of patients in stage C infiltrative and 11 of 19 (58%) with stage C immune. Patients with stage C immune disease had a significantly better survival than patients with stage C infiltrative disease, with a median survival of 7.4 versus 3.7 years, respectively (*P* .02; Figure 1). Furthermore, there was a trend for a better outcome for patients with ITP than for patients with AIHA or stage C infiltrative disease (*P* .06; Figure 2).

Survival according to clinical stages at diagnosis, including C immune as a prognostic category

The survival analysis of patients according to the Binet staging system showed the following median survivals: stage A, 10 years; stage B, 6.3 years; stage C, 3.9 years (*P* .01). When stage C immune was included in the overall analysis as a distinct category,

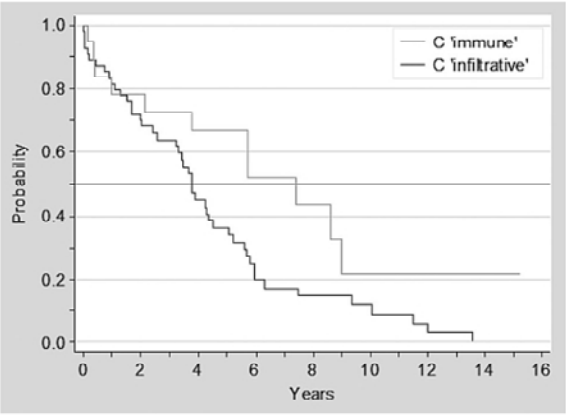


Figure 1. Survival of patients with CLL in advanced clinical stage C infiltrative versus C immune.

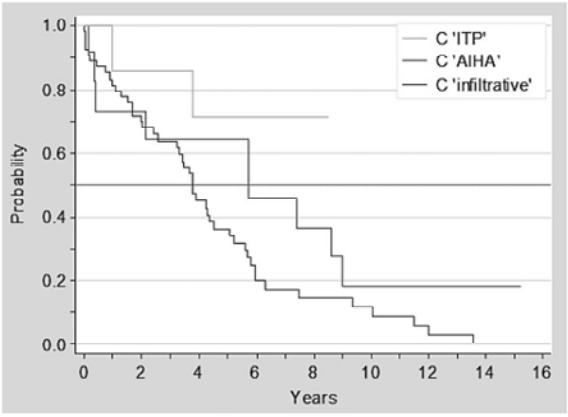


Figure 2. Survival of patients with CLL in advanced clinical stage C infiltrative versus AIHA versus ITP.

median survivals were as follows: stage A, 10.2 years; stage B, 5.6 years; stage C immune, 7.4 years; and stage C infiltrative, 3.7 years (*P* .001; Figure 3).

Discussion

The association of CLL and autoimmune cytopenia was already recognized in seminal descriptions of the disease published in the late 1960s.[15-17] The occurrence of AIHA or ITP has been reported to range from 5% to 38%, depending on patient selection, length of follow-up, and definition of autoimmune cytopenia.[3,6] In the present analysis, using strict diagnostic criteria, 70 of 960 patients with CLL were found to have AIHA or ITP at some point during the course of their disease. This results in a prevalence of 7%, which is in agreement with recent studies in which the percentage of patients with CLL and autoimmune cytopenia ranges from 4.5% to 10%.[4,5,7] Note that for ITP, besides difficulties in diagnostic criteria, only acute onset, symptomatic ITP is usually recognized; therefore, the frequency of this complication might be underestimated.

The mechanisms underlying immune cytopenia in CLL are unclear, but red blood cell autoantibodies and imbalances in immunosurveillance mechanisms, including T-regulatory cells, are considered to play a crucial role.[18-22] In addition, treatment-triggered autoimmune cytopenia was recognized in the first comprehensive descriptions of CLL.[15,17] In the early 1990s, however, there was concern that treatment with purine analogs could, through their potent immunosuppressive effect, be associated with a higher frequency of AIHA that in some instances could eventually be fatal. These cases have been mainly observed in patients with active immune cytopenia and heavily pretreated.[23-27] As a result of these observations, there is agreement that purine analogs should be avoided in patients with autoimmune cytopenia, particularly if related to purine analogs, or patients with active autoimmune cytopenia or DAT at study entry.

However, in patients with no prior history of AIHA there is evidence that the risk of developing this complication on exposure to purine analogs is not superior to that observed with other agents. In line with this notion, in our series 4% of 204 patients exposed to fludarabine-containing regimens presented autoimmune cytopenia compared with 5% of 231 patients treated with chlorambucil. This is in agreement with recent studies.[28,29] Thus, as part of the UK LRF CLL4 trial analyses, Dearden et al[29] reported on the incidence and prognostic significance of DAT positivity and overt clinically apparent AIHA in 777 patients with CLL. No differences in the percentage of patients becoming DAT after therapy (14% chlorambucil, 13% fludarabine, and 10% fludarabine plus cyclophosphamide) were observed. Notably, the incidence of AIHA was significantly lower in patients treated with fludarabine plus cyclophosphamide (5%) than in patients allocated to receive chlorambucil (12%) or fludarabine alone (11%; *P* .01), suggesting that the addition of cyclophosphamide to fludarabine might have a "protective" effect on the appearance of AIHA.[29] In another study from the M.D. Anderson group the incidence of AIHA in 300 patients (of which 8 had previously presented with AIHA) treated with fludarabine, cyclophosphamide, and rituximab was 6.5%,[28] and, in the recent German CLL8 trial, the global incidence of AIHA in 793 patients was 1% with no significant differences between patients treated with fludarabine and cyclophosphamide with or without rituximab (Michael Hallek, University of Cologne, written communication, April 26, 2010).

From the clinical standpoint, male sex, older age, a high blood lymphocyte count, and advanced disease have been classically associated with autoimmune cytopenia.[4,7,30] In conformity with these observations, in our series a high blood lymphocyte count at diagnosis and a short LDT (ie. 12 months) correlated with the risk of developing autoimmune cytopenia. More recently, some studies have found an association between autoimmune cytopenia

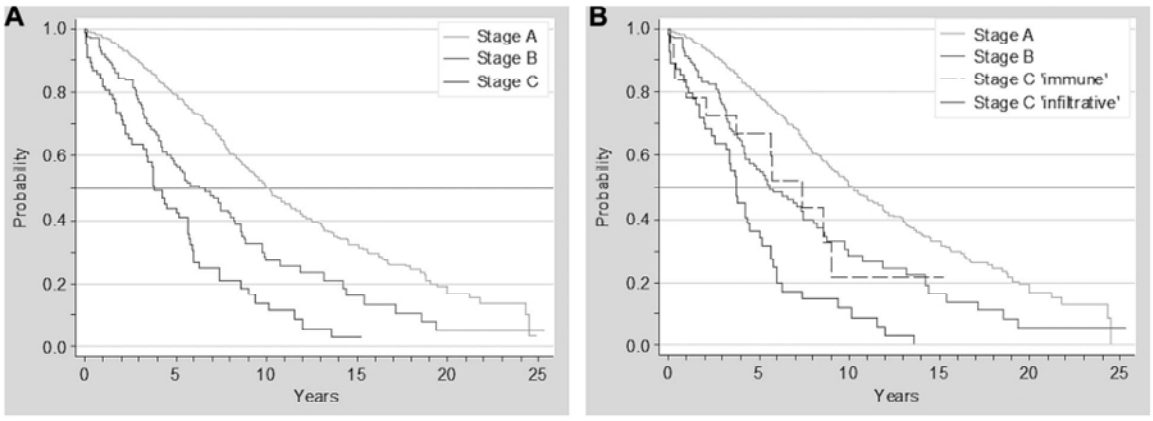


Figure 3. Survival of patients with CLL according to Binet staging system. Survival of patients with CLL according to advanced Binet staging system (A) and to a modified Binet staging system, breaking down stage C according to the origins of cytopenia (infiltrative vs immune; B).

and unfavorable biomarkers, including unmutated *IGHV* genes,[7,31-33] high expression of CD38[34] or ZAP-70,[7,35] and increased B2M serum levels.[28,29,32] In our series we also found a correlation between increased serum B2M levels, high ZAP-70 or CD38 expression, and autoimmune cytopenia. Unfortunately, because of the retrospective nature of the study limited information was available for *IGHV* mutational status and cytogenetics, with this precluding a meaningful analysis on the basis of these latter parameters.

The most controversial issue for immune cytopenia in CLL is its prognostic significance.[4,5,7,36] Mauro et al[4] reported an association between disease activity and AIHA but no effect on survival. Two other reports evaluated the prognostic significance of the origin of cytopenia, concluding that, whereas cytopenia because of bone marrow failure confers poor prognosis, autoimmune cytopenia is not an adverse prognostic factor.[5,36] More recently a multicenter study showed that patients with CLL and ITP had worse prognosis than patients who did not develop this complication probably because of a strong association between the development of ITP and poor prognostic variables (ie, unmutated *IGHV*).[7,33]

Against this background, we first investigated whether the occurrence of autoimmune cytopenia had any effect on the outcome of patients with CLL. Overall, in our series the development of autoimmune cytopenia at any time during the evolution of the disease did not significantly influence prognosis. Nevertheless, survival of patients in early stage (Binet A) and immune cytopenia was slightly worse (median, 9 years) than that of patients not presenting with this complication (median, 10 years). Although this difference was not statistically significant, it is of interest that an association between AIHA and progressive stage A disease was identified some years ago.[37] In addition, the time at which autoimmune cytopenia was noticed (ie, at diagnosis, during the course of the disease) had no prognostic effect.

Rai et al[8] and Binet et al[9] systems do not take into account the origin of cytopenia to define clinical stages. We, therefore, analyzed the outcome of patients in advanced clinical stage (Binet C) at diagnosis according to the origin of the cytopenia, and we found that patients with cytopenia because of immune mechanisms (C immune) had longer survival than patients with stage C because of bone marrow infiltration (C infiltrative). Interestingly, this observation was first made 20 years ago by Geisler and Hansen[11] in a short series of patients and has been now confirmed in larger series of patients from the Mayo Clinic[5] and our own study. The main reason for the better outcome of patients with cytopenias of immune origin relies on the response to therapy. Not surprisingly a large proportion of patients with stage C immune did respond to corticosteroids and, as a result, shifted to stage A. In contrast, only a small proportion of patients with stage C infiltrative did respond to therapy. Despite this, however, patients with stage C immune still had an inferior outcome than patients with stage A disease, their prognosis being closer to patients with stage B disease. Although the reasons for these findings are not entirely clear, autoimmune cytopenias correlated with poor prognostic variables; therefore, it is tempting to speculate that autoimmune phenomena are a fingerprint of a biologically more aggressive disease.

In conclusion, in this large, unselected and single institution series, which should therefore be considered as representative of the general population of subjects with CLL, the prevalence of autoimmune cytopenia was 7%. Autoimmune cytopenia correlated with well-known poor prognostic variables, including high blood lymphocyte count, short LDT, as well as high serum B2M level and high ZAP-70 and CD38 expressions, but not with treatment modality (fludarabine based vs alkylator based). From the prognostic point of view, the development of autoimmune cytopenia did not significantly influence prognosis in the whole group of patients. Importantly, however, patients presenting with advanced disease related to an immune mechanism had better prognosis than patients in whom advanced stage reflected a high tumor burden only. Therefore, determining the cause of cytopenia (immune vs infiltrative) is important not only for prognostic but also for therapeutic considerations. Finally, the different outcome of patients with advanced disease according to the origin of cytopenia, as shown in this study and others,[5] also makes a case for including a stage C immune group in the prognostic categorization of pa- tients with CLL.

Acknowledgments

This work was supported in part by Red Tematica de Investigacio´n Cooperativa en Cancer (grant RT 06/0020/002051), Spanish Ministry of Science, Instituto Carlos III (FISS PI080304), Generalitat de Catalunya (2009SGR1008), CLL Global Foundation, and Premi Distincio Generalitat de Catalunya (EM), and by a grant from Instituto de Salud Carlos III (PFIS; G.F.).

Authorship

Contribution: C.M. designed the study, analyzed the data, and wrote the paper; K.H. and G.F. analyzed the data and wrote part of the paper; M.E., X.F., and T.B. analyzed the data and the study results; A.P. performed the statistical analysis; and E.M. designed the study along with C.M., wrote the paper, and approved its final version.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

The current affiliation for C.M. is M. D. Anderson International, Madrid, Spain.

Correspondence: Emili Montserrat, Hematology Department, Hospital Clínic, Villarroel 170, Barcelona 08036, Spain; e-mail address: emontse@clinic.ub.es.

References

1. Rozman C, Montserrat E. Chronic lymphocytic leukemia. *N Engl J Med.* 1995;333(16):1052-1057.
2. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. *N Engl J Med.* 2005;352(8):804-815.
3. Hamblin TJ. Autoimmune complications of chronic lymphocytic leukemia. *Semin Oncol.* 2006;33(2):230-239.
4. Mauro FR, Foa R, Cerretti R, et al. Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. *Blood.* 2000;95(9):2786-2792.
5. Zent CS, Ding W, Schwager SM, et al. The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma. *Br J Haematol.* 2008;141(5):615-621.
6. Dearden C. Disease-specific complications of chronic lymphocytic leukemia. *Hematology Am Soc Hematol Educ Program.* 2008:450-456.
7. Visco C, Ruggeri M, Laura Evangelista M, et al. Impact of immune thrombocytopenia on the clinical course of chronic lymphocytic leukemia. *Blood.* 2008;111(3):1110-1116.
8. Rai KR, Sawitsky A, Cronkite EP, et al. Clinical staging of chronic lymphocytic leukemia. *Blood.* 1975;46(2):219-234.
9. Binet JL, Auquier A, Dighiero G, et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer.* 1981;48(1):198-206.
10. Moreno C, Montserrat E. New prognostic markers in chronic lymphocytic leukemia. *Blood Rev.* 2008;22(4):211-219.

11. Geisler C, Hansen MM. Chronic lymphocytic leukaemia: a test of a proposed new clinical staging system. *Scand J Haematol.* 1981;27(4):279-286.
12. Cheson BD, Bennett JM, Rai KR, et al. Guidelines for clinical protocols for chronic lymphocytic leukemia: recommendations of the National Cancer Institute-sponsored working group. *Am J Hematol.* 1988;29(3):152-163.
13. Cheson BD, Bennett JM, Grever M, et al. National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. *Blood.* 1996;87(12):4990-4997.
14. Armitage P. *Statistical Methods in Medical Research*. Malden, MA: Blackwell Science; 2001.
15. Lewis FB, Schwartz RS, Dameshek W. X-radiation and alkylating agents as possible "trigger" mechanisms in the autoimmune complications of malignant lymphoproliferative disease. *Clin Exp Immunol.* 1966;1(1):3-11.
16. Dameshek W. Chronic lymphocytic leukemia-an accumulative disease of immunologically incompetent lymphocytes. *Blood.* 1967;29(4):566-584.
17. Galton DA. The pathogenesis of chronic lymphocytic leukemia. *Can Med Assoc J.* 1966;94(19):1005-1010.
18. Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells. *Blood.* 2005;105(5):2007-2015.
19. Beyer M, Kochanek M, Darabi K, et al. Reduced frequencies and suppressive function of CD4 CD25hi regulatory T cells in patients with chronic lymphocytic leukemia after therapy with fludarabine. *Blood.* 2005;106(6):2018-2025.
20. Galletti J, Canones C, Morande P, et al. Chronic lymphocytic leukemia cells bind and present the erythrocyte protein band 3: possible role as initiators of autoimmune hemolytic anemia. *J Immunol.* 2008;181(5):3674-3683.
21. Bowen DA, Call TG, Shanafelt TD, et al. Treatment of autoimmune cytopenia complicating progressive chronic lymphocytic leukemia/small lymphocytic lymphoma with rituximab, cyclophosphamide, vincristine, and prednisone. *Leuk Lymphoma.* 2010;51(4):620-627.
22. Kaufman M, Rai K. The challenge of treating complex autoimmune cytopenias in chronic lymphocytic leukemia. *Leuk Lymphoma.* 2010;51(4):574-575.
23. Bastion Y, Coiffier B, Dumontet C, Espinouse D, Bryon PA. Severe autoimmune hemolytic anemia in two patients treated with fludarabine for chronic lymphocytic leukemia. *Ann Oncol.* 1992;3(2):171-172.
24. Myint H, Copplestone JA, Orchard J, et al. Fludarabine-related autoimmune haemolytic anaemia in patients with chronic lymphocytic leukaemia. *Br J Haematol.* 1995;91(2):341-344.
25. Byrd JC, Hertler AA, Weiss RB, et al. Fatal recurrence of autoimmune hemolytic anemia following pentostatin therapy in a patient with a history of fludarabine-associated hemolytic anemia. *Ann Oncol.* 1995;6(3):300-301.
26. Tertian G, Cartron J, Bayle C, Rudent A, Lambert T, Tchernia G. Fatal intravascular autoimmune hemolytic anemia after fludarabine treatment for chronic lymphocytic leukemia. *Hematol Cell Ther.* 1996;38(4):359-360.
27. Weiss RB, Freiman J, Kweder SL, Diehl LF, Byrd JC. Hemolytic anemia after fludarabine therapy for chronic lymphocytic leukemia. *J Clin Oncol.* 1998;16(5):1885-1889.
28. Borthakur G, O'Brien S, Wierda WG, et al. Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab; incidence and predictors. *Br J Haematol.* 2007;136(6):800-805.
29. Dearden C, Wade R, Else M, et al. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. *Blood.* 2008;111(4):1820-1826.
30. Barcellini W, Capalbo S, Agostinelli RM, et al. Relationship between autoimmune phenomena and disease stage and therapy in B-cell chronic lymphocytic leukemia. *Haematologica.* 2006;91(12):1689-1692.
31. Vanura K, Le T, Esterbauer H, et al. Autoimmune conditions and chronic infections in chronic lymphocytic leukemia patients at diagnosis are associated with unmutated IgVH genes. *Haematologica.* 2008;93(12):1912-1916.
32. Ricci F, Tedeschi A, Vismara E, et al. Positive direct antiglobulin test (DAT) in chronic lymphocytic leukemia (CLL) patients: correlation with prognostic factors [abstract]. *Blood.* 2009;114(22):Abstract 2351.
33. Visco C, Novella E, Peotta E, et al. Autoimmune haemolytic anemia in patients with chronic lymphocytic leukemia is associated with IgVH status. *Haematologica.* 2010;95(7):1230-1232.
34. Ghia P, Guida G, Stella S, et al. The pattern of CD38 expression defines a distinct subset of chronic lymphocytic leukemia (CLL) patients at risk of disease progression. *Blood.* 2003;101(4):1262-1269.
35. Zanotti R, Frattini F, Ghia P, et al. ZAP-70 expression is associated with increased risk of autoimmune cytopenias in CLL patients. *Am J Hematol.* 2010;85(7):494-498.
36. Kyasa MJ, Parrish RS, Schichman SA, Zent CS. Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma. *Am J Hematol.* 2003;74(1):1-8.
37. Hamblin TJ, Oscier DG, Young BJ. Autoimmunity in chronic lymphocytic leukaemia. *J Clin Pathol.* 1986;39(7):713-716.

Revisión 1: Chronic lymphocytic leukemia and autoimmunity: a systemic review.

Kate Hodgson*, Gerardo Ferrer*, Emili Montserrat, Carol Moreno. Haematologica. 2011; 96(5):752-61.

(*) Equal contribution.

Resumen

En este trabajo llevamos a cabo una revisión sistemática de la literatura de acuerdo con la normativa PRISMA [225] en relación a la autoinmunidad en la LLC. Además de realizar un análisis de las citopenias autoinmunes con la LLC también revisamos la autoinmunidad no hemática. De esta exhaustiva revisión, en la que todos las publicaciones pertinentes fueron estudiadas independientemente por tres de los autores (GF, KH, CM), se pudo concluir que a pesar de que en la literatura existen múltiples publicaciones (por lo general de casos aislados o de series muy pequeñas de enfermos) sobre la coexistencia de LLC y enfermedades autoinmunes no sanguíneas, los individuos con LLC no tiene un mayor riesgo de presentar enfermedades autoinmunes no hemáticas ni que, de igual modo, los pacientes con trastornos autoinmunes no hemáticas padezcan LLC con mayor frecuencia que la población general. Esta conclusión concuerda con los pocos estudios epidemiológicos llevados a cabo con rigor. En cambio, la asociación de LLC con citopenias autoinmunes está muy bien demostrada, sobre todo por lo que hace a la AHAI y a la PTI. La asociación de LLC con eritroblastopenia también es un hecho, aunque menos frecuente que la de LLC con AHAI o PTI. Sin embargo, la asociación de LLC con neutropenia autoinmune es excepcional y los pocos casos registrados, la mayor parte de ellos en publicaciones antiguas, invitan a pensar que tales neutropenias no estaban asociadas a LLC sino a otros síndromes linfoproliferativos, por ejemplo la linfocitosis de linfocitos grandes granulares.

Chronic lymphocytic leukemia and autoimmunity: a systematic review

Kate Hodgson, Gerardo Ferrer, Emili Montserrat, and Carol Moreno*

Institute of Hematology and Oncology, Department of Hematology, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), University of Barcelona, Barcelona, Spain

Current address: Department of Hematology, Hospital de la Santa Creu i Sant Pau, Barcelona, Spain.

ABSTRACT

Chronic lymphocytic leukemia is frequently associated with immune disturbances. The relationship between chronic lymphocytic leukemia and autoimmune cytopenias, particularly autoimmune hemolytic anemia and immune thrombocytopenia, is well established. The responsible mechanisms, particularly the role of leukemic cells in orchestrating the production of polyclonal autoantibodies, are increasingly well understood. Recent studies show that autoimmune cytopenia is not necessarily associated with poor prognosis. On the contrary, patients with anemia or thrombocytopenia due to immune mechanisms have a better outcome than those in whom these features are due to bone marrow infiltration by the disease. Moreover, fears about the risk of autoimmune hemolysis following single agent fludarabine may no longer be appropriate in the age of chemo-immunotherapy regimens. However, treatment of patients with active hemolysis may pose impor- tant problems needing an individualized and clinically sound approach. The concept that autoimmune cytopenia may precede the leukemia should be revisited in the light of recent data showing that autoimmune cytopenia may be observed in monoclonal B-cell lymphocytosis, a condition that can only be detected by using sensitive flow cytometry techniques. On the other hand, there is no evidence of an increased risk of non-hemic autoimmune disorders in chronic lymphocytic leukemia. Likewise, there is no epidemiological proof of an increased risk of chronic lymphocytic leukemia in patients with non-hemic autoimmunity. Finally, since immune disor- ders are an important part of chronic lymphocytic leukemia, studies aimed at revealing the mechanisms linking the neoplas- tic and the immune components of the disease should help our understanding of this form of leukemia.

Citation: Hodgson K, Ferrer G, Montserrat E, and Moreno C. Chronic lymphocytic leukemia and autoimmunity: a systematic review. Haematologica 2011;96(5):752-761. doi:10.3324/haematol.2010.036152

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of monoclonal lymphocytes with a distinctive immunophenotype (i.e. $CD5^+$, $CD19^+$, $CD20^{dim}$, $CD23^+$, $SmIg^{dim}$) in peripheral blood, bone marrow, and lymphoid tissues.[1,2] Patients with CLL frequently present with immune disturbances, which constitute a notable feature of the disease compared to other chronic lymphoproliferative disorders.[3-8] In this paper, we will review autoimmune disorders in CLL, their incidence, pathophysiological mechanisms, prognostic impact, and management.

Design and Methods

To identify studies that examined the epidemiological evidence for an association between CLL and autoimmune disease, as well as case reports and series regarding CLL and autoimmune phenomena, we searched PUBMED using the keywords that are specified in the *Online Supplementary Appendix*. The abstracts and papers linked to the PUBMED searches were scanned to identify any reports not included in this computerized search. For CLL-associated immune cytope- nia, we focused on prevalence, outcome and association with prognostic variables, and therapy. For non-hemic autoimmunity, we included all original case reports and series published in English which discussed the presentation of autoimmune phenomena in patients with CLL. The evidence of any causal association between the CLL and non-hemic autoimmune disease was independently assessed by KH and CM for each case report. The process we used to identify and report this litera- ture was modeled on the PRISMA consensus, adapted to rec- ognize the observational nature of the data and the year of publication of many of the case reports.[9]

Epidemiology

The association of CLL and autoimmune cytopenia was recognized in the late 1960s.[3,4,10,11] A positive direct antiglobu-

KH and GF contributed equally to this manuscript.
The online version of this article has a Supplementary Appendix.
Acknowledgments: the authors would like to thank Nick Chiorazzi (The Feinstein Institute for Medical Research, North Shore-Long Island Jewish Health System, Manhasset, NY, USA) for the critical review of the manuscript and helpful comments, and Professor Mariano Monzo (Medical School, University of Barcelona, Spain) for his ongoing support.
Funding: this work has been supported thanks in part to Red Temàtica de Investigación Cooperativa en Cáncer grant RT 06/0020/002051 of the Spanish Ministry of Science. Instituto Carlos III FISS PI080304, Generalitat de Catalunya 2009SGR1008 and CLL Global Foundation. GF is a recipient of a grant from Instituto de Salud Carlos III (PFIS).
Manuscript received on October 26, 2010. Revised version arrived on December 15, 2010. Manuscript accepted on January 11, 2011.
Correspondence: Emili Montserrat, M.D., Department of Hematology, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain. Phone: international +34.9.32275475. E-mail: emontse@clinic.ub.es

lin test (DAT) with or without frank AIHA is strongly associated with CLL,[12-15] as are immune thrombocytopenia (ITP)[16-18] and pure red cell aplasia (PRCA).[19] The occurrence of immune cytopenia has been reported to range from less than 5% to 38%.[14,20] In the most recent studies, the proportion of patients presenting with autoimmune cytopenia at some point during the course of their disease ranges from 4.3% to 9.7%.[12,13,15,21,22] The most common complication is AIHA (about 7%) whereas the incidence of ITP, and particularly autoimmune neutropenia and PRCA, is lower in most studies (<1-2%). There are no case reports or epidemiological studies suggesting a link between CLL and autoimmune diseases affecting the blood coagulation system, such as acquired hemophilia or acquired von Willebrand disease.

Regarding non-hemic autoimmunity, several early studies described an increased incidence of autoimmune phenomena other than autoimmune cytopenia in CLL. In line with this, in studies published in the 1980s, autoimmune disease (AID) was reported to be more common in relatives of patients with CLL than in controls.[23] Also, autoimmunity was shown to be much more common in patients with lymphoproliferative disorders, including CLL, than in patients with myeloproliferative conditions (8% *vs.* 1.7%).[16]

In more recent studies (Table 1), clinically apparent autoimmune disorders have been reported in 2% to 12% of patients whereas positive serum markers for a variety of autoimmune conditions ("serological autoimmunity") have been found in 8% to 41% of patients.[17,25,27] However, case-control studies do not suggest an increase in AID in patients with CLL.[5]

These observations have been followed up by larger studies, examining AID as a risk factor for the development of CLL and as a complication of CLL. Regarding the possibility that autoimmune conditions predispose to CLL, a Nordic case-control study looked at the risk of developing CLL in the context of personal or family history of AID. The risk of CLL was much higher in individuals with a personal history of AIHA (odds ratio, OR 104), and somewhat raised in those with pernicious anemia (PA; OR 1.94).[28] Likewise, in another large study, individuals who developed CLL had a much higher incidence of prior AIHA compared to those who did not, meaning that AIHA carried a 3.86-fold increased risk of developing CLL. No link to other autoim- mune diseases, including pernicious anemia, was observed.[29] The association between AIHA and risk of developing CLL is difficult to interpret because, as discussed later, many of these cases might harbor a CLL clone which is not easily detectable by conventional diagnostic methods.

In a large patient-control study, a positive OR (6.7) for AIHA was observed, with a trend to increased risk for ITP.[30] No increased risk of other AID was observed. In another large study looking at patients with ulcerative colitis, though an increased incidence of non-Hodgkin's lym- phoma was observed, there was no increase in CLL.[31] In a recent review of the risk of lymphoid malignancy in patients with AID, there was elevated risk of organ specific lymphoma, e.g. celiac disease and enteropathy associated

Table 1. Case series of autoimmune cytopenias in chronic lymphocytic leukemia.

Author, Date	Population	Number of patients, time interval	Outcome	Clinical/biological correlates
Hamblin 1986[5]	195 patients from a single institution	19 of 195 (9.7%), 1972-1985 15 AIHA 4 ITP	Not reported	Not reported
Kyasa 2000[24]	132 patients from a single primary care system	12 of 132 (9.1%), 1989-2001 6 AIHA 5 ITP 1 PRCA	OS not different to CLL patients without autoimmune complications	Not reported
Mauro 2000[12]	1,203 patients from a single institution	52 of 1,203 (4.3%), 1986-1996	OS not different in DAT positive anemic CLL and DAT negative non-anemic CLL patients	High WCC, advanced age, male gender, active CLL
Barcellini 2006[17]	3,150 patients from the GIEMA group, 17 institutions	194 of 3,150 (6.2%), unspecified time interval 129 AIHA 35 ITP	Not reported	AIHA associated with advanced stage active CLL, old age
Duek 2006[25]	National CLL registry of 964 patients	63 of 964 (6.55%), 1971-2006 55 DAT pos at diagnosis 9 ITP or Evans	Not reported	High B2M, high CD38
Visco 2008[18]	1,278 patients from 3 institutions	64 of 1,278 (5%) ITP, 1996-2004 47 AIHA 28 DAT pos only	OS worse in CLL patients with ITP than those who never develop ITP. OS of patients with thrombocytopenia at diagnosis significantly worse than non-thrombocytopenic CLL regardless of etiology	High WCC, unmutated *IgVH* gene, high Zap70
Zent 2008, 2009[1826]	1,750 patients from a single institution	75 of 1,750 (4.5%), 1995-2004 41 AIHA 35 ITP 9 PRCA 3 AIG	OS not different to CLL patients who never develop cytopenia. OS since cytopenia was superior in patients with immune cytopenia compared to cytopenia due to bone marrow failure	Male gender, unmutated *IgVH* gene, high Zap70, poor risk cytogenetics
Moreno 2010[26]	961 patients from a single institution	70 of 960 (7%), 1980-2008 49 AIHA 20 ITP 1 Evans	OS not different to CLL patients who never develop AID. OS of immune cytopenia at presentation superior to OS of stage C at presentation	High WCC, high LDT, high B2M, high CD38

AIHA: autoimmune hemolytic anemia; ITP: immune thrombocytopenia; PRCA: pure red cell aplasia; DAT: direct anti-globulin test; OS: overall survival; WCC: white cell count; LDT: lymphocyte doubling time; B2M: beta 2 microglobulin; IgVH: immunoglobulin heavy chain variable region.

T-cell lymphoma. However, risk of CLL was not increased.[32]

Further interest in the link between autoimmunity and CLL comes from genetic studies. Both CLL[33,34] and autoimmunity[35] are known to have a hereditary component. In the Nordic study no general increase in risk of CLL was associated with family history of AID. The lack of a link between family history of AID and CLL risk was taken to exclude an underlying genetic predisposition linking CLL and AID.[20]

Biological aspects

The biological explanation for the frequency of autoimmune cytopenia in CLL is complex and not completely understood (reviewed by Kipps and Carson,[6] Calgaris-Cappio[36] and Ghia *et al.*[37]), with neoplastic CLL cells, T cells and microenvironment cells playing a role (Figure 1).

Although it has been proposed that CLL derives from marginal zone B cells,[30,39] the normal counterpart of the CD5$^+$ B CLL cell has not been fully elucidated (reviewed in [40]). In mouse models of CLL, CD5$^+$ B cells (B1a cells) are most plentiful in the peritoneal cavity and can produce polyreactive antibodies that bind DNA and can act as rheumatoid factors, i.e. bind IgG.[41,42] However, human CD5$^+$ B cells rarely produce auto-antibodies and may not be an exact equivalent of the mouse B1 cell, at least not as the cell of origin of CLL.[37,43]

The B-cell response to antigens is mediated by the B-cell receptor (BCR). The analysis of the BCR in patients with CLL shows a stereotyped repertoire with identical or quasi-identical sequence, suggesting selection of B cells with antigen binding sites of restricted structure (reviewed in [39,44]). CLL cells, particularly those with unmutated *IGHV* gene, can present a highly polyreactive BCR which recognizes auto-antigens.[45, 45-47] Of note, the same antigens are recognized by "natural" antibodies known to be pathological in certain autoimmune diseases.[48]

However, the BCR signaling in CLL can be defective and this has been related to the low number of surface immunoglobulin molecules on CLL cells,[49] non function al assembly of the BCR,[50,51] and mutations in accessory proteins.[52] Despite this, CLL cells can produce auto-reactive antibodies *in vitro* after stimulation.[53,54] Although in rare instances CLL cells produce auto-reactive antibodies *in vivo* in sufficient quantity to cause clinical disease (e.g. cold agglutinin disease, discussed below), the autoimmune cytopenias which are a common feature of CLL are caused by polyclonal antibodies.[20] The capacity of CLL cells to function as antigen presenting cells is nearly abrogated *in vitro*, the exception to this rule being red cell antigen Rh processing.[55] An alternative red cell antigen, B3, has also been demonstrated to be processed by CLL cells, which are then able to provoke a T-cell response.[56] It has been noted that AIHA is more common in advanced CLL, where the spleen is heavily infiltrated by leukemic cells,[12] which brings CLL cells in close proximity to damaged red blood cells.[56] In this regard, the spleen also contains CD40 ligand-expressing T cells which *in vitro* are able to induce activation of CLL cells and improve antigen presentation.[57] On the other hand, CLL cells interact with T cells to modulate the immune environment, which may be important in permitting the development of autoimmunity. Thus, CLL is characterized by acquired T-cell defects including numerical increase in T cells, inversion of the CD4:CD8 ratio, production by CLL cells of the inhibitory cytokines IL-6, IL-10, TNF and TGF-β, as well as alterations in T-cell cytoskeleton formation and vesicle transportation.[58-63] Finally, it is worth mentioning that CLL is associated with impairment of the innate immune system.[64-67]

Autoimmune cytopenia in chronic lymphocytic leukemia

Clinical and biological correlates

Several clinical and biological features of CLL have been associated with an increased risk of developing autoimmune cytopenia (Table 2). In most studies, a correlation between advanced stage and the risk of AIHA has been reported.[5,17] In line with this, AIHA has also been associated with active CLL.[12] Older patients also seem to be more

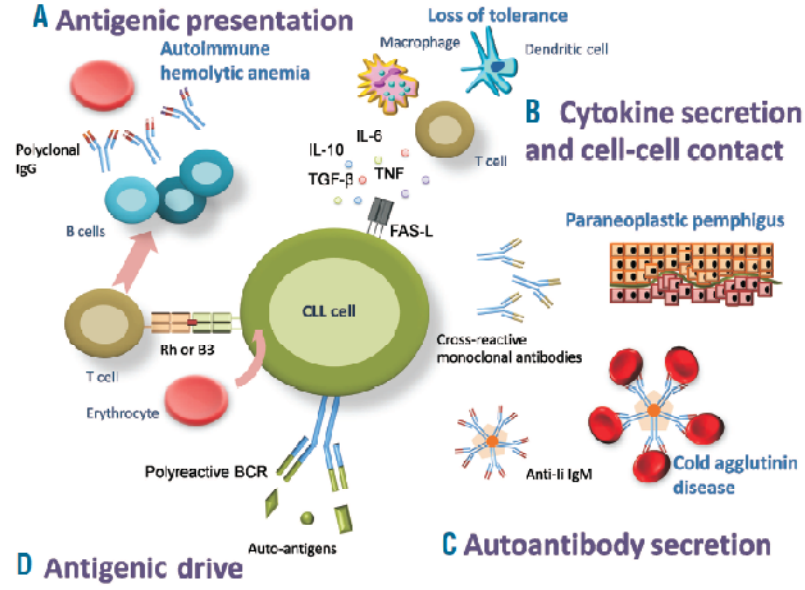


Figure 1. Mechanisms of autoimmune disease in CLL. (A) CLL cells may process red blood cell antigens and act as antigen presenting cells, inducing a T-cell response and the formation of polyclonal antibodies by normal B cells, thus indirectly provoking autoimmune hemolytic anemia. (B) CLL cells express inhibitory cytokines that alter tolerance, which may facilitate the escape of self-reactive cells. (C) Rarely CLL cells are effector cells that secrete a pathological monoclonal autoantibody. Two such diseases are paraneoplastic pemphigus, where immunoglobulins are cross-reactive with epitopes located at the dermal-epidermal junction, and cold agglutinin disease, where IgMs have anti-red cell reactivity. (D) In turn, CLL cells may be stimulated through their polyreactive BCR that recognizes auto-antigens.

prone to develop this complication, independently of CLL stage or duration.[12,17,22]

Due to the retrospective nature of most studies, the relationship between newer biological prognostic markers and autoimmune cytopenia has not been comprehensively assessed. Nevertheless, both AIHA and ITP have been associated with poor prognostic factors such as unmutated *IGHV* gene, high ZAP70 expression, and increased serum beta-2 microglobulin levels.[13,15,68] The stereotyped BCR seen in CLL may be reactive with autoantigens.[69]

Although the risk of immune cytopenia increases over the course of the disease, it can be the presenting feature of CLL and it has been classically considered that it can precede the diagnosis of CLL.[13,15,24] The association between a prior history of AIHA or ITP and the risk of presenting CLL should be interpreted with caution because peripheral blood flow cytometry is not generally performed as part of the routine diagnostic work up of AIHA. Supporting this caveat is the recent observation that the precursor condition known as monoclonal B-cell lymphocytosis (MBL) is markedly more common in patients with supposed idiopathic AIHA or ITP than in matched controls.[70] This reflects the importance of excluding CLL and other chronic lymphoproliferative diseases in patients with AIHA.[71]

The possibility that therapy could trigger autoimmune cytopenia in patients with CLL was recognized in initial descriptions of the disease.[4,11] In the early 1990s, however, there was concern that treatment with purine analogs (particularly fludarabine) could be associated with a higher frequency of autoimmune cytopenia.[72-74] This was thought to be related to prolonged suppression of $CD4^+$ T cells by fludarabine. A decrease in $CD4^+CD25^+FOXP3^+$ regulatory T cells (Tregs) has been shown to lead to AID and Tregs are highly sensitive to fludarabine (reviewed in [75]).The cases reported were mainly observed in heavily pre-treated patients with active immune cytopenia who had already received purine analogs.[72,74,76] As a result of these observations, it is now agreed that purine analogs should be avoided in patients with a history of autoimmune cytopenia, particularly if related to purine-analog therapy.

Current evidence shows that the risk of developing autoimmune cytopenia after purine analog exposure is no greater than with other agents.[21,22] In the UK CLL4 trial, no differences were observed in the percentage of patients becoming DAT-positive after therapy (14% chlorambucil, 13% fludarabine, and 10% fludarabine plus cyclophosphamide). Notably, the incidence of AIHA was significantly lower in patients treated with fludarabine plus cyclophosphamide (5%) than in those allocated to receive chlorambucil (12%) or fludarabine alone (11%) ($P<0.01$).[22] This suggests that the addition of cyclophosphamide to fludarabine might have a "protective" effect on the appearance of AIHA. An earlier smaller study supports this low incidence of AIHA in patients treated with fludarabine, cyclophosphamide and rituximab.[21] In our own experience, the incidence of AIHA was slightly lower after fludarabine-based therapy (4%) than after chlorambucil treatment (5%).[15] The most recent data comes from the German CLL 8 trial of patients with CLL requiring treatment and without clinically apparent autoimmune cytopenia. When treated with fludarabine and cyclophosphamide with or without rituximab the rate of AIHA was 1%.[77] Taken together these results demonstrate that the risk of AIHA is not higher following regimes in which fludarabine and cyclophosphamide (with or without rituximab) are given together in comparison to the risk seen after older therapies for CLL. The best approach to treatment of autoimmune cytopenia in CLL is discussed below.

Prognostic significance

The effect of autoimmune cytopenia on prognosis of patients with CLL remains uncertain. There are few studies investigating this issue in large unselected series from single institutions such as would be representative of the general population with CLL.

In a series of 1,203 patients with CLL, AIHA has been associated with active disease, but without a negative impact on survival.[12] In a cohort of 1,750 patients with CLL associated cytopenia, the relative outcome was compared between cytopenia due to bone marrow failure and immune cytopenia. Patients with immune cytopenia at diagnosis had a better outcome than those in whom cytopenia was due to bone marrow failure,[13] and the later development of autoimmune cytopenia did not result in a worse prognosis than that of patients who never developed this complication.[26] Our group has investigated the impact of autoimmune cytopenia on CLL outcome in a series of 961 patients.[15] Patients who had autoimmune cytopenia at the time of diagnosis had a clearly superior survival than those who presented with cytopenia due to bone marrow failure. Similarly, development of autoimmune cytopenia at any stage in the disease did not have an impact on survival.

The UK CLL4 trial mentioned above assessed the prognostic effect of a positive DAT in 783 patients with CLL requiring treatment for the first time. A positive DAT predicted a poorer response to treatment. Both a positive DAT and AIHA were associated with a lower overall survival.[22] The authors suggest that DAT at the time of therapy may be a prognostic indicator. It is important to note, however, that this study was performed in patients requiring therapy and, hence, with poor prognosis.

A study of ITP in 1,278 patients with CLL showed that acute ITP at diagnosis or at any time in the disease was associated with an inferior outcome compared to those patients who never developed ITP, independently of other clinical prognostic variables[18] but probably related to the association of ITP with an unmutated *IGHV* gene.[68] Interestingly, the same group has demonstrated a similar association between an unmutated *IGHV* gene and AIHA, without the same negative impact on survival.[78] PRCA and

Table 2. Prognostic factors correlated with autoimmune cytopenia in CLL.

Clinical prognostic factors	References
Advanced stage	(5, 13, 17, 24)
Older age	(12, 17, 22)
Male	(12, 13)
High white cell count	(12, 15, 18)
Short lymphocyte doubling time	(15, 17)
Biological prognostic factors	
Beta 2 microglobulin	(15, 22, 25)
High CD38	(15, 25)
High ZAP 70	(13, 18)
Unmutated *IGVH* genes	(13, 18, 68)
Poor risk cytogenetics	(13)

autoimmune neutropenia are much less common, and as such, information concerning the prognostic impact of PRCA is not considered individually.

Diagnosis and management

A high degree of suspicion is required to diagnose autoimmune cytopenia in patients with CLL. Appropriate laboratory investigations include a DAT, lactate dehydrogenase (LDH), bilirubin, haptoglobins, and reticulocyte count. A bone marrow examination (aspirate and biopsy) is particularly important to differentiate between the causes of cytopenia. The multiple possible causes of cytopenia in CLL (bone marrow failure, hypersplenism, chemotherapy, sep- sis, autoimmunity) and the possibility of two or more caus- es occurring simultaneously require careful clinical judg- ment in the management of these patients.

Most patients with CLL and AIHA will have an anemia with positive DAT in the context of reticulocytosis and raised bilirubin. Serum LDH is less discriminating as it may be elevated due to active CLL. Moreover, DAT negative AIHA has been seen, particularly in association with therapy.[21] Reticulocytosis may not be seen in the context of a bone marrow overwhelmed by leukemic cells or when there has been recent chemotherapy. Bone marrow exami- nation is essential to distinguish between therapy related causes of cytopenia.[2]

ITP causes particular diagnostic difficulties. There is no sensitive and specific test to parallel the DAT in AIHA, and thrombocytopenia in CLL is more commonly due to splenomegaly and bone marrow failure secondary to infiltration by disease. Nevertheless, thrombocytopenia in a patient with CLL can be considered immune mediated when there is a sudden large fall in platelets (>50% fall to a platelet count <100∞10⁹/L) in the absence of splenomegaly, infection or chemotherapy and with plentiful megakaryocytes in the bone marrow.[14] In advanced disease, anemia usually occurs before thrombocytopenia,[79] so isolated thrombocytopenia is more likely to be immune in origin.[80] ITP with a gradual rather than sudden decline in platelet count is seen more commonly in adults than classic acute ITP of childhood, and can present particular diagnostic difficulties. Response of thrombocytopenia to corticosteroids may be the diagnostic test.

Treatment of patients with CLL and autoimmune cytopenia is largely based on expert opinion and can be divided depending on whether the patient's CLL requires treatment at the same time.[81] In those patients with immune cytope- nia in the context of quiescent CLL, the treatment is the same as idiopathic AIHA initially with corticosteroids, and then in patients who fail to respond or relapse quickly, consideration of alternative immunosuppression (e.g. ciclosporine, mycophenylate or azathioprine) or splenectomy.[71,82] There are case reports of the use of combinations of the anti-CD20 monoclonal antibody rituximab with or without immunosuppression with good effect.[83-87] The anti-CD52 monoclonal antibody alemtuzumab has also been successfully used.[88-90] Intravenous immunoglobulin can be useful where a rapid response is needed (e.g. a patient with ITP and significant bleeding) though as a single agent it will not give lasting effects. More recently, it has been found that new thrombopoietin receptor agonists may be effec- tive in ITP associated with CLL as is the case in primary ITP.[91] Supportive care should include blood product transfu- sion as clinically indicated, folic acid in AIHA and local efforts to control bleeding in ITP. Failure of autoimmune cytopenia to respond to conventional treatment is considered an indication for anti-CLL therapy.

Given the concerns about therapy-triggered AIHA discussed above, there has been recent interest in the most appropriate treatment for patients with active CLL and immune cytopenia or a positive DAT (Table 3). Monotherapy with fludarabine is not appropriate, either in terms of risk of AIHA or efficacy in treatment of CLL. The studies discussed above suggest that treatment with current chemotherapy (e.g. fludarabine, cyclophosphamide) or c h e mo-immunoth e r a py (e .g . fluda r a bine , c yc loph osphamide, rituximab) regimens do not provoke an excess of AIHA, and that patients with a previous history of AIHA or a positive DAT might be safely treated with such regimens.[21,22,77] Indeed, optimal treatment of CLL may be the most efficient way to treat associated cytopenia.[8 1] However, patients with active AIHA or ITP are still excluded from clinical trials, and given the ongoing concerns about using fludarabine in this setting, alternative regimens which do not feature fludarabine have also been explored (Table 3).[87,92-94] Importantly, after successful treatment, patients with stage C "immune" may be "down-staged" to Binet stage A and thus no longer fulfill the criteria for initiation of treatment for CLL. This makes a clear understanding of the origin of cytopenia in a patient with CLL even more important before a decision about treatment is made.

Chronic lymphocytic leukemia-produced autoantigens and clinical disease

There are numerous case reports of other autoimmune diseases in patients with CLL (Table 4). Whilst the epidemiological evidence discussed above does not suggest an increased risk of AID in CLL, or CLL in AID except immune cytopenia, there are cases in which the CLL clone has been demonstrated to produce a clinically important autoantibody.[97,98,114-118] There are other cases in which, though CLL and an AID coexist in a patient, there is no evidence of a causal link (Table 4). In other situations, CLL associated with a monoclonal immunoglobulin or light chain causes organ damage, but by a mechanism which does not involve autoimmunity.[119-121]

Cold agglutinin disease

Cold agglutinin disease (CAD), where clonal IgM binds to erythrocytes in the cool peripheries, is associated with chronic lymphoproliferative disorders (CLPDs), most commonly Waldenstrom's macroglobulinemia, but also CLL.[116] In a patient with antecedent CAD who later developed CLL, *IGHV* gene mutations were invariable but associated with kappa light chain intra-clonal diversification, suggest- ing the CLL was derived from the CAD clone, with addi- tional genetic evolution.[115] It has also been demonstrated that the auto-antibody may have the same BCR rearrangement as the CLL cells.[118]

Paraneoplastic pemphigus

P a r a ne opla stic pe mph ig us (PNP) is a n a utoimmune mucocutaneous disease with blistering and erosion, associated with an underlying neoplasia.[122] CLL is one of the tumors most commonly associated with this disease; others include non-Hodgkin's lymphoma, Castleman's disease and Hodgkin's lymphoma.[117] There is some evidence that the antibodies that recognize multiple antigens in the epi- dermis and ultimately cause the disease may be produced by the tumor.[123] The antigens targeted appear to cross react

with the specific rearrangements of the *IGHV* gene. Other authors have suggested epitope spreading as the mechanism, i.e. the development of immune responses against endogenous epitopes during a chronic autoimmune or infectious response.[122] This theory is supported by the more recent recognition of PNP in association with treatment with fludarabine.[114] However, PNP does arise in untreated CLL, and has been successfully treated with fludarabine-containing regimens.[124] It has also been noted that dysregulated cytokine production, particularly IL-6, may be the mechanism by which tumors, including CLL, cause PNP.[117]

Neuropathies

As with myeloma-associated gammopathy, there are a few reports of polyneuropathy secondary to CLL with associated gammopathy. A monoclonal anti-MAG (myelin-associated glycoprotein) has been demonstrated[97,98] and anti-CLL therapy led to clinical improvement in neurologi- cal symptoms. Guillain-Barré syndrome has been reported in the context of stem cell collection, and after treatment with chlorambucil, but whether this was directly related to CLL or to viral reactivation in the context of immunosuppression is uncertain.[99]

Chronic lymphocytic leukemia complications which may be confused with autoimmune disease

Acquired angio-edema (AAE) is associated with CLPD, especially monoclonal gammopathy of uncertain significance (MGUS) and low grade NHL (splenic lymphoma with villous lymphocytes and lymphoplasmacytic lym- phoma), and is due to an excess of complement 1 (C1) sec- ondary to a low level of its inhibitor (C1-INH). This reduc- tion in serum C1-INH can be due to an autoantibody or to consumption by the tumor. A monoclonal autoantibody has been demonstrated in MGUS and NHL, but not in CLL.[120] Earlier reports of AAE in small lymphocytic lym- phoma describe a B-cell CLPD but with an immunopheno- type which would not now be considered CLL (FMC7 pos, sIg strong and CD5 negative).[125] So where CLL is related to AAE, it does not appear to be by an autoimmune mechanism, but rather by direct tumor consumption of C1-INH. Similarly, a monoclonal gammopathy in CLL can cause renal disease.[119,121] However, this is not due to an autoimmune mechanism, but rather to direct damage to renal tubules caused by deposition of immunoglobulins, particularly free light chains.

Table 3. Treatment approaches for autoimmune cytopenia in CLL.

Author, Date	Population	Baseline findings	Outcome	Comments
Kaufman 2009[92]	Single institution patients with steroid-refractory immune cytopenia or immune cytopenia and active CLL treated with R-CD		Cytopenia: 21 of 21 patients responded to R-CD. CLL: not reported	No response of CLL to therapy is reported.
Bowden 2010[93]	Single institution patients with immune cytopenia and active CLL treated with R-CVP		Cytopenia: 19 of 20 patients responded to R-CVP. CLL: 17 of 20 patients responded (9 CR) with median TTT 27.7	Authors note that CLL outcome is inferior to current best therapy
D'Arena 2010[97]	Multi-center patients with steroid refractory ITP in association with inactive CLL treated with single agent rituximab		Cytopenia: of 21 patients, 12 (57%) had a CR and 6 (29%) had a PR. CLL did not require treatment in any patient	Treatment well tolerated. Patients may also have failed IVIg or vincristine.
Rossignol 2010[94]	Single institution patients with immune cytopenia and CLL, either resistant to corticosteroids or with other indication for treatment with R-CD		Cytopenia: of 48 patients, 40 (83%) had a CR and 3 (6.5%) had a PR. CLL: of 20 patients with active CLL, 7 (35%) achieved a CR, with an overall response in 19 (95%) OR	Treatment well tolerated. Relapse of autoimmune disease was strongly correlated with relapse of CLL.
Borthakur 2006[21]	FCR trial, single institution	9 of 300 had AIHA at start of therapy - one had worsening of AIHA with FCR which responded to cessation of FCR and administration of steroids	19 of 300 developed AIC 14 DAT negative AIHA 3 DAT positive AIHA 2 ITP	Incidence of AIHA not different from that in historical cohort of FC patients, though would be different if only DAT positive anemia considered
Dearden 2008[22]	UK CLL4 trial - F *vs.* FC *vs.* CLB	44 of 777 (14%) DAT positive previously untreated patients with CLL now requiring treatment (clinical AIHA excluded)	77 of 777 developed AIHA 47 (12%) chlorambucil, 21 (11%) fludarabine alone, 9 (5%) FC	DAT positivity is an independent negative predictor of outcome
Hallek 2010[77]	German CLL8 - FC *vs.* FCR		7 of 800 developed AIHA, 4 (1%) FC and 3 (<1%) FCR	

R-CVP: rituximab cyclophosphamide vincristine prednisolone; R-CD: rituximab cyclophosphamide dexamethasone; F: fludarabine; FC: fludarabine cydophosphamide; FCR: fludarabine, cyclophosphamide rituximab; CR: complete remission;TTT: time to treatment; AIHA: autoimmune hemolytic anemia; ITP: immune thrombocytopenia; DAT: direct anti-globulin test

Table 4. Case reports of CLL and non-hemic autoimmune conditions.

AID	Author	Date	Type of report	Attempt to explain	Comment
Churg-Strauss					No reports in PUBMED
Pernicious anemia (PA)	Parker[95]	1976	2 LPD cases with PA	Descriptive only	Very brief abstract only
	Ruvidic[96]	1990	PA 23 years after diagnosis of treated CLL, develops gastric cancer	Descriptive only	Abstract only, in Serbian
Polyneuropathy	Drake[97]	1998	Acute polyneuropathy in CLL with IgG monoclonal protein and response to CLB	IgG paraprotein which responds to CLB as does neuron symptoms	Causal evidence presented
	Mitsui[98]	1999	Polyneuropathy in CLL and HTLV infection	IgM from CLL cells bound to gangliosides, but interaction with HTLV uncertain	Causal evidence presented, unsure of extent of role of HTLV
	D´Arena[99]	2004	GBS developing with cyclo prime prior to PBSC harvest	Descriptive with suggestions ?related to viral or autoimmune	No causal evidence, but may be linked
Raynaud's	No reports in PUBMED		Rheumatoid arthritis (RA)	Taylor[100]	1989 4 patients with
CLL in an RA	Significantly increased		Very small epidemiological population of 1,500	incidence	study, no pathological examination
	Voulgari[101]	2002	Stage 0 patient who developed RA, RA patient who developed stage 0 CLL	4 per 1,000 *vs.* 0.2 per 1,000 in general population	Very small epidemiological study, no pathological examination
	Onal[102]	2005	Single case CLL patient who developed seropositive arthritis	Arthritis improved with CLL therapy	No pathological study of RhF etc.
Sjogren's syndrome	Lehner-Netsch[103]	1969	Single case report of co-diagnosis	Descriptive only	No pathological study
	Gumpel[104]	1972	Single case report of co-diagnosis	Parotid swelling reduced with CLB but still sicca syndrome	No pathological study
	Bán[105]	1984			Not available online, no abstract
Systemic lupus erythematosus (SLE)	Ho[106]	1985	Single case report of CLL in woman after 5 years of SLE		Not available online, abstract only
	Lishner[107]	1990	Single case report of CLL in woman after 5 years of SLE		Not available online, abstract only
	Lugassy[108]	1992	2 CLL pts develop SLE, 1 SLE pt develops CLL	Descriptive and literature review only	No causal evidence presented
Thyroiditis	Haubenstock[109]	1985	Single case report		Not available online, abstract only
	Beyan[110]	2006	CLL patient gets Hashimoto's 1 year after treatment with fludarabine	Descriptive only	No causal evidence presented
Ulcerative colitis (UC)	Crispino[111]	2007	UC patient develops stage 0 CLL (5 cases with heme-onc)	Descriptive, states causal link uncertain	No causal evidence presented
Vasculitis	Mariette[112]	1993	Vasuclitis in a CLL patient	Monoclonal IgM showed anti-cardiolipin specificity, but serologically measured anti-cardiolipin Ab was IgG	Clinically relevant antibody not linked to clone, possible role for antigenic stimulation of CLL clone
	Pamuk[113]	2007	pANCA vasculitis in patient with CLL	No monoclonal spike, 141 other CLL patients did not have a pos pANCA so not false positive	Autoimmune mechanism link to CLL not established, but plausible

CLB: chlorambucil; RhF: rheumatoid factor; LPD: lymphoproliferative disease; GBS: Guillain-Barre syndrome; PBSC: peripheral blood stem cell; HTLV: human T-lymphotrophic virus.

Conclusions

Chronic lymphocytic leukemia is frequently associated with immune disturbances. Whereas the association of CLL with autoimmune cytopenias, particularly autoimmune hemolytic anemia and immune thrombocytopenia, is well established, there is no proof of an increased risk of non-hemic autoimmune disorders in CLL. The predilection in CLL for autoimmune disease attacking the formed elements of the blood is only partially understood and may be related to the ability of CLL cells to process and present antigens derived from blood cells, in contrast to their poor general performance as antigen presenting cells. The mechanisms leading to autoimmune cytopenia in CLL are complex and involve interactions between the malignant B-CLL cells, abnormally functioning T cells, the microenvironment, and the immune system.

While there has been important debate regarding the prognostic significance of immune cytopenias in patients

with CLL, recent studies show that this complication is not necessarily associated with impaired prognosis, with some of the conflicting results being likely due to differences in the patient cohorts studied. Importantly, patients with advanced disease due to an immune mechanism (Binet C "immune") have a better outcome than those in whom advanced stage reflects a high tumor burden with massive bone marrow infiltration (Binet C "infiltrative"). This highlights the importance of determining the origin of the cytopenia in patients with CLL for both prognostic and therapeutic purposes.

Given the clear link between autoimmune cytopenia and CLL, there has been sustained interest in the possibility of a relationship between CLL and other forms of autoimmunity. In most cases, however, there is not a causal link between non-hemic autoimmunity and CLL. However, in a few cases, including paraneoplastic pemphigus and cold agglutinin disease, there is evidence that the CLL clone produces the pathological antibody.

Finally, further research on mechanisms connecting the neoplastic and the immune component of CLL is clearly needed to improve our understanding about this form of leukemia and eventually improve its clinical management.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Swerdlow S, International Agency for Research on C, World Health O. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon France: International Agency for Research on Cancer 4th ed. 2008.
2. Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008;111(12):5446-56.
3. Galton DA. The pathogenesis of chronic lymphocytic leukemia. Can Med Assoc J. 1966;94(19):1005-10.
4. Dameshek W. Chronic lymphocytic leukemia--an accumulative disease of immunolgically incompetent lymphocytes. Blood. 1967;29(4):S566-84.
5. Hamblin TJ, Oscier DG, Young BJ. Autoimmunity in chronic lymphocytic leukaemia. J Clin Path. 1986;39(7):713-6.
6. Kipps TJ, Carson DA. Autoantibodies in chronic lymphocytic leukemia and related systemic autoimmune diseases. Blood. 1993;81(10):2475-87.
7. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med. 2005 24;352(8):804-15.
8. Zenz T, Mertens D, Küppers R, Döhner H, Stilgenbauer S. From pathogenesis to treatment of chronic lymphocytic leukaemia. Nat Rev Cancer. 2010;10(1):37-50.
9. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JPA, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration. Br Med J. 2009;339:b2700.
10. Ebbe S, Wittels B, Dameshek W. Autoimmune thrombocytopenic purpura ("ITP" type) with chronic lymphocytic leukemia. Blood. 1962;19:23-37.
11. Lewis FB, Schwartz RS, Dameshek W. X-radiation and alkylating agents as possible "trigger" mechanisms in the autoimmune complications of malignant lymphoproliferative disease. Clin Exp Immunol. 1966;1(1):3-11.
12. Mauro FR, Foa R, Cerretti R, Giannarelli D, Coluzzi S, Mandelli F, et al. Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. Blood. 2000;95(9):2786-92.
13. Zent CS, Ding W, Schwager SM, Reinalda MS, Hoyer JD, Jelinek DF, et al. The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma. Br J Haematol. 2008;141(5):615-21.
14. Dearden C. Disease-specific complications of chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program. 2008:450-6.
15. Moreno C, Hodgson K, Ferrer G, Elena M, Filella X, Pereira A, et al. Autoimmune cytopenias in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance. Blood. 2010;116(23):4771-6.
16. Dührsen U, Augener W, Zwingers T, Brittinger G. Spectrum and frequency of autoimmune derangements in lymphoproliferative disorders: analysis of 637 cases and comparison with myeloproliferative diseases. Br J Haematol. 1987;67(2):235-9.
17. Barcellini W, Capalbo S, Agostinelli RM, Mauro FR, Ambrosetti A, Calori R, et al. Relationship between autoimmune phenomena and disease stage and therapy in B-cell chronic lymphocytic leukemia. Haematologica. 2006;91(12):1689-92.
18. Visco C, Ruggeri M, Laura Evangelista M, Stasi R, Zanotti R, Giaretta I, et al. Impact of immune thrombocytopenia on the clinical course of chronic lymphocytic leukemia. Blood. 2008;111(3):1110-6.
19. Narra K, Borghaei H, Al-Saleem T, Höglund M, Smith MR. Pure red cell aplasia in B-cell lymphoproliferative disorder treated with rituximab: report of two cases and review of the literature. Leuk Res. 2006;30(1):109-14.
20. Hamblin TJ. Autoimmune complications of chronic lymphocytic leukemia. Semin Oncol. 2006;33(2):230-9.
21. Borthakur G, O'Brien S, Wierda WG, Thomas DA, Cortes JE, Giles FJ, et al. Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab--incidence and predictors. Br J Haematol. 2007;136(6):800-5.
22. Dearden C, Wade R, Else M, Richards S, Milligan D, Hamblin T, et al. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. Blood. 2008;111(4):1820-6.
23. Conley CL, Misiti J, Laster AJ. Genetic factors predisposing to chronic lymphocytic leukemia and to autoimmune disease. Medicine. 1980;59(5):323-34.
24. Kyasa MJ, Parrish RS, Schichman SA, Zent CS. Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma. Am J Hematol. 2003;74(1):1-8.
25. Duek A, Shvidel L, Braester A, Berrebi A. Clinical and immunologic aspects of B chronic lymphocytic leukemia associated with autoimmune disorders. Isr Med Assoc J. 2006;8(12):828-31.
26. Zent CS, Shanafelt T. Management of autoimmune cytopenia complicating chronic lymphocytic leukemia. Leukemia & Lymphoma. 2009;50(6):863-4.
27. Vanura K, Le T, Esterbauer H, Späth F, Porpaczy E, Shehata M, et al. Autoimmune conditions and chronic infections in chronic lymphocytic leukemia patients at diagnosis are associated with unmutated IgVH genes. Haematologica. 2008;93(12):1912-6.
28. Landgren O, Engels EA, Caporaso NE, Gridley G, Mellemkjaer L, Hemminki K, et al. Patterns of autoimmunity and subsequent chronic lymphocytic leukemia in Nordic countries. Blood. 2006;108(1):292-6.
29. Landgren O, Gridley G, Check D, Caporaso NE, Morris Brown L. Acquired immune-related and inflammatory conditions and subsequent chronic lymphocytic leukaemia. Br J Haematol. 2007;139(5):791-8.
30. Söderberg KC, Jonsson F, Winqvist O, Hagmar L, Feychting M. Autoimmune diseases, asthma and risk of haematological malignancies: a nationwide case-control study in Sweden. Eur J Cancer. 2006;42(17):3028-33.
31. Hemminki K, Li X, Sundquist J, Sundquist K. Cancer risks in ulcerative colitis patients. Int J Cancer. 2008;123(6):1417-21.
32. Smedby KE, Askling J, Mariette X, Baecklund E. Autoimmune and inflammatory disorders and risk of malignant lymphomas--an update. J Intern Med. 2008;264(6):514-27.
33. Slager SL, Kay NE, Fredericksen ZS, Wang

33. AH, Liebow M, Cunningham JM, et al. Susceptibility genes and B-chronic lymphocytic leukaemia. Br J Haematol. 2007;139 (5):762-71.
34. Di Bernardo MC, Crowther-Swanepoel D, Broderick P, Webb E, Sellick G, Wild R, et al. A genome-wide association study identifies six susceptibility loci for chronic lymphocytic leukemia. Nat Genet. 2008;40(10): 1204-10.
35. Lettre G, Rioux JD. Autoimmune diseases: insights from genome-wide association studies. Hum Mol Genet. 2008;17(R2): R116-21.
36. Caligaris-Cappio F. Relationship between autoimmunity and immunodeficiency in CLL. Hematol Cell Ther. 1997;39(S1):S13-6.
37. Ghia P, Scielzo C, Frenquelli M, Muzio M, Caligaris-Cappio F. From normal to clonal B cells: Chronic lymphocytic leukemia (CLL) at the crossroad between neoplasia and autoimmunity. Autoimmun Rev. 2007;7(2): 127-31.
38. Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, et al. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001;194(11):1625-38.
39. Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. Annu Rev Immunol. 2003;21:841-94.
40. Chiorazzi N, Ferrarini M. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities Blood. 2011;117(6):1781-91.
41. Phillips JA, Mehta K, Fernandez C, Raveché ES. The NZB mouse as a model for chronic lymphocytic leukemia. Cancer Res. 1992;52(2):437-43.
42. Youinou P, Renaudineau Y. The paradox of CD5-expressing B cells in systemic lupus erythematosus. Autoimmun Rev. 2007;7 (2):149-54.
43. Hervé M, Xu K, Ng Y-S, Wardemann H, Albesiano E, Messmer BT, et al. Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity. J Clin Invest. 2005;115(6): 1636-43.
44. Klein U, Dalla-Favera R. New insights into the pathogenesis of chronic lymphocytic leukemia. Semin Cancer Biol. 2010;20(6): 377-83.
45. Catera R, Silverman GJ, Hatzi K, Seiler T, Didier S, Zhang L, et al. Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation. Mol Med. 2008;14(11-12):665-74.
46. Lanemo Myhrinder A, Hellqvist E, Sidorova E, Söderberg A, Baxendale H, Dahle C, et al. A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies. Blood. 2008;111 (7):3838-48.
47. Chu CC, Catera R, Zhang L, Didier S, Agagnina BM, Damle RN, et al. Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed non-muscle myosin heavy chain IIA: implications for patient outcome and cell of ori- Blood. 2010;115(19):3907-15.
48. Elkon K, Casali P. Nature and functions of autoantibodies. Nat Clin Pract Rheumatol. 2008;4(9):491-8.
49. Ternynck T, Dighiero G, Follezou J, Binet JL. Comparison of normal and CLL lymphocyte surface Ig determinants using peroxidase-labeled antibodies. I. Detection and quantitation of light chain determinants. Blood. 1974;43(6):789-95.
50. Payelle-Brogard B, Magnac C, Alcover A, Roux P, Dighiero G. Defective assembly of the B-cell receptor chains accounts for its low expression in B-chronic lymphocytic leukaemia. Br J Haematol. 2002;118(4):976-85.
51. Vuillier F, Dumas G, Magnac C, Prevost M-C, Lalanne AI, Oppezzo P, et al. Lower levels of surface B-cell-receptor expression in chronic lymphocytic leukemia are associated with glycosylation and folding defects of the mu and CD79a chains. Blood. 2005;105(7):2933-40.
52. Gordon MS, Kato RM, Lansigan F, Thompson AA, Wall R, Rawlings DJ. Aberrant B cell receptor signaling from B29 (Igbeta, CD79b) gene mutations of chronic lymphocytic leukemia B cells. Proc Nat Acad Sci USA. 2000;97(10):5504-9.
53. Sthoeger ZM, Wakai M, Tse DB, Vinciguerra VP, Allen SL, Budman DR, et al. Production of autoantibodies by CD5-expressing B lymphocytes from patients with chronic lymphocytic leukemia. J Exp Med. 1989;169(1):255-68.
54. Beaume A, Brizard A, Dreyfus B, Preud'homme JL. High incidence of serum monoclonal Igs detected by a sensitive immunoblotting technique in B-cell chronic lymphocytic leukemia. Blood. 1994;84(4): 1216-9.
55. Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells. Blood. 2005;105(5):2007-15.
56. Galletti J, Cañones C, Morande P, Borge M, Oppezzo P, Geffner J, et al. Chronic lymphocytic leukemia cells bind and present the erythrocyte protein band 3: possible role as initiators of autoimmune hemolytic anemia. J Immunol. 2008;181(5):3674-83.
57. von Bergwelt-Baildon M, Maecker B, Schultze J, Gribben JG. CD40 activation: potential for specific immunotherapy in B-CLL. Ann Oncol. 2004;15(6):853-7.
58. Görgün G, Holderried TAW, Zahrieh D, Neuberg D, Gribben JG. Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells. J Clin Invest. 2005;115(7):1797-805.
59. Tinhofer I, Marschitz I, Kos M, Henn T, Egle A, Villunger A, et al. Differential sensitivity of CD4+ and CD8+ T lymphocytes to the killing efficacy of Fas (Apo-1/CD95) ligand+ tumor cells in B chronic lymphocytic leukemia. Blood. 1998;91(11):4273-81.
60. Sampalo A, Brieva JA. Humoral immunodeficiency in chronic lymphocytic leukemia: role of CD95/CD95L in tumoral damage and escape. Leuk Lymphoma. 2002;43(4): 881-4.
61. Rebmann V, Nückel H, Dührsen U, Grosse-Wilde H. HLA-G in B-chronic lymphocytic leukaemia: Clinical relevance and functional implications. Semin Cancer Biol. 2007;17 (6):430-5.
62. Riches JC, Ramsay AG, Gribben JG. T-cell function in chronic lymphocytic leukaemia. Semin Cancer Biol. 2010;20(6):431-8.
63. Ramsay AG, Gribben JG. Immune dysfunction in chronic lymphocytic leukemia T cells and lenalidomide as an immunomodulatory drug. Haematologica. 2009;94(9): 1198-202.
64. Schlesinger M, Broman I, Lugassy G. The complement system is defective in chronic lymphatic leukemia patients and in their healthy relatives. Leukemia 1996;10(9): 1509-13.
65. Shvidel L, Vorst E, Berrebi A. Complement values in B chronic lymphocytic leukemia: prognostic significance and correlation with cell maturation stage. Leukemia. 1998;12(4):635-6.
66. Itälä M, Vainio O, Remes K. Functional abnormalities in granulocytes predict susceptibility to bacterial infections in chronic lymphocytic leukaemia. Eur J Haematol. 1996;57(1):46-53.
67. Maki G, Hayes GM, Naji A, Tyler T, Carosella ED, Rouas-Freiss N, et al. NK resistance of tumor cells from multiple myeloma and chronic lymphocytic leukemia patients: implication of HLA-G. Leukemia. 2008;22(5):998-1006.
68. Visco C, Giaretta I, Ruggeri M, Madeo D, Tosetto A, Rodeghiero F. Un-mutated IgVH in chronic lymphocytic leukemia is associated with a higher risk of immune thrombocytopenia. Leukemia. 2007;21(5):1092-3.
69. Rossi D, Gaidano G. Biological and clinical significance of stereotyped B-cell receptors in chronic lymphocytic leukemia. Haematologica. 2010;95(12):1992-5.
70. Mittal S, Blaylock MG, Culligan DJ, Barker RN, Vickers MA. A high rate of CLL phenotype lymphocytes in autoimmune hemolytic anemia and immune thrombocytopenic purpura. Haematologica. 2008;93(1):151-2.
71. Lechner K, Jäger U. How I treat autoimmune hemolytic anemias in adults. Blood. 2010;116(11):1831-8.
72. Bastion Y, Coiffier B, Dumontet C, Espinouse D, Bryon PA. Severe autoimmune hemolytic anemia in two patients treated with fludarabine for chronic lymphocytic leukemia. Ann Oncol. 1992;3(2): 171-2.
73. Tosti S, Caruso R, D'Adamo F, Picardi A, Ali Ege M, Girelli G, et al. Severe autoimmune hemolytic anemia in a patient with chronic lymphocytic leukemia responsive to fludarabine-based treatment. Ann Hematol. 1992;65(5):238-9.
74. Myint H, Copplestone JA, Orchard J, Craig V, Curtis D, Prentice AG, et al. Fludarabine-related autoimmune haemolytic anaemia in patients with chronic lymphocytic leukaemia. Br J Haematol. 1995;91(2):341-4.
75. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol. 2010;11 (1):7-13.
76. Byrd JC, Hertler AA, Weiss RB, Freiman J, Kweder SL, Diehl LF. Fatal recurrence of autoimmune hemolytic anemia following pentostatin therapy in a patient with a history of fludarabine-associated hemolytic anemia. Ann Oncol. 1995;6(3):300-1.
77. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. Lancet. 2010;376(9747):1164-74.
78. Visco C, Novella E, Peotta E, Paolini R, Giaretta I, Rodeghiero F. Autoimmune haemolytic anemia in patients with chronic lymphocytic leukemia is associated with IgVH status. Haematologica. 2010;95(7): gin. 1230-2.
79. Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood. 1975;46(2):219-34.
80. Zent CS, Kay NE. Autoimmune Complications in Chronic Lymphocytic Leukaemia (CLL). Baillieres Best Pract Res

Clin Haematol. 2010;23(1):47-59.
81. Gribben JG. How I treat CLL up front. Blood. 2010;115(2):187-97.
82. D'Arena G, Cascavilla N. Chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. Leuk Lymphoma. 2007;48(6):1072-80.
83. Chemnitz J, Draube A, Diehl V, Wolf J. Successful treatment of steroid and cyclophosphamide-resistant hemolysis in chronic lymphocytic leukemia with rituximab. Am J Hematol. 2002;69(3):232-3.
84. Pamuk GE, Turgut B, Demir M, Tezcan F, Vural O. The successful treatment of refractory autoimmune hemolytic anemia with rituximab in a patient with chronic lymphocytic leukemia. Am J Hematol. 2006;81(8):631-3.
85. Gentile M, Lucia E, Iorio C, Vigna E, Mazzone C, Morelli R, et al. Prompt and sustained response of a steroid-refractory autoimmune hemolytic anemia to a rituximab-based therapy in a chronic lymphocytic leukemia patient. Cancer Chemother Pharmacol. 2008;62(4):741-3.
86. Garvey B. Rituximab in the treatment of autoimmune haematological disorders. Br J Haematol. 2008;141(2):149-69.
87. D'Arena G, Capalbo S, Laurenti L, Del Poeta G, Nunziata G, Deaglio S, et al. Chronic Lymphocytic Leukemia-Associated Immune Thrombocytopenia Treated with Rituximab: A Retrospective Study of 21 Patients. Eur J Haematol. 2010;85(6):502-7.
88. Karlsson C, Hansson L, Celsing F, Lundin J. Treatment of severe refractory autoimmune hemolytic anemia in B-cell chronic lymphocytic leukemia with alemtuzumab (humanized CD52 monoclonal antibody). Leukemia. 2007;21(3):511-4.
89. Laurenti L, Tarnani M, Efremov DG, Chiusolo P, De Padua L, Sica S, et al. Efficacy and safety of low-dose alemtuzumab as treatment of autoimmune hemolytic anemia in pretreated B-cell chronic lymphocytic leukemia. Leukemia. 2007;21(8):1819-21.
90. Royer B, Vaida I, Etienne A, Garidi R, Damaj G, Marolleau JP. Treatment of severe autoimmune hemolytic anemia in B-cell chronic lymphocytic leukemia with alemtuzumab. Leukemia. 2007;21(8):1841-2.
91. Koehrer S, Keating MJ, Wierda WG. Eltrombopag, a second-generation thrombopoietin receptor agonist, for chronic lymphocytic leukemia-associated ITP. Leukemia. 2010;24(5):1096-8.
92. Kaufman M, Limaye SA, Driscoll N, Johnson C, Caramanica A, Lebowicz Y, et al. A combination of rituximab, cyclophosphamide and dexamethasone effectively treats immune cytopenias of chronic lymphocytic leukemia. Leuk Lymphoma. 2009;50(6):892-9.
93. Bowen DA, Call TG, Shanafelt TD, Kay NE, Schwager SM, Reinalda MS, et al. Treatment of autoimmune cytopenia complicating progressive chronic lymphocytic leukemia/small lymphocytic lymphoma with rituximab, cyclophosphamide, vin- cristine, and prednisone. Leuk Lymphoma. 2010;51(4):620-7.
94. Rossignol J, Michallet AS, Oberic L, Picard M, Garon A, Willekens C, et al. Rituximab-cyclophosphamide-dexamethasone combination in the management of autoimmune cytopenias associated with chronic lymphocytic leukemia. Leukemia. 2010 Dec 3. [Epub ahead of print].
95. Parker AC, Bennett M. Pernicious anaemia and lymphoproliferative disease. Scand J Haematol. 1976;17(5):395-7.
96. Ruvidić R, Bosković D. [Chronic lymphocytic leukemia complicated by pernicious anemia during long-term remission]. Srpski Arhiv Za Celokupno Lekarstvo. 1990;118(11-12):495-7.
97. Drake WM, Monson JP, Trainer PJ, Sharief M, Dick JP, Kelsey SM. Acute polyneuropathy with chronic lymphocytic leukaemia and paraproteinaemia: response to chlorambucil and prednisolone. J Neurol Neurosur Psychiatr. 1998;64(4):564.
98. Mitsui Y, Kusunoki S, Hiruma S, Akamatsu M, Kihara M, Hashimoto S, et al. Sensorimotor polyneuropathy associated with chronic lymphocytic leukemia, IgM antiganglioside antibody and human T-cell leukemia virus I infection. Muscle Nerve. 1999;22(10):1461-5.
99. D'Arena G, Vigliotti ML, Pizza V, Tartarone A, Volpe G, Iodice G, et al. Guillain-Barré syndrome complicating mobilization therapy in a case of B-cell chronic lymphocytic leukemia. Leuk Lymphoma. 2004;45(7):1489-90.
100. Taylor HG, Nixon N, Sheeran TP, Dawes PT. Rheumatoid arthritis and chronic lymphatic leukaemia. Clin Exp Rheumatol. 1989;7(5):529-32.
101. Voulgari PV, Vartholomatos G, Kaiafas P, Bourantas KL, Drosos AA. Rheumatoid arthritis and B-cell chronic lymphocytic leukemia. Clin Exp Rheumatol. 2002;20(1):63-5.
102. Onal IK, Ozçakar L, Dizdar O, Büyükasik Y, Dündar S. Seropositive arthritis in chronic lymphocytic leukemia: a remark on B cell-mediated autoimmunity. Rheumatol Int. 2005;25(7):569-70.
103. Lehner-Netsch G, Barry A, Delage JM. [Leukemias and autoimmune diseases: Sjogren's syndrome and hemolytic anemia associated with chronic lymphocytic anemia]. Can Med Assoc J. 1969;100(24):1151-4.
104. Gumpel JM. Chronic lymphatic leukaemia presenting as Sjögren's syndrome. Proc R Soc Med. 1972;65(10):877-8.
105. Bán A, Ferenczy S. Sjögren's syndrome associated with chronic lymphocytic leukemia. Panminerva Medica. 1984;26(3):209-12.
106. Ho CH, Chiang YM, Chong LL, Lin HY, Hwang TS. Development of chronic lymphocytic leukaemia in a case of Sjögren's syndrome with systemic lupus erythematosus. Scand J Haematol 1985;35(2):246-8.
107. Lishner M, Hawker G, Amato D. Chronic lymphocytic leukemia in a patient with systemic lupus erythematosus. Acta Haematol. 1990;84(1):38-9.
108. Lugassy G, Lishner M, Polliack A. Systemic Lupus Erythematosus and Chronic Lymphocytic Leukemia: Rare Coexistence in Three Patients, with Comments on Pathogenesis. Leuk Lymphoma. 1992;8(3):243.
109. Haubenstock A, Zalusky R. Autoimmune hyperthyroidism and thrombocytopenia developing in a patient with chronic lymphocytic leukemia. Am J Hematol. 1985;19(3):281-3.
110. Beyan C, Kaptan K, Ifran A. Coexistence of chronic lymphocytic leukemia and Hashimoto's thyroiditis. Ann Hematol. 2006;85(11):811-2.
111. Crispino P, Pica R, Angelucci E, Consolazio A, Rivera M, Cassieri C, et al. Hematological malignancies in chronic inflammatory bowel diseases: report of five cases and review of the literature. Int J Colorectal Dis. 2007;22(5):553-8.
112. Mariette X, Levy Y, Dubreuil ML, Intrator L, Danon F, Brouet JC. Characterization of a human monoclonal autoantibody directed to cardiolipin/beta 2 glycoprotein I produced by chronic lymphocytic leukaemia B cells. Clin Exp Immunol. 1993;94(2):385-90.
113. Pamuk GE, Uyanik MS, Demir M, Tekgündüz E, Turgut B, Soy M. Systemic antineutrophil cytoplasmic antibody vasculitis in a patient with chronic lymphocytic leukemia: quite a rare diagnosis. Leuk Res. 2007;31(8):1149-51.
114. Gooptu C, Littlewood TJ, Frith P, Lyon CC, Carmichael AJ, Oliwiecki S, et al. Paraneoplastic pemphigus: an association with fludarabine? Br J Dermatol. 2001;144(6):1255-61.
115. Ruzickova S, Pruss A, Odendahl M, Wolbart K, Burmester G-R, Scholze J, et al. Chronic lymphocytic leukemia preceded by cold agglutinin disease: intraclonal immunoglobulin light-chain diversity in VH4-34 expressing single leukemic B cells. Blood. 2002;100(9):3419-22.
116. Zaja F, Vianelli N, Sperotto A, Patriarca F, Tani M, Marin L, et al. Anti-CD20 therapy for chronic lymphocytic leukemia-associated autoimmune diseases. Leuk Lymphoma. 2003;44(11):1951-5.
117. Anhalt GJ. Paraneoplastic Pemphigus. J Investig Dermatol Symp Proc. 2004;9(1):29-33.
118. Yegin ZA, Yagcl M, Haznedar R. Cold agglutinin disease with IgG monoclonal gammopathy in a case of chronic lymphocytic leukemia: An unusual presentation. Transfus Apher Sci. 2009;40(3):219-20.
119. Moulin B, Ronco PM, Mougenot B, Francois A, Fillastre JP, Mignon F. Glomerulonephritis in chronic lymphocytic leukemia and related B-cell lymphomas. Kidney Int. 1992;42(1):127-35.
120. Cicardi M, Beretta A, Colombo M, Gioffre D, Cugno M, Agostoni A. Relevance of lymphoproliferative disorders and of anti- C1 inhibitor autoantibodies in acquired angioedema. Clin Exp Immunol. 1996;106(3):475-80.
121. Mallouk A, Pham P-TT, Pham P-CT. Concurrent FSGS and Hodgkin's lymphoma: case report and literature review on the link between nephrotic glomerulopathies and hematological malignancies. Clin Exp Nephrol. 2006;10(4):284-9.
122. Zhu X, Zhang B. Paraneoplastic pemphigus. J Dermatol. 2007;34(8):503-11.
123. Wang L, Bu PD, Yang Y, Chen X, Zhu PX. Castleman's tumours and production of autoantibody in paraneoplastic pemphigus. Lancet. 2004;363(9408):525-31.
124. Taintor AR, Leiferman KM, Hashimoto T, Ishii N, Zone JJ, Hull CM. A novel case of IgA paraneoplastic pemphigus associated with chronic lymphocytic leukemia. J Am Acad Dermatol. 2007;56(5 Suppl):S73-6.
125. Bain BJ, Catovsky D, Ewan PW. Acquired angioedema as the presenting feature of lymphoproliferative disorders of mature B-lymphocytes. Cancer. 1993;72(11):3318-22.

Revisión 2: Autoimmune cytopenia in chronic lymphocytic leukaemia: diagnosis and treatment

Kate Hodgson, Gerardo Ferrer, Arturo Pereira, Carol Moreno, Emili Montserrat.

British Journal of Haematology. 2011; 154 (1): 14-22.

Resumen

Las citopenias autoinmunes son frecuentes en los pacientes con LLC. El pronóstico de estos pacientes es mejor que aquellos casos en los que la citopenia se debe por infiltración masiva de la médula ósea por la enfermedad, y su tratamiento requiere consideraciones especiales. Por estas razones en esta revisión se discute el diagnóstico y tratamiento de los pacientes con citopenias autoinmunes en pacientes con LLC, incluyendo la AHAI, PTI, eritroblastopenia y la granulocitopenia autoinmune de acuerdo con las pruebas y publicaciones existentes y se pone de relieve la necesidad de llevar a cabo estudios prospectivos en un campo complejo y en el que las recomendaciones terapéuticas hasta la fecha se han basado más en la opinión de expertos que en estudios clínicos llevados a cabo meticulosamente

bjh state of the art review

Autoimmune cytopenia in chronic lymphocytic leukaemia: diagnosis and treatment

Kate Hodgson,[1] Gerardo Ferrer,[1] Arturo Pereira,[2] Carol Moreno[1*] and Emili Montserrat[1]

[1]*Department of Haematology, Institute of Haematology and Oncology, and* [2]*Department of Haemotherapy-Haemostasis, Hospital Clínic, University of Barcelona, Institute d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*

Summary

Autoimmune cytopenia, especially autoimmune haemolytic anaemia (AIHA), appears in 5–10% of patients with chronic lymphocytic leukaemia (CLL). In these patients, the prognosis is not as poor as in those cases in which the cytopenia is due to a massive bone marrow infiltration by the disease, and their treatment requires special considerations. For these reasons, the diagnosis of autoimmune cytopenia should be entertained in any patient with CLL presenting with cytopenia. In patients with autoimmune cytopenia and CLL, treatment is as for idiopathic autoimmune cytopenia, with most patients responding to corticosteroids. For patients not responding to corticosteroids, splenectomy is a reasonable treatment choice. Monoclonal antibodies and thrombopoietin analogues have shown enough activity to support their use, especially within clinical studies, in selected cases not responding to corticosteroids and before splenectomy. In patients with resistant immune cytopenia, the most effective treatment is that of the underlying CLL. Fear of fludarabine-associated AIHA is no longer appropriate in the age of chemo-immunotherapy. Finally, prospective studies are required to better identify the optimal therapy for these patients.

Keywords: chronic lymphocytic leukaemia, immune cytopenia, immune disorders, immune haemolytic anaemia, immune thrombocytopenia.

Chronic lymphocytic leukaemia (CLL) is the most common leukaemia to affect adults in the Western world (Hallek *et al*, 2008; Swerdlow and International Agency for Research on Cancer & World Health Organisation, 2008). Patients with CLL show immune disturbances, which are more marked in this form of leukaemia than in other chronic lymphoproliferative disorders (Galton, 1966; Dameshek, 1967; Hamblin *et al*, 1986; Kipps & Carson, 1993; Chiorazzi *et al*, 2005; Zenz *et al*, 2010). Notably, CLL patients have a higher risk of autoimmune disorders, almost entirely related to blood cells in the form of autoimmune haemolytic anaemia (AIHA) or pure red cell aplasia (PRCA), immune thrombocytopenia (ITP) and, more rarely, autoimmune granulocytopenia (reviewed in Hodgson *et al*, 2011). Autoimmune cytopenia in CLL depends on complex interactions between the malignant B- CLL cells, abnormally functioning T-cells, microenvironment, and the wider immune system. A revision of these mechanisms is beyond the scope of this paper and interested readers are directed to published reviews (Kipps & Carson, 1993; Caligaris-Cappio, 1997; Ghia *et al*, 2007; Riches *et al*, 2010; Hodgson *et al*, 2011).

The proportion of patients who present with autoimmune cytopenia at some point during the course of their disease varies, from 4·3% to 9·7% (Mauro *et al*, 2000; Borthakur *et al*, 2007; Dearden *et al*, 2008; Zent *et al*, 2008; Moreno *et al*, 2010). AIHA is the most common complication, at about 7%. An additional 7–14% of patients have a positive direct antiglobulin test (DAT) without clinical evidence of haemolysis (Dearden *et al*, 2008). ITP occurs at a frequency of between 2% and 5% (Visco *et al*, 2008; Moreno *et al*, 2010) although it may be underestimated unless a bone marrow examination is performed on all patients with thrombocytopenia (Zent *et al*, 2009). The incidence of autoimmune granulocytopenia and PRCA is much lower (<1%).

Epidemiological studies have confirmed the link between CLL and AIHA, demonstrating an association between prior AIHA and subsequent CLL (Landgren *et al*, 2006, 2007; Söderberg *et al*, 2006). In contrast, there is no epidemiological evidence of a significant link between prior ITP and the later development of CLL (Landgren *et al*, 2006; Söderberg *et al*, 2006).

Autoimmune cytopenia does not necessarily confer poor prognosis in patients with CLL (Zent *et al*, 2008). In a recent study from our group, patients with immune cytopenia at

Correspondence: Professor E Montserrat, Department of Haematology, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain.
E-mail: emontse@clinic.ub.es
*Present address: Carol Moreno MD PhD, Haematology Department. Hospital de la Santa Creu i Sant Pau, Mas Casanovas 90, 08041 Barcelona, Spain

First published online 02 May 2011
doi:10.1111/j.1365-2141.2011.08707.x

diagnosis (stage C 'immune') have been shown to have a better outcome than those in whom cytopenia was due to bone marrow failure (stage C 'infiltrative'), though their survival was inferior to patients with uncomplicated stage A (Moreno *et al*, 2010). This may be related to the association observed in these series between AIHA and poor prognostic factors, such as unmutated *IGHV* gene, high ZAP70 expression, and increased serum β_2 microglobulin levels. Visco *et al* (2008) reported that acute ITP was associated with an inferior outcome, which could be explained by the association found by the same group of investigators between ITP and unmutated *IGHV* genes (Visco *et al*, 2007).

Dearden *et al* (2008) assessed the prognostic effect of a positive DAT in patients with CLL requiring treatment for the first time. A positive DAT predicted a poorer response to treatment and together with AIHA was associated with a lower overall survival. It is important to note, however, that this study was performed in a subset of patients with CLL that required treatment, which is a different group of patients from those who are diagnosed with a clinically apparent AIHA.

Diagnosis

Given the multiple possible causes of cytopenia in CLL (e.g. bone marrow failure, hypersplenism, chemotherapy, sepsis, autoimmunity), a high degree of suspicion is required to diagnose these patients. Autoimmune cytopenia will most commonly present before CLL requires therapy (Zent *et al*, 2008; Moreno *et al*, 2010). However, the highest risk is in those patients with advanced stage disease. It is thus imperative to exclude autoimmune cytopenia in the work-up of any patient with CLL and cytopenia (Hallek *et al*, 2008). For this, it is important to undertake a bone marrow biopsy, as an aspirate alone can give equivocal results. Equally, as autoimmune cytopenia can be a presenting feature of many lymphoproliferative disorders, it is important to rule out that diagnosis in patients with apparently idiopathic autoimmune cytopenia.

Table I. Diagnosis of AIHA in patients with CLL.

Diagnostic feature	Caveats
Microspherocytes on blood film	Not seen in other forms of anaemia associated with CLL
Positive DAT	'DAT negative' AIHA can be seen, especially after fludarabine
Reticulocytosis	May be absent in patients with extensive infiltration by CLL or after chemotherapy
Elevated serum LDH	May be elevated due to CLL
Elevated serum indirect bilirubin	May be normal in 40%
Reduced serum haptoglobins	More associated with intravascular haemolysis, but may be mildly low in extravascular haemolysis
BM aspirate/biopsy (erythroid hyperplasia)	BM aspirate/biopsy indicated in any patient with CLL and anaemia to exclude PRCA, CLL infiltration or effects of chemotherapy

AIHA, autoimmune haemolytic anaemia; CLL, chronic lymphocytic leukaemia; DAT, direct antiglobulin test; LDH, lactate dehydrogenase; BM, bone marrow; PRCA, pure red cell aplasia.

Autoimmune haemolytic anaemia

In patients with CLL, some of the features of AIHA may be absent or atypical (Table I). Most patients will present with symptomatic anaemia, which may be of gradual or sudden onset. Usually this will be in the context of increased production of red cells, shown by reticulocytosis, and evidence of extravascular haemolysis, with raised indirect bilirubin and lactate dehydrogenase (LDH) levels in serum. A careful examination of a peripheral blood smear will reveal spherocytes and other abnormal erythrocytes. The DAT is usually positive for red cell-bound polyclonal IgG and/or C3. Cold agglutinin disease associated with IgM produced by the clonal CLL B cells has been reported but is extremely infrequent. Haptoglobins will be reduced once the red cell turnover is significantly raised, even though AIHA is extravascular (Owen *et al*, 1960; Marchand *et al*, 1980). However, if the patient has a normally functioning liver, bilirubin levels may not rise despite clinically significant haemolysis. In patients with advanced CLL, the serum LDH level may be elevated due to active CLL and thus is less discriminating. Reticulocytosis may be absent in the context of recent chemotherapy or where bone marrow function is overwhelmed by infiltration by leukaemic cells. Hence bone marrow examination is important in this situation. AIHA and ITP may present together, a situation that is known as Evans syndrome (Evans *et al*, 1951)

A rather controversial field is DAT-negative AIHA (Borthakur *et al*, 2007). Barcellini *et al* (2002) showed that by using mitogen-stimulated DAT, a technique that can demonstrate latent anti-red blood cell (RBC) autoimmunity, 29% of CLL patients had detectable anti-RBC antibodies, compared to 4·3% by standard DAT technique. Thus it may be considered that a negative DAT in AIHA is a reflection of imperfect technical performance leading to false-negative results (Walker, 1990; Garratty & Petz, 2007).

Immune thrombocytopenia

Thrombocytopenia in CLL is less commonly due to immune causes than to splenomegaly and bone marrow failure due to disease infiltration or myelotoxicity related to therapy. In advanced disease, anaemia usually occurs before thrombocytopenia (Rai *et al*, 1975), thus isolated thrombocytopenia is more likely to be immune in origin (Zent & Kay, 2010). ITP with a gradual rather than sudden decline in platelet count is seen more commonly in adults than classic acute ITP of childhood. In the context of CLL, it is most commonly an

Table II. Diagnosis of ITP in patients with CLL.

Diagnostic feature	Caveats
Sudden profound fall in platelet count (to $<100 \times 10^9$/l and by >50%)	Fall may be gradual, more likely to be ITP if patient not anaemic
Absence of splenomegaly	Splenomegaly can be caused by disease infiltration.
No recent chemotherapy	Recovery of platelets may be slow
Not due to disease infiltration	More likely as cause if there is no anaemia
Blood film shows absent or low number of normal platelets and normal red cells	
BM aspirate/biopsy shows adequate megakaryocytes	May be difficult to appreciate in a bone marrow heavily infiltrated by CLL

ITP, immune thrombocytopenia; CLL, chronic lymphocytic leukaemia; BM, bone marrow.

incidental finding on a routine blood count, as fewer than 50% of patients will be symptomatic (Zent & Shanafelt, 2009). Diagnostic criteria and their caveats are shown in Table II. In a patient with CLL, thrombocytopenia can be considered as immune-mediated when there is a sudden, profound decrease in platelets (>50% fall to a platelet count $<100 \times 10^9$/l) in the absence of splenomegaly, infection or chemotherapy and with copious megakaryocytes in the bone marrow (Dearden, 2008). If the patient has not previously had a normal count, the initial laboratory evaluation of a low platelet count requires a careful examination of the blood smear, the assay for EDTA-dependent pseudothrombocytopenia (Sakurai *et al*, 1997), and inspection of the platelet volume curve to rule out inherited macrothrombocytopenia. Minor forms of Mediterranean macrothrombocytopenia, with large platelets and levels in the range $80–150 \times 10^9$/l, are frequent in healthy people of Southern European ancestry (Balduini *et al*, 2003). The largest platelets are missed by many automated haemocytometers, which contributes to underestimation of the already low platelet count. It is also worth determining *H. pylori* status, as active infection can be associated with ITP, which may resolve without further treatment if *H. pylori* is eradicated (Ahn, 2010). Platelet antibody assays aimed at measuring the autoantibody bound to platelets have limited diagnostic utility because of very low specificity (Beardsley & Ertem, 1998). Assays based on platelet glycoproteins show a better predictive accuracy but are mostly home-made and difficult to standardize (Berchtold *et al*, 1997). There has been little progress in this field over the past two decades and most experts agree that laboratory demonstration of platelet autoantibodies is not necessary to maintain the diagnosis of autoimmune thrombocytopenia (Provan *et al*, 2010). A bone marrow biopsy is necessary, and immunohistochemistry stains for Factor VIII may demonstrate persistence of adequate megakaryocytes despite apparently overwhelming infiltration with CLL. In patients where ambiguity of the cause remains, the response of thrombocytopenia to corticosteroids may be the ultimate diagnostic test.

Pure red cell aplasia

The diagnosis of PRCA should be considered in any patients with anaemia and reticulocytopenia (Ghazal, 2002). Diagnostic criteria and their caveats are shown in Table III. There will typically be an isolated normochromic normocytic anaemia. The expression of the recticulocyte count as a percentage of red cells can be misleadingly normal in the presence of severe anaemia and a corrected or absolute reticulocyte count must be performed. The bone marrow will show characteristic defects of erythroblast maturation, and anti-glycophorin immunohistochemistry may facilitate identification of red cell precursors when the bone marrow is overcrowded by lymphocytes. It should be noted that in patients with CLL, PRCA can be seen in association with other immune cytopenias. In addition, any patient with CLL and reticulocytopenic anaemia should be evaluated for viral infections that have been associated with PRCA, namely cytomegalovirus, Epstein–Barr virus, and parvovirus. In the presence of macrocytosis, folic acid and vitamin B12 levels should be investigated.

Autoimmune granulocytopenia

Autoimmune granulocytopenia occurs rarely in CLL, and should be suspected in patients where there is isolated neutropenia without another cause being apparent. Diagnostic criteria and their caveats are depicted in Table IV. In the Mayo Clinic series, in which bone marrow examinations were prospectively performed on all patients with CLL and cytopenia, only three out of 1750 patients were eventually diagnosed with autoimmune granulocytopenia. All suffered severe infections. Early reports of autoimmune granulocytopenia in CLL were probably due to an underlying diagnosis of large granular lymphocyte leukaemia, which, it is now understood, can cause

Table III. Diagnosis of PRCA in patients with CLL.

Diagnostic feature	Caveats
Normochromic normocytic anaemia	Multiple possible causes
Reticulocytopenia	May be a feature in patients with a bone marrow heavily infiltrated by CLL or post-chemotherapy; must be corrected for haematocrit
BM aspirate/biopsy shows characteristic defects of erythroblast maturation	May be difficult to appreciate in a bone marrow heavily infiltrated by CLL

PRCA, pure red cell aplasia; CLL, chronic lymphocytic leukaemia; BM, bone marrow.

Table IV. Diagnosis of Autoimmune granulocytopenia in patients with CLL.

Diagnostic feature	Caveats
Persistent otherwise unexplained neutropenia	Multiple possible causes of neutropenia in patients with CLL
Anti-neutrophil antibodies	Failure to detect antibodies does not rule out diagnosis. False negative and positive results
BM aspirate/biopsy is normocelular with normal differential count although maturation 'arrest' is also a possibility	May be difficult to appreciate in a bone marrow heavily infiltrated by CLL

CLL, chronic lymphocytic leukaemia; BM, bone marrow.

both B cell dyscrasias and autoimmune granulocytopenia (Viny *et al*, 2008). In patients treated with rituximab, late onset (>4 weeks after exposure) neutropenia is a relatively frequent phenomenon (Wolach *et al*, 2010). The mechanism is unknown. Voog *et al* (2003) have reported IgG antibodies to be bound to neutrophils although others consider it to be linked to disturbances provoked by B cell recovery (Dunleavy *et al*, 2010). The course is usually benign, with most cases found incidentally and resolving spontaneously in 6–20 d or in 3–5 d upon granulocyte colony-stimulating factor (G-CSF) therapy. Occasional severe infections have been reported (Wolach *et al*, 2010).

Therapy-associated AIHA

The possibility that therapy could trigger AIHA in patients with CLL was recognized in early descriptions of the disease (Galton, 1966; Lewis *et al*, 1966; Dameshek, 1967). In spite of early concerns about a possible higher incidence of AIHA in patients treated with purine analogues, particularly fludarabine but also cladribine, and pentostatin (Bastion *et al*, 1992; Tosti *et al*, 1992; Byrd *et al*, 1995; Myint *et al*, 1995), there is now evidence that the risk of developing autoimmune cytopenia after exposure to multi-drug regimens containing purine analogues is not greater than with other agents (Borthakur *et al*, 2007; Dearden *et al*, 2008; Moreno *et al*, 2010). In the UK CLL4 trial, the percentage of patients becoming DAT-positive after therapy was similar across treatment groups (14% chlorambucil, 13% fludarabine, and 10% fludarabine plus cyclophosphamide). Notably, the incidence of clinical AIHA was significantly lower in patients treated with fludarabine in combination with cyclophosphamide (5%) compared to those who received chlorambucil (12%) or fludarabine alone (11%) ($P < 0{\cdot}01$), suggesting that the addition of cyclophosphamide to fludarabine might have a 'protective' effect on the appearance of AIHA (Dearden *et al*, 2008). An earlier smaller study supported the low incidence of AIHA in patients treated with fludarabine, cyclophosphamide and rituximab (Borthakur *et al*, 2007). In our own experience, the incidence of AIHA was slightly lower after fludarabine-based therapy (4%) than after chlorambucil treatment (5%) (Moreno *et al*, 2010). In the German CLL 8 trial, which included patients without prior evidence of AIHA, the rate of AIHA was 1% after treatment with fludarabine and cyclophosphamide with or without rituximab (Hallek *et al*, 2010). Collectively, these results demonstrate that the risk of AIHA is not higher following regimens in which fludarabine and cyclophosphamide (with or without rituximab) are given together in comparison to the risk seen after older therapies for CLL.

Treatment

A major limitation when discussing therapy of patients with CLL and immune cytopenia is that it is mainly based on retrospective studies and experts' opinion. Nevertheless, whereas most patients with AIHA will have symptoms and require intervention to reduce the haemolysis, most patients with ITP will have their thrombocytopenia detected on routine blood count, and be asymptomatic (Zent *et al*, 2009). However, as patients with CLL tend to be older, and this is associated with a higher risk of subsequent bleeding, it has been recommended to initiate therapy in those patients with a platelet count under 50×10^9/l (Provan *et al*, 2010) or 30×10^9/l (British Committee for Standards in Haematology 2003).

In short, any patient with autoimmune cytopenia should be treated with conventional therapy (e.g. corticosteroids, immunosuppression); if the autoimmune cytopenia resolves and the CLL is inactive, no further therapy is needed but the patient should be regularly followed to monitor the disease evolution. If, on the contrary, no response is observed, antileukaemic therapy should be started (Hallek *et al*, 2008). With regard to the sequence in which different treatment modalities should be tried in those patients who fail corticosteroids, there is not enough evidence as to suggest a rigid treatment algorithm. Due to this reason and until more data obtained from prospective studies are available, clinicians should consider each treatment option in the light of the particular needs of each patient, his or her co-morbidity and the potential benefits of each therapy; needless to say, treatment cost is also an issue to be considered.

Immunosuppression

Treatment should be initiated with oral prednisone at a daily dose of 0·5–2 mg/kg and subsequently tapered once a response is observed over several months. It should be noted that patients not responding after 4–6 weeks of therapy are unlikely to respond. The alternative regimen of pulsed high dose dexamethasone (40 mg/d for 4 d repeated every 2 weeks), which has response rates of up to 80% in primary ITP (Mazzucconi *et al*, 2007), warrants further comparison with the standard therapy in terms of efficacy and safety. Up to 80%

of patients will respond to corticosteroids but responders may well be corticosteroid-dependent (Mauro *et al*, 2000; Kyasa *et al*, 2003; Zent *et al*, 2008). Patients who fail to respond to corticosteroids, who require high doses for maintenance or who relapse quickly on withdrawal, should be considered for alternative immunosuppression (e.g. cyclosporine, mycophenylate or azathioprine) or, as discussed below, monoclonal antibodies, targeted therapies or splenectomy. (D'Arena & Cascavilla, 2007; Lechner & Jager, 2010).

Monoclonal antibodies

The anti-CD20 monoclonal antibody rituximab has established short-term efficacy in treating AIHA and ITP, as well as anti-CLL activity either as a single agent or in combination with immunosuppressive agents (Chemnitz *et al*, 2002; Pamuk *et al*, 2006; Garvey, 2008; Gentile *et al*, 2008; D'Arena *et al*, 2010). Rituximab has been used successfully to treat patients with CLL and PRCA, both arising *de novo* and following therapy with fludarabine (Ghazal, 2002; Narra *et al*, 2006). This contrasts with reports of unsuccessful attempts to treat primary PRCA with rituximab (Dungarwalla *et al*, 2007). The anti-CD52 monoclonal antibody alemtuzumab has also been successfully used (Karlsson *et al*, 2007; Laurenti *et al*, 2007; Royer *et al*, 2007).

Splenectomy

Splenectomy remains an effective treatment, particularly for ITP. Akpek *et al* (1999) suggested in a small study that, in CLL, splenectomy for AIHA was ineffective and associated with important mortality. However, more recently, Hill *et al* (2004) showed durable complete responses and no surgical fatalities in a larger series of patients treated with laparoscopic splenectomy. The role of splenectomy is more established in ITP than in AIHA, where success rates approach 90% with two-thirds of patients having a durable response (Provan *et al*, 2010). Patients will require the appropriate immunizations and other (e.g. antibiotics) prophylactic measures.

Targeted therapies

The new thrombopoietin analogues, romiplostim and eltrombopag, are raising a lot of interest and excitement (but also controversy) regarding their role in the treatment of ITP (George, 2010). These agents act by increasing the production of platelets rather than by avoiding their premature destruction. Kuter *et al* (2010) reported a randomized trial of romiplostim performed on non-splenectomized patients that showed a reduction in the number of splenectomies performed (9% vs. 36% of those treated with standard of care). In patients who remained thrombocytopenic after splenectomy, romiplostim was able to maintain a platelet count of $>50 \times 10^9$/l in 38% (Kuter *et al*, 2008). Bussel *et al* (2009) reported a randomized controlled trial, which demonstrated that 59% of patients, irrespective of splenectomy status, increased platelet counts to $>50 \times 10^9$/l when treated with eltrombopag plus standard of care, compared to 16% of patients treated with standard of care alone. Both romiplostim and eltrombopag have been reported to be successful in case reports of patients with ITP and CLL (Koehrer *et al*, 2010; D'Arena & Cascavilla, 2011; Sinisalo *et al*, 2011; Tadmor & Polliack, 2011). Notably, these agents need to be taken indefinitely to maintain response, and although they have the advantage of not being immunosuppressive, their long-terms effects are largely unknown and, among others, marrow fibrosis has been of concern. (Kuter *et al*, 2009) Autoimmune granulocytopenia can be treated with G-CSF (Voog *et al*, 2003).

High-dose intravenous immunoglobulin

This can be useful where a rapid response is needed (e.g. ITP and significant bleeding or in combination with red cell transfusion in a patient with catastrophic haemolysis) although it will not give lasting effects as a single agent.

Transfusion management

In AIHA, the risk of blood transfusion is higher as the patient's autoantibodies react with any transfused cells. This is important clinically, and may range from an asymptomatic reduction of the survival of transfused RBCs to a dangerous acute haemolytic reaction. The latter is particularly true when relative large volumes of blood are transfused in a short period of time (Petz, 1996). In the laboratory, the autoantibody may mask the concomitant presence of alloantibodies capable of causing a haemolytic transfusion reaction, thereby reducing the reliability of the pretransfusion compatibility tests. The decision to transfuse patients with AIHA varies with the clinical setting and the degree of compatibility provided by the pretransfusion tests, and requires good communication between clinicians and the transfusion service staff. However, when transfusion is clinically indicated, the minimal volume of blood should be infused to maintain a tolerable haematocrit – even if the transfusion must be repeated some hours later – until corticosteroids or other specific therapies become effective.

In ITP, platelet transfusion is ineffective for the prophylaxis of spontaneous bleeding or before invasive procedures because of the reduced survival of transfused platelets. It should be reserved for patients with life-threatening haemorrhage in whom twice or thrice the usual dose must be transfused, followed by high dose intravenous immunoglobulin (1 g/kg × 1 d), corticosteroids, and a new high-dose platelet transfusion. In desperate cases, recombinant human activated factor VII may be effective, though there is no formal evidence supporting this off-label use of the drug (Poon, 2007). Importantly, irradiated products should be given in the context of previous therapy with fludarabine (Treleaven *et al*, 2011).

Supportive care

For patients with AIHA, folic acid is important to support increased red cell production. Local efforts to control bleeding may be necessary in ITP. Patients with CLL are already at risk of infections, and this will be higher if they are taking additional immunosuppression. Prophylaxis for *Pneumocystis jiroveci* is appropriate for those who are on long-term corticosteroids. It is important that patients have instructions about how to access appropriate care in the event of fever or other symptoms of infection. Additionally, bisphosphonates should be considered for patients who may be at risk of steroid-induced osteoporosis.

Treatment of CLL in patients with active autoimmune cytopenia

There is evidence that in patients with a prior history of AIHA while receiving fludarabine, re-treatment with this agent can re-trigger AIHA (Myint *et al*, 1995). The same, though, also applies to other purine analogues and alkylating agents (Byrd *et al*, 1995). Therefore patients who have had an episode of treatment -associated AIHA need very considered management, and may benefit from treatment with therapy that excludes the implicated agents. Regimens with some evidence in support of their use in this situation include rituximab with cyclophosphamide and dexamethasone (Kaufman *et al*, 2009; Bowen *et al*, 2010; Rossignol *et al*, 2011) and rituximab, cyclophosphamide, vincristine and prednisolone (Bowen *et al*, 2010), which incidentally include agents (e.g. corticosteroids, rituximab) that have been shown to be effective in the treatment of AIHA and ITP.

Current chemotherapy (e.g. fludarabine plus cyclophosphamide) or chemo-immunotherapy regimens (e.g. fludarabine, cyclophosphamide, rituximab) do not provoke an excess of AIHA, and patients with a previous history of non-fludarabine related AIHA or a positive DAT can be safely treated with such regimens (Borthakur *et al*, 2007; Dearden *et al*, 2008; Hallek *et al*, 2010). Indeed, the optimal therapy for the autoimmune cytopenia can be the best possible treatment for CLL (Gribben, 2010). However, in patients with active CLL and immune cytopenia, particularly AIHA, it makes sense to try to control the autoimmune process before giving antileukaemic therapy that will impair the function of a bone marrow whose function is already weakened by the disease infiltration and the autoimmune cytopenia (Zent & Shanafelt, 2009).

Conclusions

Autoimmune cytopenia complicates CLL in 5–10% of patients. Recent studies show that patients classified as having advanced disease due to an immune mechanism have a better outcome than those in whom advanced stage reflects a high tumour burden with massive bone marrow infiltration. This highlights the need to determine the origin of the cytopenia in patients with CLL for both prognostic and therapeutic purposes. The diagnosis of autoimmune cytopenia is not always straightforward and requires a high degree of clinical suspicion, an experienced laboratory, and a complete workup that should include a bone marrow examination.

Treatment of patients with CLL and autoimmune cytopenia can be challenging. Although most patients will respond to conventional treatment (i.e. corticosteroids, immunosuppressive agents, splenectomy), there is a proportion of cases in whom autoimmune cytopenia is refractory to therapy or present concomitant active CLL, situations that that require a well-balanced treatment approach based on the activity of the cytopenia and that of CLL. New anti-CLL monoclonal antibodies and targeted therapies warrant investigation in these clinical settings. Finally, in contrast to the progress made over the last two decades in the treatment of the 'neoplastic component' of CLL, which allows evidence-based treatment recommendations, therapy of the 'immune component' of the disease continues to be based on retrospective studies. Consequently, large and well designed clinical trials are needed to optimize therapy of patients with CLL and immune cytopenias and to improve their outcomes.

Acknowledgements

KH, GF, AP, CM and EM reviewed the literature, wrote the manuscript and approved the final version. The authors would like to thank Professor Mariano Monzo (Medical School, University of Barcelona, Spain) for his continuous support.

Funding

This work has been performed thanks in part to Red Temàtica de Investigación Cooperativa en Cáncer grant RT 06/0020/002051 Spanish Ministry of Science. Instituto Carlos III FISS PI080304, Generalitat de Catalunya 2009SGR1008 and, CLL Global Foundation. GF is a recipient of a grant from Instituto de Salud Carlos III (PFIS). EM is a recipient of the "August Pi i Sunyer" award.

References

Ahn, Y.S. (2010) Triple play of H pylori in ITP. *Blood*, **115,** 4155–4156.

Akpek, G., McAneny, D. & Weintraub, L. (1999) Comparative response to splenectomy in Coombs-positive autoimmune hemolytic anemia with or without associated disease. *American Journal of Hematology*, **61,** 98–102.

Balduini, C.L., Cattaneo, M., Fabris, F., Gresele, P., Iolascon, A., Pulcinelli, F.M. & Savoia, A. (2003) Inherited thrombocytopenias: a proposed diagnostic algorithm from the Italian Gruppo di Studio delle Piastrine. *Haematologica*, **88,** 582–592.

Barcellini, W., Montesano, R., Clerici, G., Zaninoni, A., Imperiali, F.G., Calori, R., Cortelezzi, A. & Zanella, A. (2002) *In vitro* production of anti-RBC antibodies and cytokines in chronic

lymphocytic leukemia. *American Journal of Hematology*, **71,** 177–183.

Bastion, Y., Coiffier, B., Dumontet, C., Espinouse, D. & Bryon, P.A. (1992) Severe autoimmune hemolytic anemia in two patients treated with fludarabine for chronic lymphocytic leukemia. *Annals of Oncology*, **3,** 171–172.

Beardsley, D.S. & Ertem, M. (1998) Platelet autoantibodies in immune thrombocytopenic purpura. *Transfusion Science*, **19,** 237–244.

Berchtold, P., Muller, D., Beardsley, D., Fujisawa, K., Kaplan, C., Kekomaki, R., Lipp, E., Morell-Kopp, M.C., Kiefel, V., McMillan, R., von dem Borne, A.E. & Imbach, P. (1997) International study to compare antigen-specific methods used for the measurement of antiplatelet autoantibodies. *British Journal of Haematology*, **96,** 477–483.

Borthakur, G., O'Brien, S., Wierda, W.G., Thomas, D.A., Cortes, J.E., Giles, F.J., Kantarjian, H.M., Lerner, S. & Keating, M.J. (2007) Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab – incidence and predictors. *British Journal of Haematology*, **136,** 800–805.

Bowen, D.A., Call, T.G., Shanafelt, T.D., Kay, N.E., Schwager, S.M., Reinalda, M.S., Rabe, K.G., Slager, S.L. & Zent, C.S. (2010) Treatment of autoimmune cytopenia complicating progressive chronic lymphocytic leukemia/small lymphocytic lymphoma with rituximab, cyclophosphamide, vincristine, and prednisone. *Leukemia & Lymphoma*, **51,** 620–627.

British Committee for Standards in Haematology (2003) Guidelines for the investigation and management of idiopathic thrombocytopenic purpura in aduts, children and in pregnancy. *British Journal of Haematology*, **120,** 574–596.

Bussel, J.B., Provan, D., Shamsi, T., Cheng, G., Psaila, B., Kovaleva, L., Salama, A., Jenkins, J.M., Roychowdhury, D., Mayer, B., Stone, N. & Arning, M. (2009) Effect of eltrombopag on platelet counts and bleeding during treatment of chronic idiopathic thrombocytopenic purpura: a randomised, double-blind, placebo-controlled trial. *Lancet*, **373,** 641–648.

Byrd, J.C., Hertler, A.A., Weiss, R.B., Freiman, J., Kweder, S.L. & Diehl, L.F. (1995) Fatal recurrence of autoimmune hemolytic anemia following pentostatin therapy in a patient with a history of fludarabine-associated hemolytic anemia. *Annals of Oncology*, **6,** 300–301.

Caligaris-Cappio, F. (1997) Relationship between autoimmunity and immunodeficiency in CLL. *Hematology and Cell Therapy*, **39,** S13–S16.

Chemnitz, J., Draube, A., Diehl, V. & Wolf, J. (2002) Successful treatment of steroid and cyclophosphamide-resistant hemolysis in chronic lymphocytic leukemia with rituximab. *American Journal of Hematology*, **69,** 232–233.

Chiorazzi, N., Rai, K.R. & Ferrarini, M. (2005) Chronic lymphocytic leukemia. *New England Journal of Medicine*, **352,** 804–815.

Dameshek, W. (1967) Chronic lymphocytic leukemia – an accumulative disease of immunolgically incompetent lymphocytes. *Blood*, **29** (Suppl.), 566–584.

D'Arena, G. & Cascavilla, N. (2007) Chronic lymphocytic leukemia-associated autoimmune hemolytic anemia. *Leukemia & Lymphoma*, **48,** 1072–1080.

D'Arena, G. & Cascavilla, N. (2011) Romiplostim for chronic lymphocytic leukemia-associated immune thrombocytopenia. *Leukemia & Lymphoma*, **52,** 701–704.

D'Arena, G., Capalbo, S., Laurenti, L., Del Poeta, G., Nunziata, G., Deaglio, S., Spinosa, G., Tamani, M., Di Padua, L., Califano, C., Ferrara, F. & Cascavilla, N. (2010) Chronic lymphocytic leukemia-associated immune thrombocytopenia treated with rituximab: a retrospective study of 21 patients. *European Journal of Haematology*, **85,** 502–507.

Dearden, C. (2008) Disease-specific complications of chronic lymphocytic leukemia. *Hematology*, 450–456.

Dearden, C., Wade, R., Else, M., Richards, S., Milligan, D., Hamblin, T. & Catovsky, D. (2008) The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. *Blood*, **111,** 1820–1826.

Dungarwalla, M., Marsh, J.C.W., Tooze, J.A., Lucas, G., Ouwehand, W., Pettengell, R., Dearden, C.E., Gordon Smith, E.C. & Elebute, M.O. (2007) Lack of clinical efficacy of rituximab in the treatment of autoimmune neutropenia and pure red cell aplasia: implications for their pathophysiology. *Annals of Hematology*, **86,** 191–197.

Dunleavy, K., Tay, K. & Wilson, W.H. (2010) Rituximab-associated neutropenia. *Seminars in Hematology*, **47,** 180–186.

Evans, R.S., Takahashi, K., Duane, R.T., Payne, R. & Liu, C. (1951) Primary thrombocytopenic purpura and acquired hemolytic anemia; evidence for a common etiology. *Archives of Internal Medicine*, **87,** 48–65.

Galton, D.A. (1966) The pathogenesis of chronic lymphocytic leukemia. *Canadian Medical Association Journal*, **94,** 1005–1010.

Garratty, G. & Petz, L.D. (2007) Direct antiglobulin test negative autoimmune haemolytic anaemia associated with fludarabine/cyclophosphamide/rituximab therapy. *British Journal of Haematology*, **139,** 622–623.

Garvey, B. (2008) Rituximab in the treatment of autoimmune haematological disorders. *British Journal of Haematology*, **141,** 149–169.

Gentile, M., Lucia, E., Iorio, C., Vigna, E., Mazzone, C., Morelli, R., Bisconte, M.G., Gentile, C. & Morabito, F. (2008) Prompt and sustained response of a steroid-refractory autoimmune hemolytic anemia to a rituximab-based therapy in a chronic lymphocytic leukemia patient. *Cancer Chemotherapy and Pharmacology*, **62,** 741–743.

George, J.N. (2010) Management of immune thrombocytopenia – something old, something new. *New England Journal of Medicine*, **363,** 1959–1961.

Ghazal, H. (2002) Successful treatment of pure red cell aplasia with rituximab in patients with chronic lymphocytic leukemia. *Blood*, **99,** 1092–1094.

Ghia, P., Scielzo, C., Frenquelli, M., Muzio, M. & Caligaris-Cappio, F. (2007) From normal to clonal B cells: chronic lymphocytic leukemia (CLL) at the crossroad between neoplasia and autoimmunity. *Autoimmunity Reviews*, **7,** 127–131.

Gribben, J.G. (2010) How I treat CLL up front. *Blood*, **115,** 187–197.

Hallek, M., Cheson, B.D., Catovsky, D., Caligaris-Cappio, F., Dighiero, G., Döhner, H., Hillmen, P., Keating, M.J., Montserrat, E., Rai, K.R. & Kipps, T.J. (2008) Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*, **111,** 5446–5456.

Hallek, M., Fischer, K., Fingerle-Rowson, G., Fink, A.M., Busch, R., Mayer, J., Hensel, M., Hopfinger, G., Hess, G., von Grunhagen, U., Bergmann, M., Catalano, J., Zinzani, P.L., Caligaris-Cappio, F., Seymour, J.F., Berrebi, A., Jager, U., Cazin, B., Trneny, M., Westermann, A., Wendtner, C.M., Eichhorst, B.F., Staib, P., Buhler, A., Winkler, D., Zenz, T., Bottcher, S., Ritgen, M., Mendila, M., Kneba, M., Dohner, H. & Stilgenbauer, S. (2010) Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet*, **376,** 1164–1174.

Hamblin, T.J., Oscier, D.G. & Young, B.J. (1986) Autoimmunity in chronic lymphocytic leukaemia. *Journal of Clinical Pathology*, **39,** 713–716.

Hill, J., Walsh, R.M., McHam, S., Brody, F. & Kalaycio, M. (2004) Laparoscopic splenectomy for autoimmune hemolytic anemia in patients with chronic lymphocytic leukemia: a case series and review of the literature. *American Journal of Hematology*, **75,** 134–138.

Hodgson, K., Ferrer, G., Montserrat, E. & Moreno, C. (2011) Chronic lymphocytic leukemia and autoimmunity: a systematic review. *Haematologica*, doi: 10.3324/haematol 2010.036152 [Epub ahead of print]

Karlsson, C., Hansson, L., Celsing, F. & Lundin, J. (2007) Treatment of severe refractory autoimmune hemolytic anemia in B-cell chronic lymphocytic leukemia with alemtuzumab (humanized CD52 monoclonal antibody). *Leukemia*, **21,** 511–514.

Kaufman, M., Limaye, S.A., Driscoll, N., Johnson, C., Caramanica, A., Lebowicz, Y., Patel, D., Kohn, N. & Rai, K. (2009) A combination of rituximab, cyclophosphamide and dexamethasone effectively treats immune cytopenias of chronic lymphocytic leukemia. *Leukemia & Lymphoma*, **50,** 892–899.

Kipps, T.J. & Carson, D.A. (1993) Autoantibodies in chronic lymphocytic leukemia and related

systemic autoimmune diseases. *Blood*, **81,** 2475–2487.

Kochrer, S., Keating, M.J. & Wierda, W.G. (2010) Eltrombopag, a second-generation thrombopoietin receptor agonist, for chronic lymphocytic leukemia-associated ITP. *Leukemia*, **24,** 1096–1098.

Kuter, D.J., Bussel, J.B., Lyons, R.M., Pullarkat, V., Gernsheimer, T.B., Senecal, F.M., Aledort, L.M., George, J.N., Kessler, C.M., Sanz, M.A., Liebman, H.A., Slovick, F.T., de Wolf, J.T., Bourgeois, E., Guthrie, Jr, T.H., Newland, A., Wasser, J.S., Hamburg, S.I., Grande, C., Lefrere, F., Lichtin, A.E., Tarantino, M.D., Terebelo, H.R., Viallard, J.F., Cuevas, F.J., Go, R.S., Henry, D.H., Redner, R.L., Rice, L., Schipperus, M.R., Guo, D.M. & Nichol, J.L. (2008) Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. *Lancet*, **371,** 395–403.

Kuter, D.J., Mufti, G.J., Bain, B.J., Hasserjian, R.P., Davis, W. & Rutstein, M. (2009) Evaluation of bone marrow reticulin formation in chronic immune thrombocytopenia patients treated with romiplostim. *Blood*, **114,** 3748–3756.

Kuter, D.J., Rummel, M., Boccia, R., Macik, B.G., Pabinger, I., Selleslag, D., Rodeghiero, F., Chong, B.H., Wang, X. & Berger, D.P. (2010) Romiplostim or standard of care in patients with immune thrombocytopenia. *New England Journal of Medicine*, **363,** 1889–1899.

Kyasa, M.J., Parrish, R.S., Schichman, S.A. & Zent, C.S. (2003) Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma. *American Journal of Hematology*, **74,** 1–8.

Landgren, O., Engels, E.A., Caporaso, N.E., Gridley, G., Mellemkjaer, L., Hemminki, K., Linet, M.S. & Goldin, L.R. (2006) Patterns of autoimmunity and subsequent chronic lymphocytic leukemia in Nordic countries. *Blood*, **108,** 292–296.

Landgren, O., Gridley, G., Check, D., Caporaso, N.E. & Morris Brown, L. (2007) Acquired immune-related and inflammatory conditions and subsequent chronic lymphocytic leukaemia. *British Journal of Haematology*, **139,** 791–798.

Laurenti, L., Tarnani, M., Efremov, D.G., Chiusolo, P., De Padua, L., Sica, S. & Leone, G. (2007) Efficacy and safety of low-dose alemtuzumab as treatment of autoimmune hemolytic anemia in pretreated B-cell chronic lymphocytic leukemia. *Leukemia*, **21,** 1819–1821.

Lechner, K. & Jager, U. (2010) How I treat autoimmune hemolytic anemias in adults. *Blood*, **116,** 1831–1838.

Lewis, F.B., Schwartz, R.S. & Dameshek, W. (1966) X-radiation and alkylating agents as possible "trigger" mechanisms in the autoimmune complications of malignant lymphophroliferative disease. *Clinical and Experimental Immunology*, **1,** 3–11.

Marchand, A., Galen, R.S. & van Lente, F. (1980) The predictive value of serum haptoglobin in haemolytic disease. *Journal of the American Medical Association*, **243,** 1909–1911.

Mauro, F.R., Foa, R., Cerretti, R., Giannarelli, D., Coluzzi, S., Mandelli, F. & Girelli, G. (2000) Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features. *Blood*, **95,** 2786–2792.

Mazzucconi, M.G., Fazi, P., Bernasconi, S., De Rossi, G., Leone, G., Gugliotta, L., Vianelli, N., Avvisati, G., Rodeghiero, F., Amendola, A., Baronci, C., Carbone, C., Quattrin, S., Fioritoni, G., D'Alfonso, G. & Mandelli, F. (2007) Therapy with high-dose dexamethasone (HD-DXM) in previously untreated patients affected by idiopathic thrombocytopenic purpura: a GIMEMA experience. *Blood*, **109,** 1401–1407.

Moreno, C., Hodgson, K., Ferrer, G., Elena, M., Filella, X., Pereira, A., Baumann, T. & Montserrat, E. (2010) Autoimmune cytopenias in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance. *Blood*, **116,** 4771–4776.

Myint, H., Copplestone, J.A., Orchard, J., Craig, V., Curtis, D., Prentice, A.G., Hamon, M.D., Oscier, D.G. & Hamblin, T.J. (1995) Fludarabine-related autoimmune haemolytic anaemia in patients with chronic lymphocytic leukaemia. *British Journal of Haematology*, **91,** 341–344.

Narra, K., Borghaei, H., Al-Saleem, T., Höglund, M. & Smith, M.R. (2006) Pure red cell aplasia in B-cell lymphoproliferative disorder treated with rituximab: report of two cases and review of the literature. *Leukemia Research*, **30,** 109–114.

Owen, J., de Gruchy, G.C. & Smith, H. (1960) Serum haptoglobins in haemolytic states. *Journal of Clinical Pathology*, **13,** 478–482.

Pamuk, G.E., Turgut, B., Demir, M., Tezcan, F. & Vural, O. (2006) The successful treatment of refractory autoimmune hemolytic anemia with rituximab in a patient with chronic lymphocytic leukemia. *American Journal of Hematology*, **81,** 631–633.

Petz, L. (1996) Blood Transfusion in the acquired hemolytic anemias. In: *Clinical practice of Transfusion Medicine* (ed. by L. Petz, S.N. Swisher, S. Kleinman, R.K. Spence & R.G. Strauss), pp. 469–499. Churchill Livingstone, New York.

Poon, M.C. (2007) The evidence for the use of recombinant human activated factor VII in the treatment of bleeding patients with quantitative and qualitative platelet disorders. *Transfusion Medicine Reviews*, **21,** 223–236.

Provan, D., Stasi, R., Newland, A.C., Blanchette, V.S., Bolton-Maggs, P., Bussel, J.B., Chong, B.H., Cines, D.B., Gernsheimer, T.B., Godeau, B., Grainger, J., Greer, I., Hunt, B.J., Imbach, P.A., Lyons, G., McMillan, R., Rodeghiero, F., Sanz, M.A., Tarantino, M., Watson, S., Young, J. & Kuter, D.J. (2010) International consensus report on the investigation and management of primary immune thrombocytopenia. *Blood*, **115,** 168–186.

Rai, K.R., Sawitsky, A., Cronkite, E.P., Chanana, A.D., Levy, R.N. & Pasternack, B.S. (1975) Clinical staging of chronic lymphocytic leukemia. *Blood*, **46,** 219–234.

Riches, J.C., Ramsay, A.G. & Gribben, J.G. (2010) T-cell function in chronic lymphocytic leukaemia. *Seminars in Cancer Biology*, **20,** 431–438.

Rossignol, J., Michallet, A.S., Oberic, L., Picard, M., Garon, A., Willekens, C., Dulery, R., Leleu, X., Cazin, B. & Ysebaert, L. (2011) Rituximab-cyclophosphamide-dexamethasone combination in the management of autoimmune cytopenias associated with chronic lymphocytic leukemia. *Leukemia*, **25,** 473–478.

Royer, B., Vaida, I., Etienne, A., Garidi, R., Damaj, G. & Marolleau, J.P. (2007) Treatment of severe autoimmune hemolytic anemia in B-cell chronic lymphocytic leukemia with alemtuzumab. *Leukemia*, **21,** 1841–1842.

Sakurai, S., Shiojima, I., Tanigawa, T. & Nakahara, K. (1997) Aminoglycosides prevent and dissociate the aggregation of platelets in patients with EDTA-dependent pseudothrombocytopenia. *British Journal of Haematology*, **99,** 817–823.

Sinisalo, M., Sankelo, M. & Itälä-Remes, M. (2011) Thrombopoietin receptor agonists can be used temporarily with patients suffering from refractory chronic lymphocytic leukemia-associated immunologic thrombocytopenia. *Leukemia and Lymphoma*, **52,** 724–725.

Söderberg, K.C., Jonsson, F., Winqvist, O., Hagmar, L. & Feychting, M. (2006) Autoimmune diseases, asthma and risk of haematological malignancies: a nationwide case-control study in Sweden. *European Journal of Cancer*, **42,** 3028–3033.

Swerdlow, S. & International Agency for Research on Cancer & World Health Organisation (2008) *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*. International Agency for Research on Cancer, Lyon, France.

Tadmor, T. & Polliack, A. (2011) Expanding the use of thrombopoietin mimetic drugs: what about chronic lymphocytic leukemia?. *Leukemia and Lymphoma*, **52,** 558–559.

Tosti, S., Caruso, R., D'Adamo, F., Picardi, A., Ali Ege, M., Girelli, G., Mauro, F.R., Maurillo, L. & Amadori, S. (1992) Severe autoimmune hemolytic anemia in a patient with chronic lymphocytic leukemia responsive to fludarabine-based treatment. *Annals of Hematology*, **65,** 238–239.

Treleaven, J., Gennery, A., Marsh, J., Norfolk, D., Page, L., Parker, A., Saran, F., Thurston, J. & Webb, D. (2011) Guidelines on the use of irradiated blood components prepared by the British Committee for Standards in Haematology blood transfusion task force. *British Journal of Haematology*, **152,** 35–51.

Viny, A.D., Lichtin, A., Pohlman, B., Loughran, T. & Maciejewski, J. (2008) Chronic B-cell dyscrasias are an important clinical feature of T-LGL leukemia. *Leukemia & Lymphoma*, **49,** 932–938.

Visco, C., Giaretta, I., Ruggeri, M., Madeo, D., Tosetto, A. & Rodeghiero, F. (2007) Un-mutated IgVH in chronic lymphocytic leukemia is associated with a higher risk of immune thrombocytopenia. *Leukemia*, **21,** 1092–1093.

Visco, C., Ruggeri, M., Laura Evangelista, M., Stasi, R., Zanotti, R., Giaretta, I., Ambrosetti, A.,

Madeo, D., Pizzolo, G. & Rodeghiero, F. (2008) Impact of immune thrombocytopenia on the clinical course of chronic lymphocytic leukemia. *Blood*, **111,** 1110–1116.

Voog, E., Morschhauser, F. & Solal-Celigny, P. (2003) Neutropenia in patients treated with rituximab. *New England Journal of Medicine*, **348,** 2691–2694.

Walker, R.H. (1990) *American Association of Blood Banks Technical Manual*. AABB, Arlington, VA.

Wolach, O., Bairey, O. & Lahav, M. (2010) Late-onset neutropenia after rituximab treatment: case series and comprehensive review of the literature. *Medicine*, **89,** 308–318.

Zent, C.S. & Kay, N.E. (2010) Autoimmune Complications in Chronic Lymphocytic Leukaemia (CLL). *Best Practice & Research Clinical Haematology*, **23,** 47–59.

Zent, C.S. & Shanafelt, T. (2009) Management of autoimmune cytopenia complicating chronic lymphocytic leukemia. *Leukemia & Lymphoma*, **50,** 863–864.

Zent, C.S., Ding, W., Schwager, S.M., Reinalda, M.S., Hoyer, J.D., Jelinek, D.F., Tschumper, R.C., Bowen, D.A., Call, T.G., Shanafelt, T.D., Kay, N.E. & Slager, S.L. (2008) The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma. *British Journal of Haematology*, **141,** 615–621.

Zent, C.S., Ding, W., Reinalda, M.S., Schwager, S.M., Hoyer, J.D., Bowen, D.A., Jelinek, D.F., Tschumper, R.C., Call, T.G., Shanafelt, T.D., Kay, N.E. & Slager, S.L. (2009) Autoimmune cytopenia in chronic lymphocytic leukemia/small lymphocytic lymphoma: changes in clinical presentation and prognosis. *Leukemia & Lymphoma*, **50,** 1261–1268.

Zenz, T., Mertens, D., Küppers, R., Döhner, H. & Stilgenbauer, S. (2010) From pathogenesis to treatment of chronic lymphocytic leukaemia. *Nature Reviews. Cancer*, **10,** 37–50.

Revisión 3: B cell activator factor and a proliferation-inducing ligand at the cross-road of chronic lymphocytic leukemia and autoimmunity

Gerardo Ferrer, Kate Hodgson, Emili Montserrat, Carol Moreno.

Leukemia and Lymphoma 2009; 50 (7):1075-82

Resumen

La acumulación de células neoplásicas y las alteraciones inmunológicas son dos hechos característicos de la LLC. La identificación de mecanismos que relacionan ambos fenómenos es importante para un mayor conocimiento de la enfermedad y eventualmente mejorar su tratamiento. En este trabajo se revisa el papel de BAFF y APRIL, dos moléculas de la familia TNF, que participan en la maduración y supervivencia de los linfocitos B. Estas dos moléculas inducen la activación de la vía NF-kB y están alteradas tanto en enfermedades autoinmunes, donde promueven el rescate de las células autoreactivas, así como en síndromes linfroproliferativos B, incluyendo la LLC, escenario en el que facilitan la supervivencia de las células neoplásicas. Ello hace, como se revisa en este trabajo, que BAFF y APRIL sean moléculas críticas que se sitúan en la encrucijada de los trastornos autoinmunes y los síndromes linfoproliferativos B, particularmente la LLC.

Leukemia & Lymphoma, July 2009; 50(7): 1075–1082

informa
healthcare

REVIEW

B cell activator factor and a proliferation-inducing ligand at the cross-road of chronic lymphocytic leukemia and autoimmunity

GERARDO FERRER, KATE HODGSON, EMILI MONTSERRAT, & CAROL MORENO

Laboratory of Translational Hematology, Department of Hematology, Hospital Clínic, Institute of Hematology and Oncology, IDIBAPS, University of Barcelona, Barcelona, Spain

(Received 3 March 2009; revised 27 April 2009; accepted 1 May 2009)

Abstract

The combination of the neoplastic accumulation of mature B lymphocytes with the presence of autoimmune phenomena is a characteristic finding in chronic B cell lymphoproliferative disorders, particularly chronic lymphocytic leukemia. Identification of mechanisms linking neoplasia to the autoimmune defects is important for a better understanding and improving the treatment of these conditions. Among such mechanisms, the B cell activator factor (BAFF) and a proliferation-inducing ligand (APRIL), two members of the tumor necrosis factor family, play an important role. BAFF and APRIL have both been associated with autoimmunity, with their underlying mechanism of action most likely being related to the rescue of autoreactive B cells. In addition, BAFF and APRIL are crucial in B cell development and homeostasis particularly via the activation of NF-κB pathway-mediated survival signals. These two proteins, therefore, constitute a paradigm of pathophysiological defects linking neoplasia and autoimmunity, thereby providing a better understanding of chronic B cell lymphoproliferative disorders.

Keywords: *Lymphoid leukemia, autoimmunity: responses to self antigen, cytokine and chemokine biology*

Introduction

Chronic lymphocytic leukemia (CLL), the most frequent form of leukemia in the Western world, is characterized by a gradual accumulation of mature B CD5+ cells in the lymphoid tissues, bone marrow, and peripheral blood. Moreover, patients with CLL frequently present a variety of immune disturbances, particularly autoimmune hemolytic anemia, which are not commonly observed in other forms of chronic lymphoproliferative disorders. Accordingly, 15–30% of patients with CLL develop autoimmune hemolytic anemia, whereas the incidence of immune thrombocytopenia, neutropenia, or pure red cell aplasia is less than 2% [1,2]. The mechanisms involved in autoimmune phenomena in CLL are still largely unknown.

The basic defect in CLL is the disruption of programmed cell death or apoptosis that is related to the increased expression of antiapoptotic proteins such as BCL-2, survivin, and MCL-1, which promote the survival of neoplastic cells [3]. Similarly, members of the tumor necrosis factor (TNF) family proteins including CD40L, B cell activator factor (BAFF), and a proliferation-inducing ligand (APRIL) are known to provide survival signals to B cells by inducing the upregulation of antiapoptotic genes and the downregulation of proapoptotic genes through the activation of the nuclear factor-κB (NF-κB) pathway [4–7].

BAFF and APRIL have already been implicated in the pathogenesis of lymphoproliferative disorders such as non-Hodgkin lymphomas, multiple myeloma, and CLL. In particular, BAFF has been shown to be a crucial survival factor for both normal and neoplastic B lymphocytes [8]. Furthermore, BAFF and APRIL have also been associated with autoimmunity and several studies have shown that these

Correspondence: Carol Moreno, MD, Department of Hematology, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain. Tel: +34-92-227-54-75. E-mail: camoreno@clinic.ub.es

ISSN 1042-8194 print/ISSN 1029-2403 online © 2009 Informa Healthcare USA, Inc.
DOI: 10.1080/10428190903013334

proteins can rescue autoreactive B cells that would be eliminated under normal conditions. Increased levels of BAFF have been observed in patients with systemic lupus erythematosus, rheumatoid arthritis, and Sjögren's syndrome [9,10], thus prompting a few clinical trials aimed at neutralizing BAFF, APRIL, or both proteins, in autoimmune disorders and chronic B cell malignancies [11–13].

In this review, we consider the structure and function of BAFF, APRIL, and their receptors, in relationship to autoimmune diseases and B cell malignancies.

B cell activator factor and a proliferation-inducing ligand

Structure, expression, and regulation

BAFF is a homotrimeric type II membrane protein which belongs to the TNF ligand family. It is also found in soluble form as a result of the cleavage of the transmembrane and receptor binding domain from the extracellular region by furin-like convertases. This protein shares 20–30% sequence homology with other TNF proteins like LT-α, CD40L, TRAIL and up to 50% similarity with APRIL [6,14]. APRIL, however, only exists as a secreted form and is cleaved in the Golgi apparatus before its release [15]. Although the crystal structure of TNF family proteins has a characteristic trimeric organization, BAFF shows a unique external loop which is thought to be important in the formation and stability of trimers and further interaction between trimers to form a symmetric complex structure [16,17].

BAFF and APRIL are mainly expressed by monocytes, macrophages, dendritic cells, and neutrophils, but are also found in non-myeloid cells [5,18–21]. In addition, APRIL is highly expressed in transformed and neoplastic cell lines [15]. BAFF expression in both membrane and soluble forms are upregulated by cytokines such as interferon-γ and interleukin-10.The soluble form of BAFF has a stimulatory effect on B cells, whereas its function as a membrane bound protein remains to be clarified [20,22].

Receptors

BAFF and APRIL ligands are able to bind two receptors of the TNF family (TNF-R), B-cell maturation antigen (BCMA), and transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI).There is a third receptor specific for BAFF known as BAFF receptor (BAFF-R) [23,24]. BCMA, TACI, and BAFF-R are type III membrane proteins with an extracellular domain, a transmembrane region and a cytoplasmic domain. The intracellular domain interacts with the protein TNF-R associated factor (TRAF) which is responsible for signal transduction to the nucleus via NF-κB [24]. The ligand binding site of the TNF-R usually contains three or four cysteine-rich domains (CRDs) stabilized by disulfide bridges in the extracellular region. Although TACI, which contains two CRDs, BCMA and BAFF-R, present only one CRD and it implies that there is an specific interaction between the ligand and its receptor [25,26]. All the above receptors are expressed on B lymphocytes although their expression depends on the stage of B cell maturation [27,28]. Thus, BAFF-R is found across the B cell lineage whereas TACI and BCMA are expressed by different subsets of B cells [29].

BCMA was first described as being involved in a chromosomal translocation t(14;16) in a human T-cell lymphoma [30]. Its expression seems to be restricted to mature B cells and is probably highest in plasma cells. Although the function of BCMA in human B cell development is uncertain, recent studies support the involvement of BCMA in the final steps of differentiation of the B cell to effector cells and in promoting the survival of plasma cells in the bone marrow after interaction with BAFF [31,32].

TACI is a receptor mostly expressed on marginal zone and memory B cells although it can also be found on the surface of activated B and T cells [27,33]. In contrast to BCMA and BAFF-R, TACI inhibits the proliferation and Ig production from B cells. Nevertheless, TACI plays a major role in Ig class switch recombination in T-cell dependent and independent humoral immune responses through its interaction with APRIL and BAFF [34–38]. Furthermore, mutations in TACI have been associated with familial and sporadic cases of common variable immunodeficiency, as well as IgA deficiency [39]. Finally, BAFF-R which is expressed on the majority of B cells including transitional, naïve, germinal center, and memory B cells, which plays an important role in B cell development and B lymphocyte long-term survival [40].

Signaling

Members of the TNF family and their ligands are critical regulators of the immune response. In particular, BAFF and APRIL are considered as important promoters of B cell survival, proliferation, and differentiation. Both proteins induce the activation of NF-κB through the interaction of their intracellular domain with the TRAF adaptor protein [9]. Two activation pathways of the NF-κB have been identified: the canonical and the alternative

pathway. The canonical NF-κB pathway is initiated by the activation of the complex IκB kinase (IKK) composed of the IKKβ and IKKγ regulatory subunit (also known as NF-κB essential modulator or NEMO). This induces the degradation of the inhibitor of NF-κBα (IκBα), and leads to the activation and nuclear translocation of NF-κB heterodimer, composed of p50, p65, and RelA. The alternative NF-κB pathway is triggered by the phosphorylation of NF-κB2/p100 by IKKα (the alternative pathway is independent of IKKγ), and permits the nuclear translocation of p52 with RelB. Not surprisingly, the canonical and the alternative NF-κB pathway induce different gene expression profiles [41]. Whereas BCMA and TACI bind TRAF2, -5, -6 molecules, thereby, inducing the activation of the canonical NF-κB pathway, BAFF-R activates the alternative pathway through TRAF3. Interestingly, TRAF3 acts as a negative regulator of BAFF-R mediated survival signals by inhibiting the activation of NF-κB pathway [42–45].

A picture of BAFF, APRIL, their receptors and the signaling pathway is presented in Figure 1.

B cell activator factor and a proliferation-inducing ligand and disease

Autoimmunity

BAFF is thought to play an important role in the induction of tolerance to self-antigens. In physiological conditions, autoreactive B cells are usually eliminated to avoid the generation of autoantibodies. In this process, normal expression of BAFF, as well as the inability of autoreactive B cells in respond to BAFF-mediated survival signals, allow the maintenance of B cell self tolerance, suggesting that BAFF upregulation can rescue self-reactive B cells, which otherwise would be deleted. This concept is supported by studies in BAFF transgenic mice showing an increase in the self-reactive B cell population when BAFF is present in excess [46]. Similarly, overproduction of BAFF has been associated with the development of autoimmune diseases such as systemic lupus eythematosus (SLE), rheumatoid arthritis, and Sjögren's syndrome (Table I). In some studies, BAFF serum levels have been correlated with the severity of the disease, and with

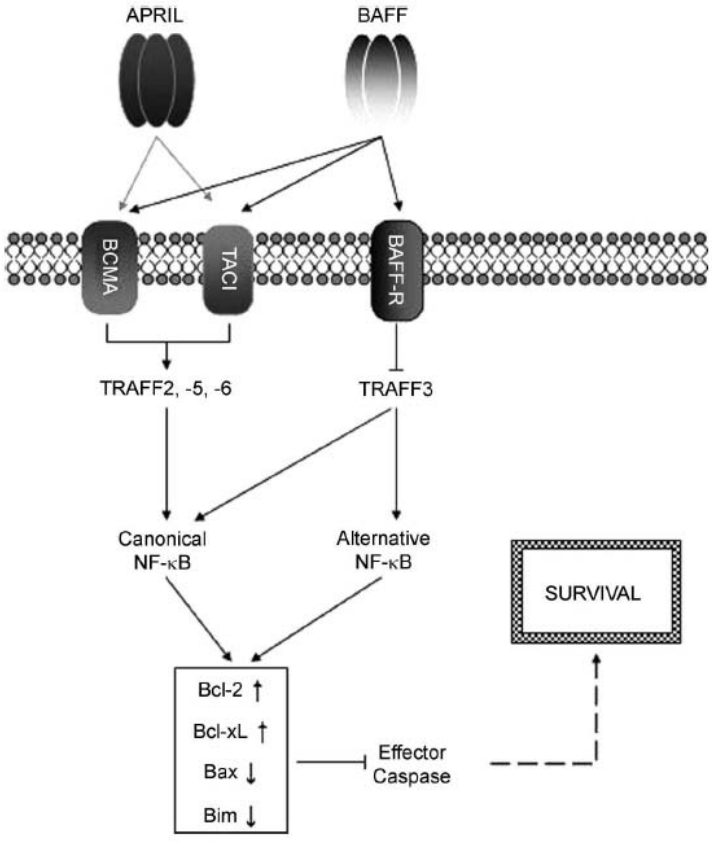


Figure 1. This diagram depicts the effects of BAFF and APRIL when interact with their receptors to induce survival through the activation of NF-κb pathway.

Table I. Autoimmune disorders with high levels of BAFF and APRIL.

Systemic lupus erythematosus
Sjögren's syndrome
Rheumatoid arthritis
Autoimmune hemolytic anemia
Immune thrombocytopenia
Myasthenia gravis
Atopic dermatitis
Coeliac disease
Autoimmune hepatitis
Bullous disease
Wegener's granulomatosis
Primary biliary cirrhosis
Adult-onset type 1 diabetes
Multiple sclerosis
Endometrosis

BAFF, B cell activator factor; APRIL, a proliferation-inducing ligand.

antibody titers. Interestingly, polymorphisms of the BAFF gene promoter region have been detected with a high frequency in patients with SLE and idiopathic thrombocytopenic purpura [47,48]. APRIL serum levels have also been found to be increased in autoimmune diseases and some polymorphisms associated with SLE [49–52].

The pathogenesis of autoimmune disorders involves several 'players', including not only B cells but also activated T cells, and abnormalities in the interaction between T and B cells. Although the participation of T cells is considered to be a key element in the development of autoimmune disorders, a recent study in murine models of SLE has shown that BAFF can trigger T cell independent immune responses through the activation of toll-like receptors (TLR) 7/9 which are upregulated in SLE [53].These studies provide further evidence for the association between BAFF and APRIL and autoimmunity [18,54–57]. The efficacy of BAFF antagonists and antibodies in some autoimmune diseases such as rheumatoid arthritis and SLE also supports the relevance of BAFF in the pathogenesis of these disorders.

B cell malignancies

Because of the critical role of BAFF and APRIL in B lymphocyte survival, the function of these proteins has been investigated in a number of lymphoproliferative disorders including non-Hodgkin lymphoma, CLL, multiple myeloma, and Waldenström's macroglobulinaemia [8,58–61]. In these malignancies, the neoplastic mature B cells are able to bind BAFF and in some cases APRIL. However, the receptor expression profile appears to be disease specific. Thus, whereas BCMA and TACI are mostly found in multiple myeloma and Hodgkin lymphoma cells, TACI, and BAFF-R are preferentially expressed in most non-Hodgkin lymphomas, CLL, and Waldenström's macroglobulinemia [62,63]. In addition, the expression of BAFF-R seem to be lower in follicular lymphoma and CLL cells than in other lymphoma subtypes such as diffuse large B cell lymphoma, mantle cell lymphoma and marginal zone lymphoma where BAFF-R expression is comparable to that found in normal B cells [64]. *In vitro* studies have shown that the binding of either BAFF or APRIL to any of these receptors promotes B cell survival, by the upregulation of antiapoptotic proteins such as BCL-2, BCL-x, MCL-1, and reduces the apoptotic rate of neoplastic cells with the downregulation of other proapoptotic proteins such as Bax [65–67]. It is of interest to note that BAFF and APRIL are not only expressed by neoplastic cells but also by other cells in the microenvironment, (e.g. bone marrow stromal cells in multiple myeloma and macrophages in patients with Burkitt lymphoma), thereby contributing to the maintenance and survival of malignant cells present in these areas [8,58,68].

From the clinical standpoint, it has already been shown that serum levels of BAFF and APRIL are higher in patients with non-Hodgkin lymphoma and Waldenström macroglobulinemia when compared with healthy individuals, supporting the concept that BAFF and APRIL are relevant in the biology of these neoplasias. In addition, a number of clinical correlates have already been established, particularly between serum BAFF and APRIL levels and prognosis [69]. It has also been shown that infection of B cells by EBV leads to the production of both BAFF and APRIL and this co-operation with the infection itself promotes cell survival [70]. Because of their pleiotropic effects on malignant B cells, BAFF and APRIL are appealing targets for novel treatment modalities [11].

B cell activator factor and a proliferation-inducing ligand in chronic lymphocytic leukemia

As promoters of B cell survival, BAFF and APRIL have also been investigated in CLL. Serum BAFF levels have been shown to be lower in CLL than in other lymphoproliferative disorders and in normal individuals [23]. Polymorphism in the promoter region of BAFF has been associated with high levels of BAFF and familial CLL [67,71,72] but no other correlations have been established between serum BAFF levels and other clinical or biological features of the disease. APRIL serum levels appear to be higher in CLL than in normal individuals, a fact that has also been associated with poor prognosis [72].

The expression of BAFF and APRIL as well as their receptors, BCMA, TACI, and BAFF-R on CLL cells indicate that both TNF proteins may play a critical role in the survival and maintenance of the neoplastic clone [58,73]. Interestingly, BAFF and APRIL are not only expressed by neoplastic cells but also by cells present in the microenvironment, particularly nurse-like cells [8,58,74–76]. *In vitro* studies have already shown that the addition of exogenous BAFF or APRIL, cocultured with or without nurse-like cells, protect CLL cells from apoptosis [75]. Thus, BAFF and APRIL may act together in an autocrine and paracrine manner resulting in the maintenance of the CLL clone. As already mentioned earlier in this review, BAFF and APRIL can activate either the canonical or the alternative NF-κB pathways through interaction with their receptors. However, it seems that activation of the canonical pathway seems to be the most critical for supporting the survival of CLL cells [77]. The complex interactions between BAFF and APRIL, stromal and neoplastic B cells in CLL are shown in summary in Figure 2.

Although an increased white blood cell count is the hallmark of CLL, neoplastic lymphocytes accumulate not only in the peripheral blood but also in other tissues like the bone marrow and lymphoid tissues [78], with an increasingly recognized major evolving role of other non-leukemic cells present in the microenvironment. It is important to recognize that the gene expression pattern of neoplastic cells may differ depending on the compartment analyzed, and especially for those genes related to migration and proliferation [79]. In line with this observation, the expression of some cytokines including BAFF and B cell receptor associated molecules appears to be higher in lymph nodes than in the peripheral blood and bone marrow, suggesting that peripheral and bone marrow CLL cells may migrate to lymph nodes and then in response to survival signals may form proliferation centers [80].

Murine models also support the relevance of BAFF in the pathogenesis of CLL. For instance, in TCL-1 and MYC transgenic mice, BAFF seems to accelerate the development of leukemia in these animals by promoting the survival of B cells [81,82]. In addition, APRIL transgenic mice are also known to develop B-1 cell neoplasia which is similar to CLL [83].

CLL is unique among B-cell lymphoproliferative disorders because of the almost constant presence of immune disturbances, particularly autoimmune cytopenias. However, little is known about the etiology of these phenomena [1,84]. Polyclonal IgG autoantibodies which may derive from residual non malignant B cells, differ from the immunoglobulins usually produced by CLL. Abnormalities in the T cell compartment have also been implicated in the pathogenesis of autoimmune phenomena. Thus, there is no clear explanation for autoimmunity in CLL. BAFF and APRIL have been implicated in the pathogenesis of different autoimmune disorders and among them, Sjögren's syndrome. In the latter syndrome B cell malignancies may develop, suggesting that BAFF and/or APRIL may play a role in the autoimmunity associated with CLL, by possibly preventing the deletion of autoreactive B cells [85].

On the basis of the fact that BAFF and APRIL can both rescue CLL cells from apoptosis as shown in earlier *in vitro* studies [75], altering the interaction between these proteins and their receptors could lead to apoptosis of CLL cells. In this regard, a phase I clinical trial based on the neutralization of BAFF and APRIL by atacicept, a soluble receptor of TACI, is currently ongoing in patients with relapsed CLL. Although well tolerated, the efficacy of this new therapy is still unknown [11]. Futhermore, the fusion of a recombinant gelonin (rGel) toxin linked to human BAFF has also been demonstrated to

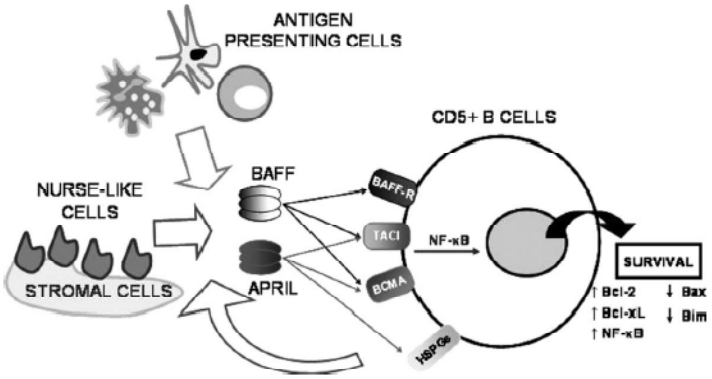


Figure 2. Schematic representation of BAFF and APRIL in CLL.

selectively cause *in vitro* apoptosis in CLL by binding specifically BAFF-R positive CLL cells suggesting that rGel/BAFF may serve as a potential therapeutic agent in CLL [86].

Conclusions

BAFF and APRIL play an important role in autoimmune disorders as well as in chronic lymphoproliferative diseases. In regard to CLL, BAFF, and APRIL are both generated in neoplastic CD5+ B lymphocytes as well as in microenvironment cells, thereby promoting survival of CLL cells via the activation of NF-κB pathway. On the basis of the presence and expression of BAFF and APRIL levels in CLL and their role in autoimmune disorders, it is feasible that these proteins act as a 'bridge' between CLL and autoimmunity. Further studies may well contribute to a better understanding of the disease. In addition, TNF family proteins are emerging as therapeutic targets in B-cell malignancies.

Acknowledgements

This work has been supported in part to Project C03/10 from 'Redes Temáticas de Investigación Cooperativa'. Ministerio de Sanidad y Consumo(2003), José Carreras International Foundation Against Leukemia (CR/08 and EM/08), (FISS 080304), CLL Global Foundation, and Fundació Farreras Valentí. G. F. is a recipient of a grant from 'Instituto de Salud Carlos III' (PFIS).

References

1. Dearden C. Disease-specific complications of chronic lymphocytic leukemia. Hematology Am Soc Hematol Educ Program 2008;2008:450–456.
2. Hamblin TJ, Oscier DG, Young BJ. Autoimmunity in chronic lymphocytic leukaemia. J Clin Pathol 1986;39:713–716.
3. Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352:804–815.
4. Hahne M, Kataoka T, Schroter M, et al. APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. J Exp Med 1998;188:1185–1190.
5. Ohata J, Zvaifler NJ, Nishio M, et al. Fibroblast-like synoviocytes of mesenchymal origin express functional B cell-activating factor of the TNF family in response to proinflammatory cytokines. J Immunol 2005;174:864–870.
6. Mackay F, Browning JL. BAFF: a fundamental survival factor for B cells. Nat Rev Immunol 2002;2:465–475.
7. Mackay F, Ambrose C. The TNF family members BAFF and APRIL: the growing complexity. Cytokine Growth Factor Rev 2003;14:311–324.
8. Tangye SG, Bryant VL, Cuss AK, Good KL. BAFF, APRIL and human B cell disorders. Semin Immunol 2006;18:305–317.
9. Mackay F, Silveira PA, Brink R. B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. Curr Opin Immunol 2007;19:327–336.
10. Ramanujam M, Davidson A. The current status of targeting BAFF/BLyS for autoimmune diseases. Arthritis Res Ther 2004;6:197–202.
11. Ansell SM, Witzig TE, Inwards DJ, et al. Phase I clinical study of atacicept in patients with relapsed and refractory B-cell non-Hodgkin's lymphoma. Clin Cancer Res 2008;14:1105–1110.
12. Kofler DM, Elter T, Gianella-Borradori A, et al. A phase Ib trial of atacicept (TACI-Ig) to neutralize APRIL and BLyS in patients with refractory or relapsed B-cell chronic lymphocytic leukemia (B-CLL) [abstract]. J Clin Oncol 2007;25:18S. Abstract 3029.
13. Ponce R, Miller D. Preclinical support for combination therapy in the treatment of autoimmunity with atacicept. Toxicol Pathol 2009;37:89–99.
14. Schneider P, Mackay F, Steiner V, et al. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med 1999;189:1747–1756.
15. Dillon SR, Gross JA, Ansell SM, Novak AJ. An APRIL to remember: novel TNF ligands as therapeutic targets. Nat Rev Drug Discov 2006;5:235–246.
16. Liu Y, Xu L, Opalka N, Kappler J, Shu HB, Zhang G. Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands. Cell 2002;108:383–394.
17. Oren DA, Li Y, Volovik Y, et al. Structural basis of BLyS receptor recognition. Nat Struct Biol 2002;9:288–292.
18. Krumbholz M, Theil D, Derfuss T, et al. BAFF is produced by astrocytes and up-regulated in multiple sclerosis lesions and primary central nervous system lymphoma. J Exp Med 2005;201:195–200.
19. Morimoto S, Nakano S, Watanabe T, et al. Expression of B-cell activating factor of the tumour necrosis factor family (BAFF) in T cells in active systemic lupus erythematosus: the role of BAFF in T cell-dependent B cell pathogenic autoantibody production. Rheumatology (Oxford) 2007;46:1083–1086.
20. Nardelli B, Belvedere O, Roschke V, et al. Synthesis and release of B-lymphocyte stimulator from myeloid cells. Blood 2001;97:198–204.
21. Scapini P, Nardelli B, Nadali G, et al. G-CSF-stimulated neutrophils are a prominent source of functional BLyS. J Exp Med 2003;197:297–302.
22. Craxton A, Magaletti D, Ryan EJ, Clark EA. Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF. Blood 2003;101:4464–4471.
23. Haiat S, Billard C, Quiney C, Ajchenbaum-Cymbalista F, Kolb JP. Role of BAFF and APRIL in human B-cell chronic lymphocytic leukaemia. Immunology 2006;118:281–292.
24. Bossen C, Schneider P. BAFF, APRIL and their receptors: structure, function and signaling. Semin Immunol 2006;18:263–275.
25. Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF superfamily. Trends Biochem Sci 2002;27:19–26.
26. Kim HM, Yu KS, Lee ME, et al. Crystal structure of the BAFF-BAFF-R complex and its implications for receptor activation. Nat Struct Biol 2003;10:342–348.
27. von Bulow GU, Bram RJ. NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily. Science 1997;278:138–141.
28. Wu Y, Bressette D, Carrell JA, et al. Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLyS. J Biol Chem 2000;275:35478–35485.
29. Reyes SL, Leon BF, Rozas VM, Gonzalez JP, Naves PR. BAFF: a regulatory cytokine of B lymphocytes involved in autoimmunity and lymphoid cancer. Rev Med Chil 2006;134:1175–1184.

30. Laabi Y, Gras MP, Carbonnel F, et al. A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma. EMBO J 1992;11:3897–3904.
31. O'Connor BP, Raman VS, Erickson LD, et al. BCMA is essential for the survival of long-lived bone marrow plasma cells. J Exp Med 2004;199:91–98.
32. Darce JR, Arendt BK, Wu X, Jelinek DF. Regulated expression of BAFF-binding receptors during human B cell differentiation. J Immunol 2007;179:7276–7286.
33. Salzer U, Jennings S, Grimbacher B. To switch or not to switch – the opposing roles of TACI in terminal B cell differentiation. Eur J Immunol 2007;37:17–20.
34. Xia XZ, Treanor J, Senaldi G, et al. TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation. J Exp Med 2000;192:137–143.
35. Xu S, Lam KP. B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses. Mol Cell Biol 2001;21:4067–4074.
36. Yan M, Wang H, Chan B, et al. Activation and accumulation of B cells in TACI-deficient mice. Nat Immunol 2001;2:638–643.
37. Fagarasan S, Honjo T. T-Independent immune response: new aspects of B cell biology. Science 2000;290:89–92.
38. Castigli E, Wilson SA, Scott S, et al. TACI and BAFF-R mediate isotype switching in B cells. J Exp Med 2005;201:35–39.
39. Castigli E, Wilson SA, Garibyan L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. Nat Genet 2005;37:829–834.
40. Darce JR, Arendt BK, Wu X, Jelinek DF. Regulated expression of BAFF-binding receptors during human B cell differentiation. J Immunol 2007;179:7276–7286.
41. Bonizzi G, Karin M. The two NF-κB activation pathways and their role in innate and adaptive immunity. Trends Immunol 2004;25:280–288.
42. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003;3:745–756.
43. He JQ, Zarnegar B, Oganesyan G, et al. Rescue of TRAF3-null mice by p100 NF-κB deficiency. J Exp Med 2006;203:2413–2418.
44. Xu Y, Cheng G, Baltimore D. Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses. Immunity 1996;5:407–415.
45. Hauer J, Puschner S, Ramakrishnan P, et al. TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-κB pathway by TRAF-binding TNFRs. Proc Natl Acad Sci USA 2005;102:2874–2879.
46. Thien M, Phan TG, Gardam S, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. Immunity 2004;20:785–798.
47. Emmerich F, Bal G, Barakat A, et al. High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura. Br J Haematol 2007;136:309–314.
48. Kawasaki A, Tsuchiya N, Fukazawa T, Hashimoto H, Tokunaga K. Analysis on the association of human BLYS (BAFF, TNFSF13B) polymorphisms with systemic lupus erythematosus and rheumatoid arthritis. Genes Immun 2002;3:424–429.
49. Koyama T, Tsukamoto H, Masumoto K, et al. A novel polymorphism of the human APRIL gene is associated with systemic lupus erythematosus. Rheumatology (Oxford) 2003;42:980–985.
50. Koyama T, Tsukamoto H, Miyagi Y, et al. Raised serum APRIL levels in patients with systemic lupus erythematosus. Ann Rheum Dis 2005;64:1065–1067.
51. Lee YH, Ota F, Kim-Howard X, Kaufman KM, Nath SK. APRIL polymorphism and systemic lupus erythematosus (SLE) susceptibility. Rheumatology (Oxford) 2007;46:1274–1276.
52. Tan SM, Xu D, Roschke V, et al. Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis. Arthritis Rheum 2003;48:982–992.
53. Groom J, Kalled SL, Cutler AH, et al. Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome. J Clin Invest 2002;109:59–68.
54. Hever A, Roth RB, Hevezi P, et al. Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator. Proc Natl Acad Sci USA 2007;104:12451–12456.
55. Kim JY, Yang Y, Moon JS, et al. Serum BAFF expression in patients with myasthenia gravis. J Neuroimmunol 2008;199:151–154.
56. Mackay IR, Groom J, Mackay CR. Levels of BAFF in serum in primary biliary cirrhosis and autoimmune diabetes. Autoimmunity 2002;35:551–553.
57. Cohen SB. Updates from B Cell trials: efficacy. J Rheumatol Suppl 2006;77:12–17.
58. Kern C, Cornuel JF, Billard C, et al. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. Blood 2004;103:679–688.
59. Kim SJ, Lee SJ, Choi IY, et al. Serum BAFF predicts prognosis better than APRIL in diffuse large B-cell lymphoma patients treated with rituximab plus CHOP chemotherapy. Eur J Haematol 2008;81:177–184.
60. Schwaller J, Went P, Matthes T, et al. Paracrine promotion of tumor development by the TNF ligand APRIL in Hodgkin's disease. Leukemia 2007;21:1324–1327.
61. Schwaller J, Schneider P, Mhawech-Fauceglia P, et al. Neutrophil-derived APRIL concentrated in tumor lesions by proteoglycans correlates with human B-cell lymphoma aggressiveness. Blood 2007;109:331–338.
62. Chiu A, Xu W, He B, et al. Hodgkin lymphoma cells express TACI and BCMA receptors and generate survival and proliferation signals in response to BAFF and APRIL. Blood 2007;109:729–739.
63. Ng LG, Sutherland AP, Newton R, et al. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol 2004;173:807.
64. Briones J, Timmerman JM, Hilbert DM, Levy R. BLyS and BLyS receptor expression in non-Hodgkin's lymphoma. Exp Hematol 2002;30:135–141.
65. He B, Chadburn A, Jou E, et al. Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL. J Immunol 2004;172:3268–3279.
66. Moreaux J, Legouffe E, Jourdan E, et al. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood 2004;103:3148–3157.
67. Novak AJ, Grote DM, Ziesmer SC, et al. Elevated serum B-lymphocyte stimulator levels in patients with familial lymphoproliferative disorders. J Clin Oncol 2006;24:983–987.
68. Herreros B, Sanchez-Aguilera A, Piris MA. Lymphoma microenvironment: culprit or innocent? Leukemia 2008;22:49–58.
69. Novak AJ, Grote DM, Stenson M, et al. Expression of BLyS and its receptors in B-cell non-Hodgkin lymphoma: correlation with disease activity and patient outcome. Blood 2004;104:2247–2253.

70. He B, Raab-Traub N, Casali P, Cerutti A. EBV-encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell-independent Ig heavy chain class switching. J Immunol 2003;171:5215–5224.
71. Molica S, Digiesi G, Mauro F, et al. Increased serum BAFF (B-cell activating factor of the TNF family) level is a peculiar feature associated with familial chronic lymphocytic leukemia. Leuk Res 2009;33:162–165.
72. Planelles L, Castillo-Gutierrez S, Medema JP, et al. APRIL but not BLyS serum levels are increased in chronic lymphocytic leukemia: prognostic relevance of APRIL for survival. Haematologica 2007;92:1284–1285.
73. Novak AJ, Bram RJ, Kay NE, Jelinek DF. Aberrant expression of B-lymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. Blood 2002;100: 2973–2979.
74. Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol 2003;123:380–388.
75. Nishio M, Endo T, Tsukada N, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1α. Blood 2005;106:1012–1020.
76. Burger JA, Tsukada N, Burger M, et al. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 2000;96:2655–2663.
77. Endo T, Nishio M, Enzler T, et al. BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-κB pathway. Blood 2007;109:703–710.
78. Rozman C, Montserrat E. Chronic lymphocytic leukemia. N Engl J Med 1995;333:1052–1057.
79. Smit LA, Hallaert DY, Spijker R, et al. Differential Noxa/Mcl-1 balance in peripheral *versus* lymph node chronic lymphocytic leukemia cells correlates with survival capacity. Blood 2007;109:1660–1668.
80. Mittal AK, Iqbal J, Nordgren TM, et al. Molecular basis of proliferation/survival and migration of CLL in peripheral blood, bone marrow and lymph nodes [abstract]. Blood 2008;112. Abstract 546.
81. Enzler T, Widhopf GF, Lee J, et al. BAFF Accelerates development of chronic lymphocytic leukemia in TCL1 transgenic mice [abstract]. Blood 2007;110. Abstract 1117.
82. Enzler T, Zhang W, Kater AP, et al. Constitutive Baff signalling plays a key role in CLL development by promoting tumor cell survival [abstract]. Blood 2008;112. Abstract 28.
83. Planelles L, Carvalho-Pinto CE, Hardenberg G, et al. APRIL promotes B-1 cell-associated neoplasm. Cancer Cell 2004;6: 399–408.
84. Vanura K, Le T, Esterbauer H, et al. Autoimmune conditions and chronic infections in chronic lymphocytic leukemia patients at diagnosis are associated with unmutated IgVH genes. Haematologica 2008;93:1912–1916.
85. Ekstrom SK, Vajdic CM, Falster M, et al. Autoimmune disorders and risk of non-Hodgkin lymphoma subtypes: a pooled analysis within the InterLymph Consortium. Blood 2008;111:4029–4038.
86. Nimmanapalli R, Lyu MA, Du M, et al. The growth factor fusion construct containing B-lymphocyte stimulator (BLyS) and the toxin rGel induces apoptosis specifically in BAFF-R-positive CLL cells. Blood 2007;109:2557–2564.

Artículo 2: The combined analysis of serum BAFF and APRIL levels is a robust predictor of disease progression in patients with chronic lymphocytic leukemia.

Gerardo Ferrer, Kate Hodgson, Arturo Pereira, Manel Juan, Montse Elena, Dolors Colomer, Gael Roué, Marta Aymerich, Tycho Baumman, Emili Montserrat, Carol Moreno.

Leukemia and Lymphoma 2011; 52 (11): 2064-8.

Resumen

BAFF y APRIL son dos miembros de la familia de proteínas TNF que son esenciales en el desarrollo y supervivencia de los linfocitos B. En este estudio analizamos el papel de estas dos proteínas en la LLC, relacionando sus niveles proteicos en suero y su expresión en forma de ARNm en las células neoplásicas con factores clínico-biológicos y la progresión de la enfermedad. En pacientes con LLC, los niveles séricos de BAFF fueron significativamente inferiores a los controles (0.64 ng/mL *vs.* 0.77 ng/mL, p = 0.014); en cambio, los niveles séricos de APRIL estaban significativamente más elevados (4.10 ng/mL *vs.* 1.84 ng/mL, p = 0.041). Tanto las células neoplásicas como los controles presentaban ARNm para las dos proteínas, aunque APRIL en la LLC de forma significativamente más baja que en las células B normales (p=0.018). Los niveles bajos de BAFF en suero se asociaron con un recuento de linfocitos en sangre elevado y fases avanzadas de la enfermedad, mientras que los niveles de APRIL se correlacionaron con la expresión del marcador CD38. En el análisis multivariado, el análisis conjunto de BAFF y APRIL en suero resultó ser un marcador pronóstico independiente del riesgo de progresión de la enfermedad (p=0.001).

Leukemia & Lymphoma, 2011; Early Online, 1-5

ISSN: 1042-8194 print / 1029-2403 online
DOI: 10.3109/10428194.2011.591008

informa
healthcare

ORIGINAL ARTICLE: RESEARCH

Combined analysis of levels of serum B-cell activating factor and a proliferation-inducing ligand as predictor of disease progression in patients with chronic lymphocytic leukemia

Gerardo Ferrer[1], Kate Hodgson[1], Arturo Pereira[2], Manel Juan[3], Montse Elena[4], Dolors Colomer[5], Gael Roué[5], Marta Aymerich[5], Tycho Baumann[1], Emili Montserrat[1] & Carol Moreno[1]

[1]*Institute of Hematology and Oncology, Department of Hematology,* [2]*Department of Hemotherapy-Hemostasis,* [3]*Department of Immunology,* [4]*Department of Clinical Biochemistry and Molecular Genetics, and* [5]*Hematopathology Unit, Department of Pathology, Hospital Clínic, University of Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain*

Abstract

B-cellactivatingfactor(BAFF)andaproliferation-inducingligand (APRIL) are regulators of normal B-cell development and survival. We investigated their role in chronic lymphocyticleukemia (CLL) by relating serum protein levels and CLL cell mRNA expression with clinical factors and disease progression. In patients with CLL, BAFF serum levels were significantly lower than in controls (0.64 ng/mL vs. 0.77 ng/mL, p 0.014), and APRIL serum levels were significantly higher (4.10 ng/mL vs. 1.84 ng/mL, p 0.041). CLL cells expressed BAFF and APRIL mRNA at lower levels than normal B-cells. Low BAFF serum levels were significantly correlated with a high blood lymphocyte count and advanced clinical stage, whereas APRIL levels were correlated with CD38 expression. In a multivariate analysis, the combined analysis of BAFF and APRIL serum levels emerged as an independent predictor of disease progression.

Keywords: Lymphoid leukemia, prognostication, cytokine production and paraneoplastic conditions

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of monoclonal B-cells with a distinctive immunophenotype (i.e. CD5 CD19 , $CD20^{dim}$, CD23 , $SmIg^{dim}$) in peripheral blood, bone marrow, and the lymphoid tissues [1]. This accumulation is mainly due to a defective regulation of programmed cell death [2]. CLL cells express high levels of antiapoptotic proteins such as Bcl-2 and Bcl-xL [3]. However, CLL cells undergo spontaneous apoptosis *in vitro*, which reflects the requirement of external factors (i.e. microenvironment signals) for survival of the CLL clone [4].

Currently, most patients with CLL are diagnosed in early, stable phases of their disease, but all of them even- tually progress. Factors that predict disease progression not only are clinically useful but also pinpoint biological mechanisms that underlie the disease process and which may be amenable to therapeutic intervention.

Members of the tumor necrosis factor (TNF) family of proteins, including CD40L, B-cell activating factor (BAFF), and a proliferation-inducing ligand (APRIL), are known to provide survival signals to B-cells by influencing the balance between anti- and pro-apoptotic genes, through activation of the nuclear factor-κB (NF-κB) pathway [5-7]. Abnormalities in these proteins have been observed in several B-cell diseases, including autoimmune phenomena, malignancies, and immunodeficiency. In addition, these proteins have been correlated with disease activity in autoimmune disor- ders, and also with poor prognosis in some B-cell malignan- cies [8,9]. Several studies have analyzed the prognostic value of either BAFF or APRIL in CLL [6,10-14], indicating that they can be predictors of disease progression. These two biomarkers, however, have not yet been analyzed in combi- nation. We hypothesized that the prognostic value of BAFF and APRIL could be additive and provide more reliable and powerful prognostic information than that offered by each of them considered independently.

This study has been performed with two aims: first, to validate the prognostic value of serum BAFF and APRIL as well as their mRNA expression in CLL cells; second, to investigate the power of combining information about BAFF and APRIL to identify those patients at a higher risk of progression.

Correspondence: Carol Moreno, MD, PhD, Hematology Department, Hospital de la Santa Creu i Sant Pau, Mas Casanovas, 90, 08041 Barcelona, Spain. Tel: 34-93-556-5647. E-mail: cmorenoa@santpau.cat

(Received 31 March 2011; revised 12 May 2011; accepted 19 May 2011)

Materials and methods

Patients

The study population comprised 60 unselected patients with CLL who had frozen serum samples available from the time of diagnosis. CLL was diagnosed according to the World Health Organization (WHO) classification [15]. Progression of CLL was defined following the International Workshop on Chronic Lymphocytic Leukemia (IW-CLL) guidelines [1], i.e. a new or 50% increase in a pathologi- cal lymph node, new or 50% increase in splenomegaly or hepatomegaly, 50% increase in blood lymphocyte count, histological transformation, or onset of CLL associated non- immune cytopenia. Clinical characteristics and prognostic markers measured at the time of diagnosis are presented in Table I. Thirty-five age- and sex-matched healthy donors were used as a control population. All patients provided informed consent in accordance with the Hospital Clinic Ethics Committee and with the Declaration of Helsinki.

BAFF and APRIL ELISA

Commercial enzyme-linked immunoassay (ELISA) kits (Quantikine Human BAFF Immunoassay→; R&D Systems, Minneapolis, MN; and Human APRIL ELISA BMS2008; BenderMedSystems GmbH, Vienna, Austria) were used to quantify BAFF and APRIL in duplicate in frozen serum samples, according to the manufacturers' recommenda- tions. Absorbance was measured in a μQuant™ microplate spectrophotometer (BioTek Instruments, Inc., Winooski, VT). Results are reported in ng/mL. Interassay coefficients of variation were 8.62% and 9.81% in BAFF and APRIL ELISA, respectively.

Isolation of B-cells

B-cells were isolated from cryopreserved peripheral blood mononuclear cells by negative selection using the B-cell isolation Kit (Miltenyi Biotec, Auburn, CA), according to the manufacturer's procedure. Cell viability assessed by trypan blue was above 85% in all samples. Isolated B-cells from healthy donors and patients with CLL were stained with fluorescein isothiocyanate (FITC) anti-CD45, phycoerythrin (PE) anti-CD14, peridinin-chlorophyll proteins conjugated with Cy5.5 (PerCP-Cy5.5) anti-CD19, and allophycocyanin (APC) anti-CD5 (BD, San Jose, CA). B-cell purity of at least 95% in all samples was confirmed by flow cytometry using a FACSCanto II (Becton Dickinson).

Table I. Clinical characteristics of patients with CLL at diagnosis.

Number of patients	60
Age, years	66 (35-86)
Male gender (%)	62
Binet stage, *n*	
A	49
B	65
C	11 (5.3-182.5)
Blood lymphocyte count (10^6/mL)	351 (251-887)
LDH (U/L)	2.2 (1.3-8.0)
β_2-Microglobulin (mg/L)	21/49
Unmutated *IGVH*	19/54
ZAP-70 20%	14/49
CD38 30%	8/60
High-risk genetics*	

*17p , 11q , trisomy 12, or three or more cytogenetic abnormalities.

CLL, chronic lymphocytic leukemia; LDH, lactate dehydrogenase; *IGVH*, immunoglobulin variable heavy chain.

Real time PCR

RNA was extracted from B-cell enriched samples using TRIzol reagent (Invitrogen, Carlsbad, CA). The myelo-monocyte cell line HL60 was used as a positive control. cDNA was obtained from total RNA using TaqMan→ Reverse Transcription Reagents (N8080234; Applied Biosystems, Foster City, CA) as previously described [16]. Real time polymerase chain reaction (PCR) was performed using an Applied Biosystems 7500 Sequence Detection system. The specific primers for BAFF, APRIL, and GUSB (β-glucuronidase; endogenous control) were provided by Applied Biosystems (Hs00198106_m1, Hs00601664_g1, and Hs99999908_m1). Expression levels were calculated by linear regression of the control sample and are presented as a percentage.

Statistical analysis

Groups of patients were compared using the Mann-Whitney *U*-test and Spearman's rank correlation. The Maxstat package for R-2.8.0 was used to optimize a cut-off point of the studied variables [17]. According to this, a cut-off equal to 0.63 ng/mL and 6.29 ng/mL was established to categorize patients with low and high BAFF and APRIL levels, respec- tively. Progression-free survival (PFS) was calculated from the date of diagnosis to the date of progression [1]. PFS curves were calculated by the Kaplan-Meier method and compari- son between groups was performed by the log-rank test. The independent association of variables with PFS was analyzed by Cox regression. Statistical analysis was performed using SPSS software (version 15; SPSS, Inc., Chicago, IL). A two- sided p-value 0.05 was considered statistically significant.

Results

BAFF and APRIL serum levels

In patients with CLL the median serum BAFF level was 0.64 ng/mL (range: 0.11-1.91), significantly lower than in healthy controls [0.77 ng/mL (range: 0.57-1.55); p 0.014], whereas serum APRIL was significantly higher than in healthy controls [4.10 ng/mL (0.20-19.70) vs. 1.84 ng/mL (0.43-14.18), p 0.041].

BAFF and APRIL mRNA expression by CLL and normal B-cells

Of the 60 patients, 21 had cryopreserved cells available from the time of diagnosis, in which we measured the expression of BAFF and APRIL mRNA. The mean values of BAFF and APRIL mRNA obtained in purified CLL cells were 7.53 4.22% and 14.77 9.66%, respectively, whereas in normal B-cells (n 10) the expression was 12.13 7.0% for BAFF and 45.53 30.8% for APRIL (p 0.119 and p 0.018, respectively). Those CLL cells that expressed high levels of BAFF mRNA also expressed high levels of APRIL mRNA (r^2 0.338, p 0.046). This correlation was not seen in normal B-cells. Additionally, a high expression of BAFF mRNA

in leukemic cells was inversely correlated with serum BAFF levels in patients with CLL (r^2 0.177, p 0.019).

BAFF and APRIL and clinical correlations in patients with CLL

Lower serum BAFF levels were significantly correlated with high blood lymphocyte count and advanced clinical stage (p 0.03, r^2 0.104 and p 0.03, respectively). However, no relationship was found with other prognostic variables (i.e. gender, immunoglobulin variable heavy chain [*IGVH*] mutational status, cytogenetics, β_2-microglobulin, ZAP-70 and CD38 expression). As for APRIL, high serum levels were correlated with a high expression of CD38 (p 0.04).

With respect to disease progression, along with other well-established prognostic factors (i.e. high blood lym- phocyte count, unmutated *IGVH*, high ZAP-70 and CD38, and poor cytogenetic abnormalities), both low BAFF (0.63 ng/mL) and high APRIL (6.29 ng/mL) serum levels were predictive of progression (p 0.04 and 0.019, respec- tively) [Figures 1(A) and 1(B)]. Furthermore, a higher risk of progression was also observed in those patients whose CLL cells expressed higher BAFF and APRIL mRNA levels (p 0.010 and p 0.053, respectively) [Figures 1(C) and 1(D)].

When the risk of progression was assessed by combining BAFF and APRIL serum levels, three different groups could be identified: a low-risk group (i.e. high BAFF and low APRIL), a high-risk group (i.e. low BAFF and high APRIL), and an intermediate-risk group (i.e. patients not fitting the two former categories). The median PFS was 20 months for the high-risk group and was not reached for the intermediate- and low-risk groups (p 0.001) (Figure 2). The combination of BAFF and APRIL was independently predictive of shorter PFS after adjustment for the blood lymphocyte count, clinical stage, and expression of CD38 (hazard rate: 4.6, 95% confidence interval [CI] 1.2-18.7; p 0.03), but not BAFF or APRIL alone.

Discussion

Recent years have witnessed increasing interest in BAFF and APRIL as B-cell survival factors implicated in the pathogenesis of several B-cell malignancies [11,18]. In CLL, the biological relevance of both molecules has been demonstrated in *in vitro* studies, which show that the addition of exog- enous BAFF and APRIL protects neoplastic CLL cells from apoptosis [19-21]. Furthermore, abnormal BAFF and APRIL serum levels are found in CLL and other B-cell malignancies [11,18,22]. Not surprisingly, there is considerable interest in finding effective ways to target this system as a new treatment modality in B-cell lymphoproliferative disorders [23,24].

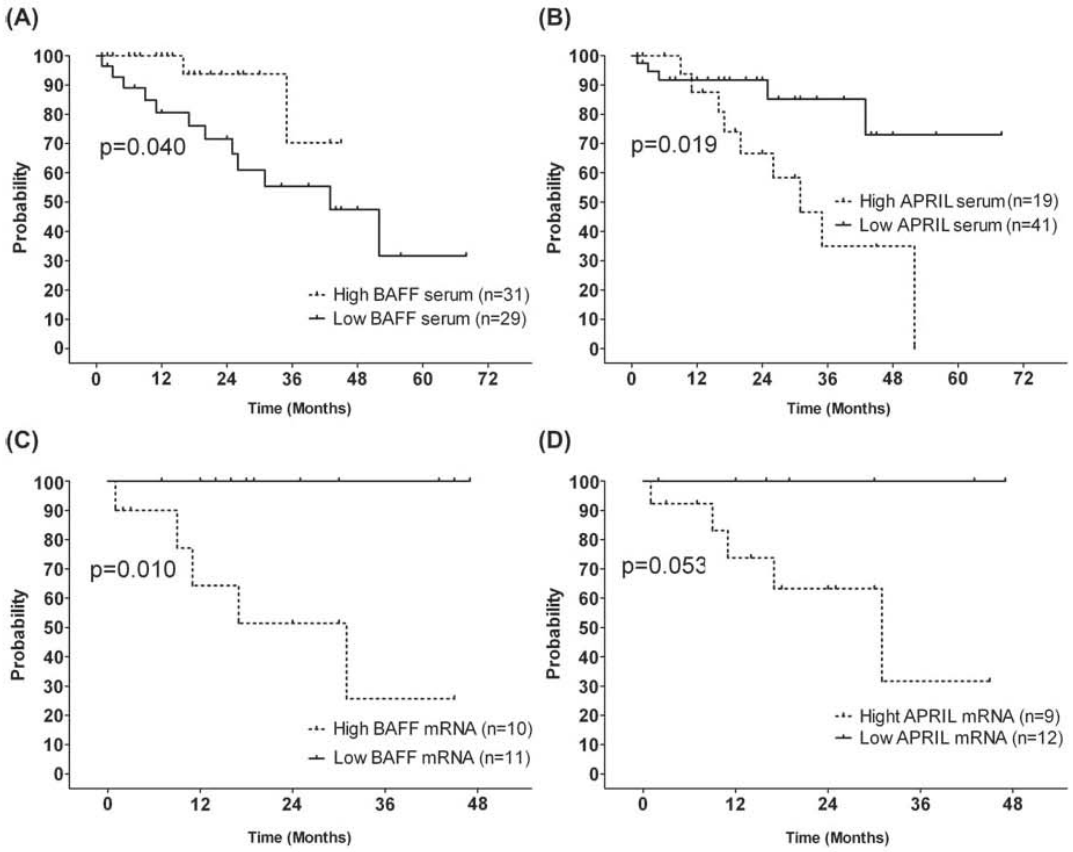


Figure 1. Progression-free survival in patients with CLL according to serum and mRNA expression of BAFF and APRIL. Cut-offs were calculated using the Maxstat R package; the value in each graph is as follows: (A) BAFF serum level 0.63 ng/mL; (B) APRIL serum level 6.29 ng/mL; (C) BAFF mRNA expression 6.24%; (D) APRIL mRNA expression 9.15%.

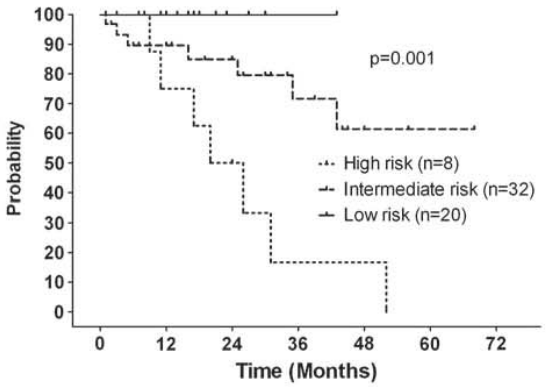


Figure 2. Progression-free survival according to combination of BAFF and APRIL serum levels. Patients were classified into three groups according to BAFF and APRIL serum levels: low-risk, high BAFF and low APRIL; high-risk, low BAFF and high APRIL; intermediate-risk, cases not fitting either of the former categories.

In this study, patients with CLL had lower BAFF but higher APRIL serum levels than normal controls. Despite the discrepancy in absolute serum levels, which are most likely due to methodological reasons (e.g. BAFF and APRIL commercial ELISA kits, populations submitted to study), these results are in line with those obtained in previous publications [6,10-14,22]. We also observed that circulating CLL cells produce on their own BAFF and APRIL mRNA, although at significantly lower levels than normal B-cells, in agreement with the observation that the microenvironment (e.g. nurse-like cells) is a major source of BAFF and APRIL in CLL [25].

In addition, we found a clear association between low BAFF levels and high blood lymphocyte counts and advanced clinical stage. However, no relationship was found with *IGVH* mutational status, CD38 and ZAP-70 expression, or cytogenetics; such associations have been found in some but not all published studies [6,10,12-14]. In agreement with the observation that serum BAFF was inversely related to lymphocyte count, we observed that among patients with follicular lymphoma, those with circulating neoplastic B-cells had lower serum BAFF compared to those without detectable neoplastic cells in the peripheral blood (data not shown). This suggests that serum BAFF may be a marker of tumor burden. It is worth noting, however, that in the majority of studies previously published, blood lymphocyte count has not been correlated with BAFF or APRIL serum levels [6,11-13,26]; in other work in which no correlation was observed between BAFF and lymphocyte counts, it was speculated, however, that this could be related to the absorp- tion of BAFF by CLL cells [14]; finally, a positive correla-
tion between the levels of BAFF and the number of CD5 B-lymphocytes in blood has also been described [10]. The reasons for these discrepancies are unclear and probably reflect methodological issues.

In our study, APRIL serum levels were significantly increased in those cases with high CD38 expression but were not associated with other variables. This may reflect the dependence on CD38 of the cross-talk between CLL cells and stromal or nurse-like cells, which are an important source of BAFF and APRIL [25].

Previous studies have suggested that either BAFF or APRIL serum levels correlate with disease progression and survival [6,10,12]. Our results not only validate these findings but demonstrate that the combined analysis of BAFF and APRIL may provide more accurate predictive information by separating patients into three risk groups. Thus, subjects showing low BAFF and high APRIL serum levels had the highest risk of disease progression whereas those with high BAFF and low APRIL had a very low progression risk. Additionally, higher mRNA expression of BAFF and APRIL was correlated with poor prognosis and could support a role for autocrine stimulation, as previous studies have indicated [10,11,20].

In summary, in this study we found that serum levels of APRIL were elevated in patients with CLL as compared to healthy controls. In turn, serum BAFF levels were lower than those of APRIL and were correlated with blood lym- phocyte count and advanced clinical stage, this suggesting that BAFF could be a marker of leukemic tumor burden. On the other hand, patients with high CD38 expression were those showing higher APRIL serum levels, in keeping with the known interaction between CD38 and APRIL. From the clinical standpoint, the new and most relevant finding of our study is that the combined analysis of serum BAFF and APRIL seems to be a better predictor of progression than either factor alone, and is independent of tumor burden. Taken together, these results provide additional evidence of the significance of BAFF and APRIL in CLL from both a biologic and a clinical perspective. However, the importance of the combined analysis of BAFF and APRIL in predicting disease progression should be validated in further studies including larger series of patients. Moreover, standardization and functional analyses are warranted to better understand the biological role of this system in CLL and to integrate the different results that we and others have reported in a unique logical framework.

Acknowledgements

This work has been supported in part by Red Temàtica de Investigación Cooperativa en Cáncer, grant RT 06/0020/ 002051, the Spanish Ministry of Science, Instituto Carlos III FISS PI080304, Generalitat de Catalunya 2009SGR1008, and the CLL Global Foundation. G.F. is a recipient of a grant from Instituto de Salud Carlos III (PFIS). E.M. is a recipient of the August Pi i Sunyer research award. Thanks are also due to Professor Mariano Monzó, MD, for his continuous support.

Potential con?ict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

References

[1] Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic

Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446-5456.

[2] Chiorazzi N, Rai KR, Ferrarini M. Chronic lymphocytic leukemia. N Engl J Med 2005;352:804-815.

[3] Schena M, Larsson LG, Gottardi D, et al. Growth- and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. Blood 1992;79:2981-2989.

[4] Collins RJ, Verschuer LA, Harmon BV, Prentice RL, Pope JH, Kerr JF. Spontaneous programmed death (apoptosis) of B-chronic lymphocytic leukaemia cells following their culture in vitro. Br J Haematol 1989;71:343-350.

[5] Mackay F, Schneider P, Rennert P, Browning J. BAFF AND APRIL: a tutorial on B cell survival. Annu Rev Immunol 2003;21:231-264.

[6] Planelles L, Castillo-Gutierrez S, Medema JP, Morales-Luque A, Merle-Beral H, Hahne M. APRIL but not BLyS serum levels are increased in chronic lymphocytic leukemia: prognostic relevance of APRIL for survival. Haematologica 2007;92:1284-1285.

[7] Thien M, Phan TG, Gardam S, et al. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. Immunity 2004;20:785-798.

[8] Mackay F, Silveira PA, Brink R. B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. Curr Opin Immunol 2007;19:327-336.

[9] Tangye SG, Bryant VL, Cuss AK, Good KL. BAFF, APRIL and human B cell disorders. Semin Immunol 2006;18:305-317.

[10] Bojarska-Junak A, Hus I, Chocholska S, et al. BAFF and APRIL expression in B-cell chronic lymphocytic leukemia: correlation with biological and clinical features. Leuk Res 2009;33: 1319-1327.

[11] Haiat S, Billard C, Quiney C, Ajchenbaum-Cymbalista F, Kolb JP. Role of BAFF and APRIL in human B-cell chronic lymphocytic leukaemia. Immunology 2006;118:281-292.

[12] Molica S, Digiesi G, Mauro F, et al. Increased serum BAFF (B-cell activating factor of the TNF family) level is a peculiar feature associated with familial chronic lymphocytic leukemia. Leuk Res 2009;33:162-165.

[13] Novak AJ, Grote DM, Ziesmer SC, et al. Elevated serum B-lymphocyte stimulator levels in patients with familial lymphoproliferative disorders. J Clin Oncol 2006;24:2882-2888.

[14] Molica S, Digiesi G, Battaglia C, et al. Baff serum level predicts time to first treatment in early chronic lymphocytic leukemia. Eur J Haematol 2010;85:314-320.

[15] Müller-Hermelink HK, Montserrat E, Catovsky D, et al. Chronic lymphocytic leukaemia/small lymphocytic lymphoma. In: Swerdlow HS, Campo E, Harris NL, et al., editors. WHO classification of tumours of haematopoietic and lymphoid tissues. Lyon: IARC; 2008. p. 180-182.

[16] Monzo M, Moreno I, Artells R, et al. Sonic hedgehog mRNA expression by real-time quantitative PCR in normal and tumor tissues from colorectal cancer patients. Cancer Lett 2006;233: 117-123.

[17] Hothorn T, Lausen B. On the exact distribution of maximally selected rank statistics. Comput Stat Data Anal 2003;43:121-137.

[18] Ferrer G, Hodgson K, Montserrat E, Moreno C. B cell activator factor and a proliferation-inducing ligand at the cross-road of chronic lymphocytic leukemia and autoimmunity. Leuk Lymphoma 2009;50:1075-1082.

[19] Endo T, Nishio M, Enzler T, et al. BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway. Blood 2007;109:703-710.

[20] Kern C, Cornuel JF, Billard C, et al. Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway. Blood 2004;103:679-688.

[21] Novak AJ, Bram RJ, Kay NE, Jelinek DF. Aberrant expression of B-lymphocyte stimulator by B chronic lymphocytic leukemia cells: a mechanism for survival. Blood 2002;100:2973-2979.

[22] Sawicka-Powierza J, Jablonska E, Kloczko J, Piszcz J, Garley M, Ratajczk-Wrona W. Evaluation of TNF superfamily molecules release by neutrophils and B leukemic cells of patients with chronic B-cell lymphocytic leukemia. Neoplasma 2011;58:45-50.

[23] Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The micro-environment in mature B-cell malignancies: a target for new treatment strategies. Blood 2009;114:3367-3375.

[24] Guadagnoli M, Kimberley F, Phan U, et al. Development and characterization of APRIL antagonistic monoclonal antibodies for treatment of B-cell lymphomas. Blood 2011 May 6. [Epub ahead of print]

[25] Nishio M, Endo T, Tsukada N, et al. Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha. Blood 2005;106:1012-1020.

[26] Novak AJ, Grote DM, Stenson M, et al. Expression of BLyS and its receptors in B-cell non-Hodgkin lymphoma: correlation with disease activity and patient outcome. Blood 2004;104: 2247-2253.

Carta 1: Comment on "Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors"

Gerardo Ferrer, Carol Moreno, Emili Montserrat.

Journal of Immunology 2012; 188 (7): 2930-1

Resumen

Esta contribución se mandó al Journal of Immunology, donde fue publicada, para confirmar la hipótesis de Kreuzaler et al. quienes acababan de publicar en esta misma revista (188(1):497-503; 2012) que los niveles de BAFF en suero parecían estar relacionados con el número de células B circulantes y la capacidad de estas para unirse al BAFF soluble. Dichos autores, sin embargo, no pudieron correlacionar los niveles séricos de BAFF con la cifra total de linfocitos B en sangre de pacientes con síndromes linfoproliferativos, cosa que nosotros sí podíamos hacer dados nuestros estudios sobre BAFF (y APRIL). En este sentido, demostramos que los niveles séricos de BAFF se correlacionaban de forma inversa con el recuento linfocitario cualquiera que fuese la enfermedad. Por ejemplo, en pacientes con linfoma folicular los niveles séricos de BAFF eran más bajos en los casos con células tumorales en sangre. También mostramos la existencia de una relación inversamente proporcional entre la cifra de linfocitos en sangre y los niveles de BAFF tanto en pacientes con LLC como en aquellos con linfoma.

References

1. Ryzhov, S., S. V. Novitskiy, A. E. Goldstein, A. Biktasova, M. R. Blackburn, I. Biaggioni, M. M. Dikov, and I. Feoktistov. 2011. Adenosinergic regulation of the expansion and immunosuppressive activity of CD11b^{1}Gr1 cells. J. Immunol. 187: 6120-6129.
2. Kato, M., M. Takahashi, A. A. Akhand, W. Liu, Y. Dai, S. Shimizu, T. Iwamoto, H. Suzuki, and I. Nakashima. 1998. Transgenic mouse model for skin malignant melanoma. Oncogene 17: 1885-1888.
3. Umansky, V., O. Abschuetz, W. Osen, M. Ramacher, F. Zhao, M. Kato, and D. Schadendorf. 2008. Melanoma-specific memory T cells are functionally active in ret transgenic mice without macroscopic tumors. Cancer Res. 68: 9451-9458.
4. Serba, S., J. Schmidt, N. Wentzensen, E. Ryschich, and A. Marten. 2008. Transfection with CD40L induces tumour suppression by dendritic cell activation in an orthotopic mouse model of pancreatic adenocarcinoma. Gut 57: 344-351.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1290007

Response to Comment on "Adenosinergic Regulation of the Expansion and Immunosuppressive Activity of CD11b^{1}Gr1^{1} Cells"

In our recent publication in The Journal of Immunology, we reported that the granulocytic CD11b^{1}Gr1^{high} subset of myeloid-derived suppressor cells (MDSCs) is significantly decreased in tumors growing in animals lacking A_{2B} adenosine receptors, suggesting that this adenosine receptor subtype plays a role in MDSC expansion (1). We then employed an in vitro model of MDSC differentiation previously described by Youn et al. (2) to explore potential pathways involved and found high levels of ecto-5′-nucleotidase (CD73) expression on the granulocytic CD11b^{1}Gr1^{high} subset of MDSCs differentiated in vitro. It is reassuring that our in vitro findings of increased expression of CD73 in CD11b^{1}Gr1^{high} cells are now elegantly confirmed by Dr. Shevchenko et al. in two clinically relevant tumor models in vivo. This provides a mechanism whereby generation of adenosine by granulocytic CD11b^{1}Gr1^{high} tumor-infiltrating MDSCs promotes their further expansion from myeloid progenitors and represents a positive feedback loop that potentially contributes to tumor growth.

Sergey Ryzhov,* Sergey V. Novitskiy,† Italo Biaggioni,‡,x Mikhail M. Dikov,† and Igor Feoktistov*,x

*Division of Cardiovascular Medicine, Department of Medicine, Vanderbilt University, Nashville, TN 37232; †Department of Cancer Biology, Vanderbilt University, Nashville, TN 37232; ‡Division of Clinical Pharmacology, Department of Medicine, Vanderbilt University, Nashville, TN 37232; and xDepartment of Pharmacology, Vanderbilt University, Nashville, TN 37232

Address correspondence and reprint requests to Dr. Igor Feoktistov, Department of Medicine, Vanderbilt University, 360 PRB, 2220 Pierce Avenue, Nashville, TN 37232. Email address: igor.feoktistov@vanderbilt.edu

References

1. Ryzhov, S., S. V. Novitskiy, A. E. Goldstein, A. Biktasova, M. R. Blackburn, I. Biaggioni, M. M. Dikov, and I. Feoktistov. 2011. Adenosinergic regulation of the expansion and immunosuppressive activity of CD11b^{1}Gr1 cells. J. Immunol. 187: 6120-6129.
2. Youn, J. I., S. Nagaraj, M. Collazo, and D. I. Gabrilovich. 2008. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J. Immunol. 181: 5791-5802.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1290008

Comment on "Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors"

Kreuzaler et al. (1) report on the presence of high BAFF serum levels in patients with primary Ab deficiency and other conditions, including a small group of patients with chronic lymphocytic leukemia (CLL). They elegantly show that BAFF serum levels are in?uenced by the number of circulating B cells and the capacity of these cells to bind to soluble BAFF (1).

Given their variable blood involvement, chronic lymphoproliferative disorders are a good model to investigate the correlation between the number of B lymphocytes in blood and BAFF serum levels.

In support of the findings and interpretation of Kreuzaler et al., we observe a correlation between blood lymphocyte counts and BAFF serum levels in 60 patients with CLL: the higher the lymphocyte count, the lower the BAFF serum levels (2). Interestingly, we have also observed lower BAFF serum levels in patients with follicular lymphoma with leukemic expression ($n = 18$) as compared with those without blood spillover ($n = 7$) (Fig. 1A). Moreover, in our series including 85 patients with either CLL or follicular lymphoma, we have found a globally significant negative correlation between blood lymphocyte count and BAFF serum levels (Fig. 1B). Collectively, these results highlight, as concluded by Kreuzaler et al., the central role of the number of B cells (and BAFF binding receptors) in the concentration of BAFF in serum.

Gerardo Ferrer,* Carol Moreno,† and Emili Montserrat*

*Institute of Hematology and Oncology. Hospital Clínic, The August Pi i Sunyer Biomedical Research Institute, University of Barcelona, 08036 Barcelona, Spain; and †Department of Hematology, Santa Creu i Sant Pau Hospital, Autonomous University of Barcelona, 08025 Barcelona, Spain

Address correspondence and reprint requests to Dr. Emili Montserrat, Department of Hematology, Hospital Clínic, Villarroel 170, 08036 Barcelona, Spain. E-mail address: emontse@clinic.ub.es

References

1. Kreuzaler, M., M. Rauch, U. Salzer, J. Birmelin, M. Rizzi, B. Grimbacher, A. Plebani, V. Lougaris, I. Quinti, V. Thon, et al. 2012. Soluble BAFF levels inversely correlate with peripheral B cell numbers and the expression of BAFF receptors. J. Immunol. 188: 497-503.
2. Ferrer, G., K. Hodgson, A. Pereira, M. Juan, M. Elena, D. Colomer, G. Roue, M. Aymerich, T. Baumann, E. Montserrat, and C. Moreno. 2011. Combined an- alysis of levels of serum B-cell activating factor and a proliferation-inducing li- gand as predictor of disease progression in patients with chronic lymphocytic leukemia. Leuk. Lymphoma 52: 2064-2068.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1290009

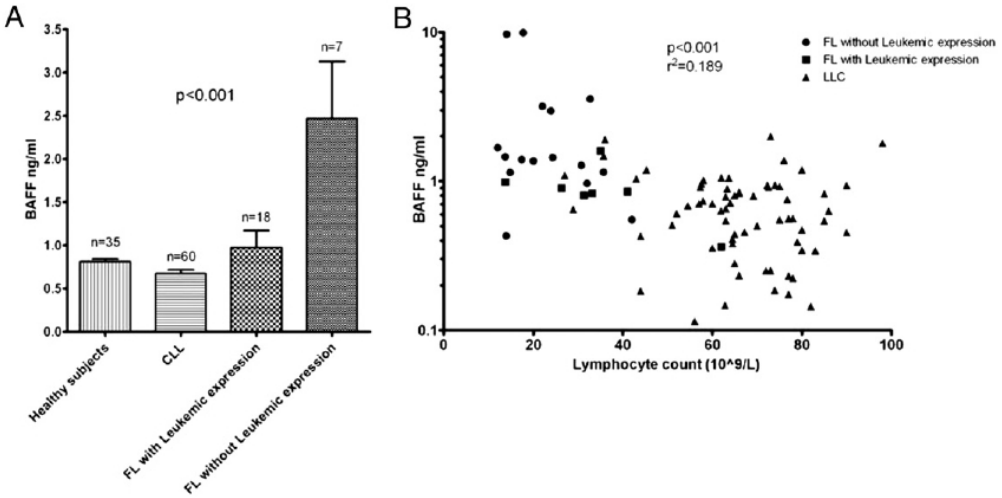


FIGURE 1. (A) BAFF serum levels in healthy subjects (n 5 35), patients with chronic lymphocytic leukemia (n 5 60), and patients with follicular lymphoma [with (n 5 18) and without (n 5 7) leukemic expression]; ANOVA test. (B) Correlation between BAFF serum levels and blood lymphocyte count in patients with CLL (n 5 60) and patients with follicular lymphoma (n 5 25). Spearman test.

Response to Comment on "Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors"

Matthias Kreuzaler,* Antonius G. Rolink,*,[1] and Hermann Eibel[†],[1]

*Division of Developmental and Molecular Immunology, Department of Biomedicine, University of Basel, 4058 Basel, Switzerland; and [†]Centre of Chronic Immunodeficiencies, University Medical Centre Freiburg, 79106 Freiburg, Germany

[1]A.G.R. and H.E. contributed equally.

Address correspondence and reprint requests to Prof. Hermann Eibel or Prof. Antonius G. Rolink, Center of Chronic Immunodeficiency, University Medical Center Freiburg, Breisacher Strasse 66, 79106 Freiburg, Germany (H.E.) or Department of Biomedicine, University of Basel, Mattenstrasse 28, 4058 Basel, Switzerland (A.G.R.). E-mail ad- dresses: hermann.eibel@uniklinik-freiburg.de (H.E.) and antonius.rolink@unibas.ch (A.G.R.)

In a recent publication, Ferrer et al. (1) reported lower BAFF levels and higher concentrations of the closely related ligand APRIL in sera of chronic lymphocytic leukemia (CLL) patients than in corresponding samples of healthy controls. They also reported an inverse correlation between BAFF concentrations, blood lymphocyte counts, clinical stage of CLL, and disease progression. Their findings strongly support our results, which demonstrated an inverse correlation between soluble BAFF concentrations and B cell numbers in patients suffering from primary Ab deficiencies as well as in CLL patients (2). Therefore, binding of BAFF to its receptors expressed on normal or malignant B cells followed by "consumption" through the target cells seems to be one of the major mechanisms regulating soluble BAFF levels.

References

1. Ferrer, G., K. Hodgson, A. Pereira, M. Juan, M. Elena, D. Colomer, G. Roue, M. Aymerich, T. Baumann, E. Montserrat, and C. Moreno. 2011. Combined analysis of levels of serum B-cell activating factor and a proliferation-inducing ligand as predictor of disease progression in patients with chronic lymphocytic leukemia. Leuk. Lymphoma 52: 2064-2068.
2. Kreuzaler, M., M. Rauch, U. Salzer, J. Birmelin, M. Rizzi, B. Grimbacher, A. Plebani, V. Lougaris, I. Quinti, V. Thon, et al. 2012. Soluble BAFF levels inversely correlate with peripheral B cell numbers and the expression of BAFF receptors. J. Immunol. 188: 497-503.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1290010

Discusión

Los objetivos fundamentales de esta tesis han sido determinar la prevalencia, correlaciones clínico-biológicas y pronóstico de citopenias autoinmunes en una amplia serie de pacientes no seleccionados de LLC e investigar las moléculas BAFF y APRIL en relación a las manifestaciones clínicas de la enfermedad, pronóstico y fenómenos autoinmunes.

La asociación de la LLC con citopenias autoinmunes es conocida desde antiguo. Ya en las primeras publicaciones "canónicas" de la enfermedad, por pioneros en el estudio de la hematología moderna como William Dameshek o David Galton [149, 226], hacían hincapié en que la LLC podía asociarse a fenómenos autoinmunes, particularmente la AHAI. La relación entre LLC y fenómenos autoinmunes que no tienen como diana a las células hemáticas es, sin embargo, mucho más controvertida. Aunque muchos autores mencionan su existencia, la misma se basa mucho más en un "estado de opinión" y en publicaciones de casos sueltos o de pequeñas series (sabida es la tendencia a publicar "asociaciones raras" en el campo de la Medicina) que en series amplias y bien documentadas. Por ello, y para tener una base firme sobre la sustentar los ulteriores trabajos de esta tesis, procedimos en primer lugar a una revisión meticulosa, basada en las recomendaciones PRISMA [225], de toda la literatura publicada sobre LLC y autoinmunidad. Como resultado de esta revisión, concluimos que los individuos con LLC no tienen un mayor riesgo de presentar enfermedades autoinmunes no hemáticas, con la excepción del pénfigo bulloso, ni que los pacientes con trastornos autoinmunes no hemáticos padezcan LLC con mayor frecuencia que la población general aunque sí es cierto que existen casos en los que se ha demostrado la producción de autoanticuerpos por parte de la clona de LLC [227-232]. Esta conclusión concuerda con los pocos estudios epidemiológicos llevados a cabo con rigor [108-112]. En cambio, la asociación de LLC con citopenias autoinmunes está muy bien demostrada, sobre todo por lo que hace a la AHAI y a la PTI. La asociación de LLC con eritroblastopenia también es un hecho, aunque menos frecuente que la de LLC con AHAI o PTI. Sin embargo, la asociación de LLC con

neutropenia autoinmune es excepcional y los pocos casos registrados, sobre todo en publicaciones antiguas, invitan a pensar que tales neutropenias no estaban asociadas a LLC sino a otros síndromes linfoproliferativos, por ejemplo la linfocitosis de linfocitos grandes granulares.

El objetivo fundamental de esta tesis fue analizar no todos los trastornos autoinmunes que se pueden observar en la LLC, sino las dos enfermedades autoinmunes que de forma más frecuente acompañan a la LLC, es decir la AHAI y la PTI. En nuestra serie, 70 de 960 pacientes habían presentado AHAI o PTI en algún momento a lo largo de la enfermedad. La prevalencia del 7% está en concordancia con estudios recientes donde se han detectado citopenias autoinmunes en el 4.5% - 10% de los pacientes [140, 144, 157].

La asociación entre el tratamiento y la aparición de AHAI en la LLC es un hecho conocido de antiguo y que ya se citaba en las primeras descripciones rigurosas de la enfermedad [149, 226], como antiguo es también el debate sobre si determinados medicamentos pueden precipitar la aparición de AHAI con más frecuencia que otros (103, 105). En los años 90, coincidiendo con la introducción de los análogos de las purinas como terapia para la LLC, se suscitó el temor de que estos fármacos pudiesen desencadenar AHAI con una frecuencia hasta entonces desconocida. Dichos temores – apoyados en estudios casuísticos muy seleccionados, sin grupo control y poco rigurosos – eran infundados. En un estudio reciente se comprobó que la incidencia de citopenia autoinmune en enfermos tratados con clorambucil es similar (14%) a la observada en los sujetos tratados con fludarabina (13%) [154]. Más aún, en estudios prospectivos se ha visto que la incidencia de AIHA es significativamente menor en pacientes tratados con fludarabina más ciclofosfamida (5%) que en aquellos que reciben clorambucil (12%) o fludarabina (11%) [143]. Cabe resaltar que nuestros resultados, que reflejan lo que ocurre en una población no seleccionada de enfermos con LLC y, por tanto, lo que ocurre en el “mundo real”, confirman plenamente estas

observaciones, puesto que la incidencia de AHAI por nosotros observada fue del 5% en los enfermos tratados con clorambucil y del 4% en los que recibieron análogos de las purinas sólo o combinados con otros agentes [155].

Las citopenias autoinmunes se asocian con frecuencia con marcadores de mal pronóstico entre los que destacan el recuento de linfocitos en sangre elevado, el estadio avanzado de la enfermedad, la ausencia de mutaciones de los genes IGHV, la expresión elevada de ZAP-70 y CD38 en los linfocitos neoplásicos, así como niveles altos de B2M en suero [140, 142, 157]. En nuestra serie, estas correlaciones se ven confirmadas con la única excepción de las mutaciones de *IGHV*, lo que podría deberse a los pocos casos en los que esta variable pudo estudiarse.

Uno de los puntos más controvertidos de las citopenias autoinmunes es su valor pronóstico [140, 144, 145, 157, 233, 234]. En los pacientes por nosotros estudiados la aparición de una citopenia autoinmune en cualquier momento de la enfermedad no influenció significativamente en el pronóstico. Puesto que ni los estadios de Rai ni de Binet [72, 73], tienen en cuenta el origen de la citopenia, analizamos la influencia de las citopenias en el grupo de pacientes con estadio avanzado (Binet C) al diagnóstico. A este respecto, observamos que los pacientes en estadio C de origen inmune tenían una supervivencia significativamente más larga (7.4 años) que aquellos en estadio avanzado por infiltración medular (3.7 años). Es interesante señalar que han tenido que pasar más de 20 años para que esta observación, inicialmente hecha por un grupo de investigadores escandinavos [235], se haya visto confirmada tanto por nosotros como por la Clínica Mayo [144]. La principal causa de la mejor supervivencia de los enfermos con estadio avanzado de origen inmune es la respuesta al tratamiento ya que suelen responder muy bien al tratamiento con glucocorticoides y, debido a ello, muchos casos inicialmente considerados en estadio avanzado pasan a estadio precoz. En cambio, los pacientes en estadio C por infiltración medular responden mal al tratamiento

esteroideo. Como colofón de esta parte, cabe resaltar que a raíz de estas publicaciones el Profesor Finbar Cotter, editor de British Journal of Haematology, nos invitó a escribir una revisión sobre el diagnóstico y tratamiento de las citopenias autoinmunes en la LLC, la cual fue publicada después de la consiguiente revisión por pares (revisión 2).

Al igual que en el caso de las citopenias autoinmunes, antes de acometer el estudio de las moléculas BAF y APRIL en la LLC, procedimos a una revisión exhaustiva de la literatura. BAFF y APRIL son dos moléculas que intervienen en la LLC y la autoinmunidad [168]. En el estudio de estas moléculas observamos que, en comparación con los sujetos normales, los pacientes con LLC presentaban niveles bajos de BAFF pero altos de APRIL en el suero. A pesar de las discrepancias en sus valores absolutos, probablemente debido a razones metodológicas, estos resultados están en consonancia con otros previamente publicados [14, 21, 170, 213, 214, 236, 237]. Además pudimos observar que las propias células de LLC eran capaces de producir ARNm de BAFF y de APRIL, aunque a niveles más bajos que las células B normales, lo que por otra parte concuerda con el hecho de que la principal fuente de estas dos moléculas en la LLC son las células del microambiente [71].

También pudimos comprobar que los niveles bajos de BAFF en suero estaban asociados al recuento linfocitario y estadio avanzado, así como con AHAI (observaciones no publicadas). Sin embargo, no hallamos ninguna correlación entre los niveles de BAFF y otros marcadores como las mutaciones de los genes de *IGHV*, la expresión de CD38, ZAP-70 o la citogenética, lo que constituye una diferencia con respecto a otros estudios [20, 21, 213, 214, 236]. Recientemente se ha descrito que los niveles de BAFF en suero están principalmente ligados a dos factores: el número de células B circulantes y capacidad de los receptores (principalmente expresados en células B) para unirse a BAFF [238]. En concordancia con este estudio observamos una relación inversa entre el recuento linfocitario y los niveles de BAFF en el suero en los pacientes de LLC y linfoma folicular.

La correlación entre los niveles de APRIL en suero y la expresión en las células neoplásicas del marcador CD38 es un hecho interesante. El CD38 es un marcador de activación celular que se ha visto asociado con una mayor recirculación de las células de LLC en los ganglios linfáticos donde interactúa con las células del microambiente y promueve la producción de APRIL [239]. A su vez, las células de la LLC facilitan transformación de las células CD14+ a células *nurse-like*, las cuales expresan fuertemente APRIL y contribuyen a la supervivencia de las células de la LLC [71]. Es plausible por ello pensar que en los casos CD38 positivos la mayor recirculación de las células leucémicas en los ganglios linfáticos facilita una expresión más elevada de APRIL por parte del microambiente.

En estudios previos se ha observado la asociación de tanto BAFF como APRIL con la progresión de la enfermedad y supervivencia de los enfermos con LLC [213, 214, 236], cosa que nuestros resultados apoyan. Además, nuestro estudio ha puesto de manifiesto que el valor pronóstico de BAFF y APRIL aumenta cuando ambas moléculas se valoran conjuntamente. Así, los pacientes con niveles bajos de BAFF y altos de APRIL presentaban el mayor riesgo de progresión, mientras que aquellos con niveles altos de BAFF y bajos de APRIL tenían un riesgo de progresión muy bajo.

En resumen, así como las citopenias autoinmunes están claramente asociadas a la LLC, no se puede asegurar lo mismo de otras enfermedades autoinmunes. En el primer artículo en la amplia serie analizada de pacientes de un único centro observamos una prevalencia de las citopenias autoinmunes del 7%. Estos pacientes tenían factores de mal pronóstico como el recuento linfocitario elevado, un tiempo de duplicación linfocitario rápido, así como una B2M sérica, ZAP-70 y CD38 elevadas, pero no con el tipo de tratamiento recibido. Por lo que hace al pronóstico de la enfermedad, los pacientes con citopenias autoinmunes se comportaron de forma parecida al resto. Pero, de forma sumamente importante, los enfermos con estadio avanzado atribuible a un origen inmune tenían un pronóstico mejor que aquellos en los que la

fase avanzada de la enfermedad reflejaba una alta carga tumoral. Ello invita, de acuerdo con el estudio de la Clínica Mayo, y de la Editorial en *Blood* que acompañó a nuestro artículo, a diferenciar dentro de los pacientes con LLC en estadio avanzado aquellos en los que la citopenia tiene un origen autoinmune (C "inmune") y los que la anemia se debe al fallo de la medula ósea a causa de la infiltración linfocitaria (C "infiltrativo").

En relación con BAFF y APRIL, los pacientes con LLC presentan niveles altos de APRIL y bajos de BAFF en comparación con sujetos sanos. Además, los niveles de BAFF se correlacionan con el recuento linfocitario en sangre, estadio avanzado y la presencia de AHAI. Por último, la valoración conjunta de BAFF y APRIL ofrece mayor información pronóstica que la que se deriva del análisis de estas moléculas por separado.

Por último, el doctorando desea subrayar que los resultados presentados en esta Tesis Doctoral no podrían haberse obtenido sin la sólida base de la investigación sobre muy diversos aspectos, tanto clínicos como biológicos, de la LLC en el Servicio de Hematología del Hospital Clínico desde hace más de treinta años. Los trabajos aquí presentados no pretenden otra cosa que ser una modesta contribución a esta línea de trabajo. Y también el testimonio de admiración y afecto a todos aquellos que me han guiado, enseñado y han sido fuente de inspiración durante mis años en el Servicio de Hematología del Hospital Clínic, el Laboratorio de Embriología y Oncología Molecular de la Facultad de Medicina y la Universidad de Barcelona. Para ellos, mi mayor reconocimiento y gratitud.

Conclusiones

Las principales conclusiones de esta tesis son:

1. La LLC presenta con frecuencia citopenias autoinmunes, pero no se asocia a síndromes autoinmunes no hemáticos.

2. Debido a sus implicaciones pronósticas y terapéuticas, es importante subclasificar las LLC en estadio avanzado en función de la causa de la citopenia: autoinmune o infiltrativa, puesto que:

2.1. Los pacientes con estadio avanzado debido a una citopenia autoinmune (7% de los enfermos en nuestra serie) tienen mucho mejor pronóstico que aquellos cuya citopenia se debe a la infiltración de la medula ósea.

2.2. Este hecho se debe a que los pacientes con citopenia autoinmune, particularmente aquellos con PTI, suelen responder muy bien al tratamiento con glucocorticoides y a menudo pasan de estadio avanzado (C, III, IV) a estadio precoz (A, 0) con tan sólo este tratamiento.

3. Con respecto a BAFF/APRIL, se confirma la importancia de este sistema en la LLC ya que:

3.1. En comparación con personas sanas, los pacientes con LLC presentan niveles séricos bajos de BAFF y elevados de APRIL.

3.2. Los niveles séricos de BAFF se hallan inversamente correlacionados con el recuento de linfocitos y la fase de la enfermedad, de modo que son tanto más bajos cuanto más alta es la cifra de linfocitos y más avanzado el estadio clínico.

3.3. Los niveles bajos de BAFF y altos de APRIL en suero se correlacionan con la progresión de la enfermedad.

3.4. El análisis conjunto de BAFF y APRIL en suero resultó ser un marcador pronóstico para el riesgo de progresión de la enfermedad más preciso y de valor independiente que el de estas dos moléculas por separado.

Bibliografía

1. Hallek, M., et al., *Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines.* Blood, 2008. **111**(12): p. 5446-5456.
2. Müller-Hermelink, H.K., et al., *Chronic lymphocytic leukaemia / small lymphocytic lymphoma*, in *WHO classification of tumours of haematopoietic and lymphoid tissues*, H.S. Swerdlow, et al., Editors. 2008, International Agency for Research on Cancer: Lyon France. p. 180-182.
3. Weiss, N.S., *Geographical variation in the incidence of the leukemias and lymphomas.* Natl Cancer Inst Monogr, 1979(53): p. 139-42.
4. Finch, S.C. and M.S. Linet, *Chronic leukaemias.* Baillieres Clin Haematol, 1992. **5**(1): p. 27-56.
5. Goldin, L.R. and S.L. Slager, *Familial CLL: genes and environment.* Hematology Am Soc Hematol Educ Program, 2007: p. 339-45.
6. Goldin, L.R., et al., *Anticipation in familial chronic lymphocytic leukemia.* Am J Hum Genet, 1999. **65**(1): p. 265-9.
7. Rawstron, A.C., et al., *Monoclonal B lymphocytes with the characteristics of "indolent" chronic lymphocytic leukemia are present in 3.5% of adults with normal blood counts.* Blood, 2002. **100**(2): p. 635-639.
8. Marti, G.E., et al., *B-cell monoclonal lymphocytosis and B-cell abnormalities in the setting of familial B-cell chronic lymphocytic leukemia.* Cytometry B Clin Cytom, 2003. **52**(1): p. 1-12.
9. Slager, S.L., et al., *Genome-wide association study identifies a novel susceptibility locus at 6p21.3 among familial CLL.* Blood, 2011. **117**(6): p. 1911-6.
10. Chiorazzi, N., K.R. Rai, and M. Ferrarini, *Chronic lymphocytic leukemia.* N.Engl.J.Med., 2005. **352**(8): p. 804-815.
11. Dighiero, G. and T.J. Hamblin, *Chronic lymphocytic leukaemia.* Lancet, 2008. **371**(9617): p. 1017-29.
12. Chiorazzi, N. and M. Ferrarini, *Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities.* Blood, 2011. **117**(6): p. 1781-91.
13. Lanasa, M.C., *Novel insights into the biology of CLL.* Hematology.Am.Soc.Hematol.Educ.Program., 2010. **2010**: p. 70-76.
14. Molica, S., et al., *Increased serum BAFF (B-cell activating factor of the TNF family) level is a peculiar feature associated with familial chronic lymphocytic leukemia.* Leukemia Research, 2009. **33**(1): p. 162-165.
15. Crowther-Swanepoel, D. and R.S. Houlston, *Genetic variation and risk of chronic lymphocytic leukaemia.* Seminars in Cancer Biology, 2010.
16. Kikushige, Y., et al., *Self-renewing hematopoietic stem cell is the primary target in pathogenesis of human chronic lymphocytic leukemia.* Cancer Cell, 2011. **20**(2): p. 246-59.
17. Klein, U., et al., *The DLEU2/miR-15a/16-1 cluster controls B cell proliferation and its deletion leads to chronic lymphocytic leukemia.* Cancer Cell, 2010. **17**(1): p. 28-40.
18. Calin, G.A., et al., *Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15524-9.
19. Calin, G.A. and C.M. Croce, *Chronic lymphocytic leukemia: interplay between noncoding RNAs and protein-coding genes.* Blood, 2009. **114**(23): p. 4761-70.

20. Molica, S., et al., *Increased serum BAFF (B-cell activating factor of the TNF family) level is a peculiar feature associated with familial chronic lymphocytic leukemia.* Leuk.Res., 2009. **33**(1): p. 162-165.
21. Novak, A.J., et al., *Elevated serum B-lymphocyte stimulator levels in patients with familial lymphoproliferative disorders.* J Clin Oncol, 2006. **24**(6): p. 2882-2888.
22. Novak, A.J., et al., *Genetic variation in B-cell-activating factor is associated with an increased risk of developing B-cell non-Hodgkin lymphoma.* Cancer Research, 2009. **69**(10): p. 4217-4224.
23. Enzler, T., et al., *BAFF Accelerates Development of Chronic Lymphocytic Leukemia in TCL1 Transgenic Mice.* Blood, 2007. **110**: p. Abstract 1117.
24. Enzler, T., et al., *Constitutive Baff Signalling Plays a Key Role in CLL Development by Promoting Tumor Cell Survival.* Blood, 2008. **112**: p. Abstract 28.
25. Messmer, B.T., et al., *In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells.* J Clin Invest, 2005. **115**(3): p. 755-64.
26. Damle, R.N., et al., *Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia.* Blood, 1999. **94**(6): p. 1840-7.
27. Hamblin, T.J., et al., *Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia.* Blood, 1999. **94**(6): p. 1848-54.
28. Rozman, C. and E. Montserrat, *Chronic lymphocytic leukemia.* N Engl J Med, 1995. **333**(16): p. 1052-7.
29. Rawstron, A.C., et al., *Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia.* N.Engl.J Med, 2008. **359**(6): p. 575-583.
30. Rozman, C., E. Montserrat, and N. Vinolas, *Serum immunoglobulins in B-chronic lymphocytic leukemia. Natural history and prognostic significance.* Cancer, 1988. **61**(2): p. 279-83.
31. Molica, S., *Infections in chronic lymphocytic leukemia: risk factors, and impact on survival, and treatment.* Leuk Lymphoma, 1994. **13**(3-4): p. 203-14.
32. Wadhwa, P.D. and V.A. Morrison, *Infectious complications of chronic lymphocytic leukemia.* Semin Oncol, 2006. **33**(2): p. 240-9.
33. Richter, M.N., *Generalized Reticular Cell Sarcoma of Lymph Nodes Associated with Lymphatic Leukemia.* Am J Pathol, 1928. **4**(4): p. 285-292 7.
34. Robertson, L.E., et al., *Richter's syndrome: a report on 39 patients.* J Clin Oncol, 1993. **11**(10): p. 1985-9.
35. Cobo, F., et al., *Multiple cell cycle regulator alterations in Richter's transformation of chronic lymphocytic leukemia.* Leukemia, 2002. **16**(6): p. 1028-34.
36. Greene, M.H., R.N. Hoover, and J.F. Fraumeni, Jr., *Subsequent cancer in patients with chronic lymphocytic leukemia--a possible immunologic mechanism.* J Natl Cancer Inst, 1978. **61**(2): p. 337-40.
37. Hisada, M., et al., *Solid tumors after chronic lymphocytic leukemia.* Blood, 2001. **98**(6): p. 1979-81.
38. Goldin, L.R., et al., *Familial risk of lymphoproliferative tumors in families of patients with chronic lymphocytic leukemia: results from the Swedish Family-Cancer Database.* Blood, 2004. **104**(6): p. 1850-4.
39. Kaae, J., et al., *Merkel cell carcinoma: incidence, mortality, and risk of other cancers.* J Natl Cancer Inst, 2010. **102**(11): p. 793-801.
40. Tobin, G., et al., *Chronic lymphocytic leukemias utilizing the VH3-21 gene display highly restricted Vlambda2-14 gene use and homologous CDR3s: implicating recognition of a common antigen epitope.* Blood, 2003. **101**(12): p. 4952-7.

41. Messmer, B.T., et al., *Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia.* J Exp Med, 2004. **200**(4): p. 519-25.
42. Murray, F., et al., *Stereotyped patterns of somatic hypermutation in subsets of patients with chronic lymphocytic leukemia: implications for the role of antigen selection in leukemogenesis.* Blood, 2008. **111**(3): p. 1524-33.
43. Chen, L., et al., *Expression of ZAP-70 is associated with increased B-cell receptor signaling in chronic lymphocytic leukemia.* Blood, 2002. **100**(13): p. 4609-14.
44. Chen, L., et al., *ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia.* Blood, 2005. **105**(5): p. 2036-41.
45. Stevenson, F.K. and F. Caligaris-Cappio, *Chronic lymphocytic leukemia: revelations from the B-cell receptor.* Blood, 2004. **103**(12): p. 4389-95.
46. Richardson, S.J., et al., *ZAP-70 expression is associated with enhanced ability to respond to migratory and survival signals in B-cell chronic lymphocytic leukemia (B-CLL).* Blood, 2006. **107**(9): p. 3584-92.
47. Migliazza, A., et al., *Nucleotide sequence, transcription map, and mutation analysis of the 13q14 chromosomal region deleted in B-cell chronic lymphocytic leukemia.* Blood, 2001. **97**(7): p. 2098-104.
48. Bullrich, F., et al., *Characterization of the 13q14 tumor suppressor locus in CLL: identification of ALT1, an alternative splice variant of the LEU2 gene.* Cancer Res, 2001. **61**(18): p. 6640-8.
49. Dohner, H., et al., *Genomic aberrations and survival in chronic lymphocytic leukemia.* N Engl J Med, 2000. **343**(26): p. 1910-6.
50. Dohner, H., et al., *Cytogenetic and molecular cytogenetic analysis of B cell chronic lymphocytic leukemia: specific chromosome aberrations identify prognostic subgroups of patients and point to loci of candidate genes.* Leukemia, 1997. **11 Suppl 2**: p. S19-24.
51. Zenz, T., et al., *TP53 mutation and survival in chronic lymphocytic leukemia.* J Clin Oncol, 2010. **28**(29): p. 4473-9.
52. Gonzalez, D., et al., *Mutational status of the TP53 gene as a predictor of response and survival in patients with chronic lymphocytic leukemia: results from the LRF CLL4 trial.* J Clin Oncol, 2011. **29**(16): p. 2223-9.
53. Montserrat, E. and C. Moreno, *Genetic lesions in chronic lymphocytic leukemia: clinical implications.* Curr Opin Oncol, 2009. **21**(6): p. 609-14.
54. Puente, X.S., et al., *Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia.* Nature, 2011. **475**(7354): p. 101-5.
55. Wang, L., et al., *SF3B1 and other novel cancer genes in chronic lymphocytic leukemia.* N Engl J Med, 2011. **365**(26): p. 2497-506.
56. Rossi, D., et al., *Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia.* Blood, 2012. **119**(2): p. 521-9.
57. Rossi, D., et al., *Disruption of BIRC3 associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia.* Blood, 2012. **119**(12): p. 2854-62.
58. Gilson, E. and V. Geli, *How telomeres are replicated.* Nat Rev Mol Cell Biol, 2007. **8**(10): p. 825-38.
59. Greider, C.W. and E.H. Blackburn, *Identification of a specific telomere terminal transferase activity in Tetrahymena extracts.* Cell, 1985. **43**(2 Pt 1): p. 405-13.
60. Kim, N.W., et al., *Specific association of human telomerase activity with immortal cells and cancer.* Science, 1994. **266**(5193): p. 2011-5.
61. Augereau, A., et al., *Telomeric damage in early stage of chronic lymphocytic leukemia correlates with shelterin dysregulation.* Blood, 2011. **118**(5): p. 1316-22.

62. Lanasa, M.C., *Novel insights into the biology of CLL.* Hematology Am Soc Hematol Educ Program, 2010. **2010**: p. 70-6.
63. Raval, A., et al., *Downregulation of death-associated protein kinase 1 (DAPK1) in chronic lymphocytic leukemia.* Cell, 2007. **129**(5): p. 879-90.
64. Kanduri, M., et al., *Differential genome-wide array-based methylation profiles in prognostic subsets of chronic lymphocytic leukemia.* Blood, 2010. **115**(2): p. 296-305.
65. Cimmino, A., et al., *miR-15 and miR-16 induce apoptosis by targeting BCL2.* Proc Natl Acad Sci U S A, 2005. **102**(39): p. 13944-9.
66. Pekarsky, Y., et al., *Tcl1 expression in chronic lymphocytic leukemia is regulated by miR-29 and miR-181.* Cancer Res, 2006. **66**(24): p. 11590-3.
67. Mott, J.L., et al., *mir-29 regulates Mcl-1 protein expression and apoptosis.* Oncogene, 2007. **26**(42): p. 6133-40.
68. Zhao, H., et al., *MicroRNA and leukemia: tiny molecule, great function.* Crit Rev Oncol Hematol, 2010. **74**(3): p. 149-55.
69. Zenz, T., et al., *miR-34a as part of the resistance network in chronic lymphocytic leukemia.* Blood, 2009. **113**(16): p. 3801-8.
70. Burger, J.A., et al., *Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1.* Blood, 2000. **96**(8): p. 2655-2663.
71. Nishio, M., et al., *Nurselike cells express BAFF and APRIL, which can promote survival of chronic lymphocytic leukemia cells via a paracrine pathway distinct from that of SDF-1alpha.* Blood, 2005. **106**(3): p. 1012-1020.
72. Binet, J.L., et al., *A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis.* Cancer, 1981. **48**(1).
73. Rai, K.R., et al., *Clinical staging of chronic lymphocytic leukemia.* Blood, 1975. **46**(2).
74. Rozman, C., et al., *Bone marrow histologic pattern--the best single prognostic parameter in chronic lymphocytic leukemia: a multivariate survival analysis of 329 cases.* Blood, 1984. **64**(3): p. 642-8.
75. Montserrat, E., et al., *Bone marrow assessment in B-cell chronic lymphocytic leukaemia: aspirate or biopsy? A comparative study in 258 patients.* Br J Haematol, 1996. **93**(1): p. 111-6.
76. Vallespi, T., E. Montserrat, and M.A. Sanz, *Chronic lymphocytic leukaemia: prognostic value of lymphocyte morphological subtypes. A multivariate survival analysis in 146 patients.* Br J Haematol, 1991. **77**(4): p. 478-85.
77. Montserrat, E., *Classical and new prognostic factors in chronic lymphocytic leukemia: where to now?* Hematol J, 2002. **3**(1): p. 7-9.
78. Rozman, C., F. Bosch, and E. Montserrat, *Chronic lymphocytic leukemia: a changing natural history?* Leukemia, 1997. **11**(6): p. 775-8.
79. Montserrat, E., *Treatment options in chronic lymphocytic leukemia.* Hematol J, 2004. **5 Suppl 1**: p. S2-9.
80. Stilgenbauer, S., et al., *Incidence and clinical significance of 6q deletions in B cell chronic lymphocytic leukemia.* Leukemia, 1999. **13**(9): p. 1331-4.
81. Hamblin, T.J., et al., *CD38 expression and immunoglobulin variable region mutations are independent prognostic variables in chronic lymphocytic leukemia, but CD38 expression may vary during the course of the disease.* Blood, 2002. **99**(3): p. 1023-9.
82. Crespo, M., et al., *ZAP-70 expression as a surrogate for immunoglobulin-variable-region mutations in chronic lymphocytic leukemia.* N Engl J Med, 2003. **348**(18): p. 1764-75.
83. Orchard, J.A., et al., *ZAP-70 expression and prognosis in chronic lymphocytic leukaemia.* Lancet, 2004. **363**(9403): p. 105-11.

84. Rassenti, L.Z., et al., *ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia.* N Engl J Med, 2004. **351**(9): p. 893-901.
85. Heintel, D., et al., *High expression of lipoprotein lipase in poor risk B-cell chronic lymphocytic leukemia.* Leukemia, 2005. **19**(7): p. 1216-23.
86. Calin, G.A., et al., *A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia.* N Engl J Med, 2005. **353**(17): p. 1793-801.
87. Grabowski, P., et al., *Telomere length as a prognostic parameter in chronic lymphocytic leukemia with special reference to VH gene mutation status.* Blood, 2005. **105**(12): p. 4807-12.
88. Moreno, C., et al., *Allogeneic stem-cell transplantation may overcome the adverse prognosis of unmutated VH gene in patients with chronic lymphocytic leukemia.* J Clin Oncol, 2005. **23**(15): p. 3433-8.
89. Dohner, H., et al., *p53 gene deletion predicts for poor survival and non-response to therapy with purine analogs in chronic B-cell leukemias.* Blood, 1995. **85**(6): p. 1580-9.
90. Ritgen, M., et al., *Unmutated immunoglobulin variable heavy-chain gene status remains an adverse prognostic factor after autologous stem cell transplantation for chronic lymphocytic leukemia.* Blood, 2003. **101**(5): p. 2049-53.
91. Byrd, J.C., et al., *Select high-risk genetic features predict earlier progression following chemoimmunotherapy with fludarabine and rituximab in chronic lymphocytic leukemia: justification for risk-adapted therapy.* J Clin Oncol, 2006. **24**(3): p. 437-43.
92. Rai, K.R., et al., *Fludarabine compared with chlorambucil as primary therapy for chronic lymphocytic leukemia.* N Engl J Med, 2000. **343**(24): p. 1750-7.
93. Catovsky, D., et al., *Assessment of fludarabine plus cyclophosphamide for patients with chronic lymphocytic leukaemia (the LRF CLL4 Trial): a randomised controlled trial.* Lancet, 2007. **370**(9583): p. 230-9.
94. Hallek, M., et al., *Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial.* Lancet, 2010. **376**(9747): p. 1164-74.
95. Keating, M.J., et al., *Early results of a chemoimmunotherapy regimen of fludarabine, cyclophosphamide, and rituximab as initial therapy for chronic lymphocytic leukemia.* J Clin Oncol, 2005. **23**(18): p. 4079-88.
96. Tam, C.S., et al., *Long-term results of the fludarabine, cyclophosphamide, and rituximab regimen as initial therapy of chronic lymphocytic leukemia.* Blood, 2008. **112**(4): p. 975-80.
97. Caballero, D., et al., *Allogeneic transplant with reduced intensity conditioning regimens may overcome the poor prognosis of B-cell chronic lymphocytic leukemia with unmutated immunoglobulin variable heavy-chain gene and chromosomal abnormalities (11q- and 17p-).* Clin Cancer Res, 2005. **11**(21): p. 7757-63.
98. Sorror, M.L., et al., *Five-year follow-up of patients with advanced chronic lymphocytic leukemia treated with allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning.* J Clin Oncol, 2008. **26**(30): p. 4912-20.
99. Delgado, J., J. Briones, and J. Sierra, *Emerging therapies for patients with advanced chronic lymphocytic leukaemia.* Blood Rev, 2009. **23**(5): p. 217-24.
100. Burger, J.A., et al., *The microenvironment in mature B-cell malignancies: a target for new treatment strategies.* Blood, 2009. **114**(16): p. 3367-75.
101. Gribben, J.G. and S. O'Brien, *Update on therapy of chronic lymphocytic leukemia.* J Clin Oncol, 2011. **29**(5): p. 544-50.
102. Hamblin, T.J., D.G. Oscier, and B.J. Young, *Autoimmunity in chronic lymphocytic leukaemia.* J Clin Pathol, 1986. **39**(7): p. 713-6.

103. Galton, D.A., *The pathogenesis of chronic lymphocytic leukemia.* Can Med Assoc J, 1966. **94**(19): p. 1005-10.
104. Ebbe, S., B. Wittels, and W. Dameshek, *Autoimmune thrombocytopenic purpura ("ITP" type) with chronic lymphocytic leukemia.* Blood, 1962. **19**: p. 23-37.
105. Lewis, F.B., R.S. Schwartz, and W. Dameshek, *X-radiation and alkylating agents as possible "trigger" mechanisms in the autoimmune complications of malignant lymphophroliferative disease.* Clin Exp Immunol, 1966. **1**(1): p. 3-11.
106. Hodgson, K., et al., *Chronic lymphocytic leukemia and autoimmunity: a systematic review.* Haematologica, 2011. **96**(5): p. 752-61.
107. Conley, C.L., J. Misiti, and A.J. Laster, *Genetic factors predisposing to chronic lymphocytic leukemia and to autoimmune disease.* Medicine (Baltimore), 1980. **59**(5): p. 323-34.
108. Landgren, O., et al., *Patterns of autoimmunity and subsequent chronic lymphocytic leukemia in Nordic countries.* Blood, 2006. **108**(1): p. 292-6.
109. Di Bernardo, M.C., et al., *A genome-wide association study identifies six susceptibility loci for chronic lymphocytic leukemia.* Nat Genet, 2008. **40**(10): p. 1204-10.
110. Slager, S.L., et al., *Susceptibility genes and B-chronic lymphocytic leukaemia.* Br J Haematol, 2007. **139**(5): p. 762-71.
111. Lettre, G. and J.D. Rioux, *Autoimmune diseases: insights from genome-wide association studies.* Hum Mol Genet, 2008. **17**(R2): p. R116-21.
112. Soderberg, K.C., et al., *Autoimmune diseases, asthma and risk of haematological malignancies: a nationwide case-control study in Sweden.* Eur J Cancer, 2006. **42**(17): p. 3028-33.
113. Tinhofer, I., et al., *Differential sensitivity of CD4+ and CD8+ T lymphocytes to the killing efficacy of Fas (Apo-1/CD95) ligand+ tumor cells in B chronic lymphocytic leukemia.* Blood, 1998. **91**(11): p. 4273-81.
114. Gorgun, G., et al., *Chronic lymphocytic leukemia cells induce changes in gene expression of CD4 and CD8 T cells.* J Clin Invest, 2005. **115**(7): p. 1797-805.
115. Sampalo, A. and J.A. Brieva, *Humoral immunodeficiency in chronic lymphocytic leukemia: role of CD95/CD95L in tumoral damage and escape.* Leuk Lymphoma, 2002. **43**(4): p. 881-4.
116. Rebmann, V., et al., *HLA-G in B-chronic lymphocytic leukaemia: clinical relevance and functional implications.* Semin Cancer Biol, 2007. **17**(6): p. 430-5.
117. Riches, J.C., A.G. Ramsay, and J.G. Gribben, *T-cell function in chronic lymphocytic leukaemia.* Semin Cancer Biol, 2010. **20**(6): p. 431-8.
118. Ramsay, A.G. and J.G. Gribben, *Immune dysfunction in chronic lymphocytic leukemia T cells and lenalidomide as an immunomodulatory drug.* Haematologica, 2009. **94**(9): p. 1198-202.
119. Schlesinger, M., I. Broman, and G. Lugassy, *The complement system is defective in chronic lymphatic leukemia patients and in their healthy relatives.* Leukemia, 1996. **10**(9): p. 1509-13.
120. Shvidel, L., E. Vorst, and A. Berrebi, *Complement values in B chronic lymphocytic leukemia: prognostic significance and correlation with cell maturation stage.* Leukemia, 1998. **12**(4): p. 635-6.
121. Itala, M., O. Vainio, and K. Remes, *Functional abnormalities in granulocytes predict susceptibility to bacterial infections in chronic lymphocytic leukaemia.* Eur J Haematol, 1996. **57**(1): p. 46-53.
122. Maki, G., et al., *NK resistance of tumor cells from multiple myeloma and chronic lymphocytic leukemia patients: implication of HLA-G.* Leukemia, 2008. **22**(5): p. 998-1006.

123. Kipps, T.J. and D.A. Carson, *Autoantibodies in chronic lymphocytic leukemia and related systemic autoimmune diseases.* Blood, 1993. **81**(10): p. 2475-87.
124. Caligaris-Cappio, F., *Relationship between autoimmunity and immunodeficiency in CLL.* Hematol Cell Ther, 1997. **39 Suppl 1**: p. S13-6.
125. Ghia, P., et al., *From normal to clonal B cells: Chronic lymphocytic leukemia (CLL) at the crossroad between neoplasia and autoimmunity.* Autoimmun Rev, 2007. **7**(2): p. 127-31.
126. Phillips, J.A., et al., *The NZB mouse as a model for chronic lymphocytic leukemia.* Cancer Res, 1992. **52**(2): p. 437-43.
127. Youinou, P. and Y. Renaudineau, *The paradox of CD5-expressing B cells in systemic lupus erythematosus.* Autoimmun Rev, 2007. **7**(2): p. 149-54.
128. Herve, M., et al., *Unmutated and mutated chronic lymphocytic leukemias derive from self-reactive B cell precursors despite expressing different antibody reactivity.* J Clin Invest, 2005. **115**(6): p. 1636-43.
129. Catera, R., et al., *Chronic lymphocytic leukemia cells recognize conserved epitopes associated with apoptosis and oxidation.* Mol Med, 2008. **14**(11-12): p. 665-74.
130. Lanemo Myhrinder, A., et al., *A new perspective: molecular motifs on oxidized LDL, apoptotic cells, and bacteria are targets for chronic lymphocytic leukemia antibodies.* Blood, 2008. **111**(7): p. 3838-3848
131. Chu, C.C., et al., *Many chronic lymphocytic leukemia antibodies recognize apoptotic cells with exposed nonmuscle myosin heavy chain IIA: implications for patient outcome and cell of origin.* Blood, 2010. **115**(19): p. 3907-15.
132. Elkon, K. and P. Casali, *Nature and functions of autoantibodies.* Nat Clin Pract Rheumatol, 2008. **4**(9): p. 491-8.
133. Ternynck, T., et al., *Comparison of normal and CLL lymphocyte surface Ig determinants using peroxidase-labeled antibodies. I. Detection and quantitation of light chain determinants.* Blood, 1974. **43**(6): p. 789-95.
134. Payelle-Brogard, B., et al., *Defective assembly of the B-cell receptor chains accounts for its low expression in B-chronic lymphocytic leukaemia.* Br J Haematol, 2002. **118**(4): p. 976-85.
135. Vuillier, F., et al., *Lower levels of surface B-cell-receptor expression in chronic lymphocytic leukemia are associated with glycosylation and folding defects of the mu and CD79a chains.* Blood, 2005. **105**(7): p. 2933-40.
136. Gordon, M.S., et al., *Aberrant B cell receptor signaling from B29 (Igbeta, CD79b) gene mutations of chronic lymphocytic leukemia B cells.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5504-9.
137. Hamblin, T.J., *Autoimmune complications of chronic lymphocytic leukemia.* Semin Oncol, 2006. **33**(2).
138. Hall, A.M., et al., *Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells.* Blood, 2005. **105**(5): p. 2007-15.
139. Galletti, J., et al., *Chronic lymphocytic leukemia cells bind and present the erythrocyte protein band 3: possible role as initiators of autoimmune hemolytic anemia.* J Immunol, 2008. **181**(5): p. 3674-83.
140. Mauro, F.R., et al., *Autoimmune hemolytic anemia in chronic lymphocytic leukemia: clinical, therapeutic, and prognostic features.* Blood, 2000. **95**(9): p. 2786-92.
141. von Bergwelt-Baildon, M., et al., *CD40 activation: potential for specific immunotherapy in B-CLL.* Annals of Oncology: Official Journal of the European Society for Medical Oncology / ESMO, 2004. **15**(6): p. 853-857

142. Barcellini, W., et al., *Relationship between autoimmune phenomena and disease stage and therapy in B-cell chronic lymphocytic leukemia.* Haematologica, 2006. **91**(12): p. 1689-92.
143. Dearden, C., *Disease-specific complications of chronic lymphocytic leukemia.* Hematology Am Soc Hematol Educ Program, 2008: p. 450-6.
144. Zent, C.S., et al., *The prognostic significance of cytopenia in chronic lymphocytic leukaemia/small lymphocytic lymphoma.* Br J Haematol, 2008. **141**(5): p. 615-21.
145. Visco, C., et al., *Un-mutated IgVH in chronic lymphocytic leukemia is associated with a higher risk of immune thrombocytopenia.* Leukemia, 2007. **21**(5): p. 1092-3.
146. Kyasa, M.J., et al., *Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma.* Am J Hematol, 2003. **74**(1): p. 1-8.
147. Mittal, S., et al., *A high rate of CLL phenotype lymphocytes in autoimmune hemolytic anemia and immune thrombocytopenic purpura.* Haematologica, 2008. **93**(1): p. 151-2.
148. Lechner, K. and U. Jager, *How I treat autoimmune hemolytic anemias in adults.* Blood, 2010. **116**(11): p. 1831-8.
149. Dameshek, W., *Chronic lymphocytic leukemia--an accumulative disease of immunolgically incompetent lymphocytes.* Blood, 1967. **29**(4): p. Suppl:566-84.
150. Bastion, Y., et al., *Severe autoimmune hemolytic anemia in two patients treated with fludarabine for chronic lymphocytic leukemia.* Ann Oncol, 1992. **3**(2): p. 171-2.
151. Tosti, S., et al., *Severe autoimmune hemolytic anemia in a patient with chronic lymphocytic leukemia responsive to fludarabine-based treatment.* Ann Hematol, 1992. **65**(5): p. 238-9.
152. Myint, H., et al., *Fludarabine-related autoimmune haemolytic anaemia in patients with chronic lymphocytic leukaemia.* Br J Haematol, 1995. **91**(2): p. 341-4.
153. Wing, K. and S. Sakaguchi, *Regulatory T cells exert checks and balances on self tolerance and autoimmunity.* Nat Immunol, 2010. **11**(1): p. 7-13.
154. Borthakur, G., et al., *Immune anaemias in patients with chronic lymphocytic leukaemia treated with fludarabine, cyclophosphamide and rituximab--incidence and predictors.* Br J Haematol, 2007. **136**(6): p. 800-5.
155. Moreno, C., et al., *Autoimmune cytopenia in chronic lymphocytic leukemia: prevalence, clinical associations, and prognostic significance.* Blood, 2010. **116**(23): p. 4771-6.
156. Ghazal, H., *Successful treatment of pure red cell aplasia with rituximab in patients with chronic lymphocytic leukemia.* Blood, 2002. **99**(3): p. 1092-4.
157. Visco, C., et al., *Impact of immune thrombocytopenia on the clinical course of chronic lymphocytic leukemia.* Blood, 2008. **111**(3): p. 1110-6.
158. Mackay, F. and J.L. Browning, *BAFF: a fundamental survival factor for B cells.* Nat Rev Immunol, 2002. **2**(7).
159. Schneider, P., et al., *BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth.* J Exp Med, 1999. **189**(11).
160. Dillon, S.R., et al., *An APRIL to remember: novel TNF ligands as therapeutic targets.* Nat.Rev.Drug Discov., 2006. **5**(3): p. 235-246.
161. Liu, Y., et al., *Crystal structure of sTALL-1 reveals a virus-like assembly of TNF family ligands.* Cell, 2002. **108**(3).
162. Oren, D.A., et al., *Structural basis of BLyS receptor recognition.* Nat Struct Biol, 2002. **9**(4).
163. Krumbholz, M., et al., *BAFF is produced by astrocytes and up-regulated in multiple sclerosis lesions and primary central nervous system lymphoma.* J Exp Med, 2005. **201**(2).
164. Morimoto, S., et al., *Expression of B-cell activating factor of the tumour necrosis factor family (BAFF) in T cells in active systemic lupus erythematosus: the role of BAFF in T*

cell-dependent B cell pathogenic autoantibody production. Rheumatology (Oxford), 2007. **46**(7).
165. Nardelli, B., et al., *Synthesis and release of B-lymphocyte stimulator from myeloid cells.* Blood, 2001. **97**(1): p. 198-204.
166. Ohata, J., et al., *Fibroblast-like synoviocytes of mesenchymal origin express functional B cell-activating factor of the TNF family in response to proinflammatory cytokines.* J Immunol, 2005. **174**(2).
167. Scapini, P., et al., *G-CSF-stimulated neutrophils are a prominent source of functional BLyS.* J Exp Med, 2003. **197**(3).
168. Ferrer, G., et al., *B cell activator factor and a proliferation-inducing ligand at the cross-road of chronic lymphocytic leukemia and autoimmunity.* Leuk.Lymphoma, 2009. **50**(7): p. 1075-1082.
169. Bossen, C. and P. Schneider, *BAFF, APRIL and their receptors: structure, function and signaling.* Semin.Immunol., 2006. **18**(5): p. 263-275.
170. Haiat, S., et al., *Role of BAFF and APRIL in human B-cell chronic lymphocytic leukaemia.* Immunology, 2006. **118**(3): p. 281-292.
171. von Bulow, G.U. and R.J. Bram, *NF-AT activation induced by a CAML-interacting member of the tumor necrosis factor receptor superfamily.* Science, 1997. **278**(5335).
172. Wu, Y., et al., *Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLyS.* J Biol Chem, 2000. **275**(45).
173. Laabi, Y., et al., *A new gene, BCM, on chromosome 16 is fused to the interleukin 2 gene by a t(4;16)(q26;p13) translocation in a malignant T cell lymphoma.* Embo J, 1992. **11**(11).
174. Darce, J.R., et al., *Regulated expression of BAFF-binding receptors during human B cell differentiation.* J.Immunol., 2007. **179**(11): p. 7276-7286.
175. O'Connor, B.P., et al., *BCMA is essential for the survival of long-lived bone marrow plasma cells.* J.Exp.Med., 2004. **199**(1): p. 91-98.
176. Salzer, U., S. Jennings, and B. Grimbacher, *To switch or not to switch--the opposing roles of TACI in terminal B cell differentiation.* Eur.J.Immunol., 2007. **37**(1): p. 17-20.
177. Fagarasan, S. and T. Honjo, *T-Independent immune response: new aspects of B cell biology.* Science, 2000. **290**(5489).
178. Xia, X.Z., et al., *TACI is a TRAF-interacting receptor for TALL-1, a tumor necrosis factor family member involved in B cell regulation.* J Exp Med, 2000. **192**(1).
179. Xu, S. and K.P. Lam, *B-cell maturation protein, which binds the tumor necrosis factor family members BAFF and APRIL, is dispensable for humoral immune responses.* Mol Cell Biol, 2001. **21**(12).
180. Yan, M., et al., *Activation and accumulation of B cells in TACI-deficient mice.* Nat.Immunol., 2001. **2**(7): p. 638-343.
181. Castigli, E., et al., *TACI and BAFF-R mediate isotype switching in B cells.* J.Exp.Med., 2005. **201**(1): p. 35-39.
182. Mackay, F., P.A. Silveira, and R. Brink, *B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling.* Curr.Opin.Immunol., 2007. **19**(3): p. 327-336.
183. Bonizzi, G. and M. Karin, *The two NF-kappaB activation pathways and their role in innate and adaptive immunity.* Trends Immunol, 2004. **25**(6).
184. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword.* Nat Rev Immunol, 2003. **3**(9).
185. Hauer, J., et al., *TNF receptor (TNFR)-associated factor (TRAF) 3 serves as an inhibitor of TRAF2/5-mediated activation of the noncanonical NF-kappaB pathway by TRAF-binding TNFRs.* Proc Natl Acad Sci U S A, 2005. **102**(8).

186. He, J.Q., et al., *Rescue of TRAF3-null mice by p100 NF-kappa B deficiency.* J Exp Med, 2006. **203**(11).
187. Xu, Y., G. Cheng, and D. Baltimore, *Targeted disruption of TRAF3 leads to postnatal lethality and defective T-dependent immune responses.* Immunity, 1996. **5**(5).
188. Thien, M., et al., *Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches.* Immunity., 2004. **20**(6): p. 785-798.
189. Emmerich, F., et al., *High-level serum B-cell activating factor and promoter polymorphisms in patients with idiopathic thrombocytopenic purpura.* Br J Haematol, 2007. **136**(2).
190. Kawasaki, A., et al., *Analysis on the association of human BLYS (BAFF, TNFSF13B) polymorphisms with systemic lupus erythematosus and rheumatoid arthritis.* Genes Immun, 2002. **3**(7).
191. Koyama, T., et al., *A novel polymorphism of the human APRIL gene is associated with systemic lupus erythematosus.* Rheumatology (Oxford), 2003. **42**(8).
192. Koyama, T., et al., *Raised serum APRIL levels in patients with systemic lupus erythematosus.* Ann Rheum Dis, 2005. **64**(7).
193. Lee, Y.H., et al., *APRIL polymorphism and systemic lupus erythematosus (SLE) susceptibility.* Rheumatology (Oxford), 2007. **46**(8).
194. Tan, S.M., et al., *Local production of B lymphocyte stimulator protein and APRIL in arthritic joints of patients with inflammatory arthritis.* Arthritis Rheum, 2003. **48**(4).
195. Groom, J., et al., *Association of BAFF/BLyS overexpression and altered B cell differentiation with Sjogren's syndrome.* J Clin Invest, 2002. **109**(1).
196. Cohen, S.B., *Updates from B Cell Trials: Efficacy.* J Rheumatol Suppl, 2006. **77**.
197. Hever, A., et al., *Human endometriosis is associated with plasma cells and overexpression of B lymphocyte stimulator.* Proc.Natl.Acad.Sci.U.S.A, 2007. **104**(30): p. 12451-12456.
198. Kim, J.Y., et al., *Serum BAFF expression in patients with myasthenia gravis.* J.Neuroimmunol., 2008. **199**(1-2): p. 151-154.
199. Mackay, I.R., J. Groom, and C.R. Mackay, *Levels of BAFF in serum in primary biliary cirrhosis and autoimmune diabetes.* Autoimmunity, 2002. **35**(8): p. 551-553.
200. Liu, Z. and A. Davidson, *BAFF and selection of autoreactive B cells.* Trends Immunol, 2011. **32**(8): p. 388-94.
201. Kern, C., et al., *Involvement of BAFF and APRIL in the resistance to apoptosis of B-CLL through an autocrine pathway.* Blood, 2004. **103**(2): p. 679-688.
202. Kim, S.J., et al., *Serum BAFF predicts prognosis better than APRIL in diffuse large B-cell lymphoma patients treated with rituximab plus CHOP chemotherapy.* Eur.J.Haematol., 2008. **81**(3): p. 177-184.
203. Schwaller, J., et al., *Neutrophil-derived APRIL concentrated in tumor lesions by proteoglycans correlates with human B-cell lymphoma aggressiveness.* Blood, 2007. **109**(1): p. 331-338.
204. Schwaller, J., et al., *Paracrine promotion of tumor development by the TNF ligand APRIL in Hodgkin's Disease.* Leukemia, 2007. **21**(6): p. 1324-1327.
205. Tangye, S.G., et al., *BAFF, APRIL and human B cell disorders.* Semin.Immunol., 2006. **18**(5): p. 305-317.
206. Chiu, A., et al., *Hodgkin lymphoma cells express TACI and BCMA receptors and generate survival and proliferation signals in response to BAFF and APRIL.* Blood, 2007. **109**(2): p. 729-739.

207. Ng, L.G., et al., *B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells.* J Immunol, 2004. **173**(2).
208. He, B., et al., *Lymphoma B cells evade apoptosis through the TNF family members BAFF/BLyS and APRIL.* J.Immunol., 2004. **172**(5): p. 3268-3279.
209. Moreaux, J., et al., *BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone.* Blood, 2004. **103**(8).
210. Novak, A.J., et al., *Expression of BLyS and its receptors in B-cell non-Hodgkin lymphoma: correlation with disease activity and patient outcome.* Blood, 2004. **104**(8): p. 2247-2253.
211. He, B., et al., *EBV-encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell-independent Ig heavy chain class switching.* J.Immunol., 2003. **171**(10): p. 5215-5224.
212. Ansell, S.M., et al., *Phase I clinical study of atacicept in patients with relapsed and refractory B-cell non-Hodgkin's lymphoma.* Clin.Cancer Res., 2008. **14**(4): p. 1105-1110.
213. Bojarska-Junak, A., et al., *BAFF and APRIL expression in B-cell chronic lymphocytic leukemia: correlation with biological and clinical features.* Leukemia Research, 2009. **33**(10): p. 1319-1327.
214. Planelles, L., et al., *APRIL but not BLyS serum levels are increased in chronic lymphocytic leukemia: prognostic relevance of APRIL for survival.* Haematologica, 2007. **92**(9): p. 1284-1285.
215. Caligaris-Cappio, F., *Role of the microenvironment in chronic lymphocytic leukaemia.* Br J Haematol, 2003. **123**(3).
216. Endo, T., et al., *BAFF and APRIL support chronic lymphocytic leukemia B-cell survival through activation of the canonical NF-kappaB pathway.* Blood, 2007. **109**(2): p. 703-710.
217. Rozman, C. and E. Montserrat, *Chronic lymphocytic leukemia.* N.Engl.J.Med., 1995. **333**(16): p. 1052-1057.
218. Smit, L.A., et al., *Differential Noxa/Mcl-1 balance in peripheral versus lymph node chronic lymphocytic leukemia cells correlates with survival capacity.* Blood, 2007. **109**(4): p. 1660-8.
219. Mittal, A.K., et al., *Molecular basis of proliferation/survival and migration of CLL in peripheral blood, bone marrow and lymph nodes.* Blood, 2008. **112**: p. Abstract 546.
220. Planelles, L., et al., *APRIL promotes B-1 cell-associated neoplasm.* Cancer Cell, 2004. **6**(4): p. 399-408.
221. Zhang, W., et al., *B-cell activating factor and v-Myc myelocytomatosis viral oncogene homolog (c-Myc) influence progression of chronic lymphocytic leukemia.* Proc Natl Acad Sci U S A, 2010. **107**(44): p. 18956-60.
222. Guadagnoli, M., et al., *Development and characterization of APRIL antagonistic monoclonal antibodies for treatment of B-cell lymphomas.* Blood, 2011. **117**(25): p. 6856-65.
223. Nimmanapalli, R., et al., *The growth factor fusion construct containing B-lymphocyte stimulator (BLyS) and the toxin rGel induces apoptosis specifically in BAFF-R-positive CLL cells.* Blood, 2007. **109**(6): p. 2557-64.
224. Liberati, A., et al., *The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate healthcare interventions: explanation and elaboration.* BMJ, 2009. **339**: p. b2700.
225. Galton, D.A., et al., *The use of chlorambucil and steroids in the treatment of chronic lymphocytic leukaemia.* Br J Haematol, 1961. **7**: p. 73-98.

226. Drake, W.M., et al., *Acute polyneuropathy with chronic lymphocytic leukaemia and paraproteinaemia: response to chlorambucil and prednisolone.* J Neurol Neurosurg Psychiatry, 1998. **64**(4): p. 564.
227. Mitsui, Y., et al., *Sensorimotor polyneuropathy associated with chronic lymphocytic leukemia, IgM antigangliosides antibody and human T-cell leukemia virus I infection.* Muscle Nerve, 1999. **22**(10): p. 1461-5.
228. Gooptu, C., et al., *Paraneoplastic pemphigus: an association with fludarabine?* Br J Dermatol, 2001. **144**(6): p. 1255-61.
229. Ruzickova, S., et al., *Chronic lymphocytic leukemia preceded by cold agglutinin disease: intraclonal immunoglobulin light-chain diversity in V(H)4-34 expressing single leukemic B cells.* Blood, 2002. **100**(9): p. 3419-22.
230. Anhalt, G.J., *Paraneoplastic pemphigus.* J Investig Dermatol Symp Proc, 2004. **9**(1): p. 29-33.
231. Yegin, Z.A., M. Yagci, and R. Haznedar, *Cold agglutinin disease with IgG monoclonal gammopathy in a case of chronic lymphocytic leukemia: an unusual presentation.* Transfus Apher Sci, 2009. **40**(3): p. 219-20.
232. Kyasa, M.J., et al., *Autoimmune cytopenia does not predict poor prognosis in chronic lymphocytic leukemia/small lymphocytic lymphoma.* American Journal of Hematology, 2003. **74**(1): p. 1-8.
233. Visco, C., et al., *Immune Thrombocytopenia in patients with Chronic Lymphocytic Leukemia is associated with Stereotyped B-Cell Receptors.* Clin Cancer Res, 2012.
234. Geisler, C. and M.M. Hansen, *Chronic lymphocytic leukaemia: a test of a proposed new clinical staging system.* Scand J Haematol, 1981. **27**(4): p. 279-86.
235. Molica, S., et al., *Baff serum level predicts time to first treatment in early chronic lymphocytic leukemia.* European Journal of Haematology, 2010. **85**(4): p. 314-320.
236. Sawicka-Powierza, J., et al., *Evaluation of TNF superfamily molecules release by neutrophils and B leukemic cells of patients with chronic B - cell lymphocytic leukemia.* Neoplasma, 2011. **58**(1): p. 45-50.
237. Kreuzaler, M., et al., *Soluble BAFF Levels Inversely Correlate with Peripheral B Cell Numbers and the Expression of BAFF Receptors.* Journal of Immunology (Baltimore, Md.: 1950), 2012. **188**(1): p. 497-503.
238. Malavasi, F., et al., *CD38 and chronic lymphocytic leukemia: a decade later.* Blood, 2011. **118**(13): p. 3470-3478.
239. Hodgson, K., et al., *Autoimmune cytopenia in chronic lymphocytic leukaemia: diagnosis and treatment.* Br J Haematol, 2011. **154**(1): p. 14-22.
240. Caligaris-Cappio, F. and P. Ghia, *Novel insights in chronic lymphocytic leukemia: are we getting closer to understanding the pathogenesis of the disease?* J Clin Oncol, 2008. **26**(27): p. 4497-503.
241. Zenz, T., et al., *From pathogenesis to treatment of chronic lymphocytic leukaemia.* Nat Rev Cancer, 2010. **10**(1): p. 37-50.
242. Stevenson, F.K., et al., *B-cell receptor signaling in chronic lymphocytic leukemia.* Blood, 2011. **118**(16): p. 4313-20.
243. Strati, P. and F. Caligaris-Cappio, *A matter of debate in chronic lymphocytic leukemia: is the occurrence of autoimmune disorders an indicator of chronic lymphocytic leukemia therapy?* Curr Opin Oncol, 2011. **23**(5): p. 455-60.
244. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions.* Cell, 2009. **136**(2): p. 215-33.
245. Grimson, A., et al., *MicroRNA targeting specificity in mammals: determinants beyond seed pairing.* Mol Cell, 2007. **27**(1): p. 91-105.

246. Vasudevan, S., Y. Tong, and J.A. Steitz, *Cell-cycle control of microRNA-mediated translation regulation.* Cell Cycle, 2008. **7**(11): p. 1545-9.
247. Garzon, R., G.A. Calin, and C.M. Croce, *MicroRNAs in Cancer.* Annu Rev Med, 2009. **60**: p. 167-79.
248. Bartel, D.P., *MicroRNAs: genomics, biogenesis, mechanism, and function.* Cell, 2004. **116**(2): p. 281-97.
249. Contreras, J. and D.S. Rao, *MicroRNAs in inflammation and immune responses.* Leukemia, 2012. **26**(3): p. 404-13.
250. Lu, J., et al., *MicroRNA expression profiles classify human cancers.* Nature, 2005. **435**(7043): p. 834-8.
251. O'Connell, R.M., et al., *Physiological and pathological roles for microRNAs in the immune system.* Nat Rev Immunol, 2010. **10**(2): p. 111-22.
252. Visone, R., et al., *Karyotype-specific microRNA signature in chronic lymphocytic leukemia.* Blood, 2009. **114**(18): p. 3872-3879.
253. Li, S., et al., *MicroRNA expression profiling identifies activated B cell status in chronic lymphocytic leukemia cells.* PLoS ONE, 2011. **6**(3): p. e16956.
254. Pan, W., et al., *MicroRNA-21 and microRNA-148a contribute to DNA hypomethylation in lupus CD4+ T cells by directly and indirectly targeting DNA methyltransferase 1.* J Immunol, 2010. **184**(12): p. 6773-81.
255. Stanczyk, J., et al., *Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis.* Arthritis Rheum, 2008. **58**(4): p. 1001-9.
256. Taganov, K.D., et al., *NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12481-6.
257. Tang, Y., et al., *MicroRNA-146A contributes to abnormal activation of the type I interferon pathway in human lupus by targeting the key signaling proteins.* Arthritis Rheum, 2009. **60**(4): p. 1065-75.
258. Peach, R.J., et al., *Both extracellular immunoglobin-like domains of CD80 contain residues critical for binding T cell surface receptors CTLA-4 and CD28.* J Biol Chem, 1995. **270**(36): p. 21181-7.
259. Yellin, M.J., et al., *T lymphocyte T cell-B cell-activating molecule/CD40-L molecules induce normal B cells or chronic lymphocytic leukemia B cells to express CD80 (B7/BB-1) and enhance their costimulatory activity.* J Immunol, 1994. **153**(2): p. 666-74.
260. Ranheim, E.A. and T.J. Kipps, *Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal.* J Exp Med, 1993. **177**(4): p. 925-35.
261. Aue, G., et al., *Lenalidomide-induced upregulation of CD80 on tumor cells correlates with T-cell activation, the rapid onset of a cytokine release syndrome and leukemic cell clearance in chronic lymphocytic leukemia.* Haematologica, 2009. **94**(9): p. 1266-73.
262. Ramsay, A.G., et al., *Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug.* J Clin Invest, 2008. **118**(7): p. 2427-37.
263. Cheson, B.D., et al., *National Cancer Institute-sponsored Working Group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment.* Blood, 1996. **87**(12): p. 4990-7.
264. Navarro, A., et al., *Regulation of JAK2 by miR-135a: prognostic impact in classic Hodgkin lymphoma.* Blood, 2009. **114**(14): p. 2945-51.
265. Bargou, R.C., et al., *Characterization of a novel Hodgkin cell line, HD-MyZ, with myelomonocytic features mimicking Hodgkin's disease in severe combined immunodeficient mice.* J Exp Med, 1993. **177**(5): p. 1257-68.

266. Klein, G., et al., *An EBV-genome-negative cell line established from an American Burkitt lymphoma; receptor characteristics. EBV infectibility and permanent conversion into EBV-positive sublines by in vitro infection.* Intervirology, 1975. **5**(6): p. 319-34.
267. Abrisqueta, P., et al., *Improving survival in patients with chronic lymphocytic leukemia (1980-2008): the Hospital Clinic of Barcelona experience.* Blood, 2009. **114**(10): p. 2044-50.
268. Nana-Sinkam, S.P. and C.M. Croce, *MicroRNA in chronic lymphocytic leukemia: transitioning from laboratory-based investigation to clinical application.* Cancer Genet Cytogenet, 2010. **203**(2): p. 127-33.
269. Tomankova, T., et al., *MicroRNAs: emerging regulators of immune-mediated diseases.* Scand J Immunol, 2011.
270. Cox, M.B., et al., *MicroRNAs miR-17 and miR-20a inhibit T cell activation genes and are under-expressed in MS whole blood.* PLoS ONE, 2010. **5**(8): p. e12132.
271. Laine, J., et al., *The protooncogene TCL1 is an Akt kinase coactivator.* Mol Cell, 2000. **6**(2): p. 395-407.
272. Pekarsky, Y., et al., *Tcl1 enhances Akt kinase activity and mediates its nuclear translocation.* Proc Natl Acad Sci U S A, 2000. **97**(7): p. 3028-33.
273. Xu, H., et al., *MicroRNA miR-29 modulates expression of immunoinhibitory molecule B7-H3: potential implications for immune based therapy of human solid tumors.* Cancer Res, 2009. **69**(15): p. 6275-81.
274. Pauley, K.M., et al., *Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients.* Arthritis Res Ther, 2008. **10**(4): p. R101.
275. Wang, G., et al., *Serum and urinary cell-free MiR-146a and MiR-155 in patients with systemic lupus erythematosus.* J Rheumatol, 2010. **37**(12): p. 2516-22.
276. Pauley, K.M., et al., *Altered miR-146a expression in Sjogren's syndrome and its functional role in innate immunity.* Eur J Immunol, 2011. **41**(7): p. 2029-39.
277. Jones, S.W., et al., *The identification of differentially expressed microRNA in osteoarthritic tissue that modulate the production of TNF-alpha and MMP13.* Osteoarthritis Cartilage, 2009. **17**(4): p. 464-72.
278. Wong, C.K., et al., *Aberrant production of soluble costimulatory molecules CTLA-4, CD28, CD80 and CD86 in patients with systemic lupus erythematosus.* Rheumatology (Oxford), 2005. **44**(8): p. 989-94.
279. Genc, K., D.L. Dona, and A.T. Reder, *Increased CD80(+) B cells in active multiple sclerosis and reversal by interferon beta-1b therapy.* J Clin Invest, 1997. **99**(11): p. 2664-71.
280. Folzenlogen, D., et al., *Analysis of CD80 and CD86 expression on peripheral blood B lymphocytes reveals increased expression of CD86 in lupus patients.* Clin Immunol Immunopathol, 1997. **83**(3): p. 199-204.
281. Summers, K.L., et al., *Expression and function of CD80 and CD86 costimulator molecules on synovial dendritic cells in chronic arthritis.* Arthritis Rheum, 1996. **39**(8): p. 1287-91.
282. Thomas, R. and C. Quinn, *Functional differentiation of dendritic cells in rheumatoid arthritis: role of CD86 in the synovium.* J Immunol, 1996. **156**(8): p. 3074-86.
283. Nakasa, T., et al., *Expression of microRNA-146 in rheumatoid arthritis synovial tissue.* Arthritis Rheum, 2008. **58**(5): p. 1284-92.
284. Papadopoulos, G.L., et al., *The database of experimentally supported targets: a functional update of TarBase.* Nucleic Acids Res, 2009. **37**(Database issue): p. D155-8.
285. Kozomara, A. and S. Griffiths-Jones, *miRBase: integrating microRNA annotation and deep-sequencing data.* Nucleic Acids Res, 2011. **39**(Database issue): p. D152-7.

Anexo

Otras publicaciones durante el período de tesis

Publicaciones directamente relacionadas con la tesis doctoral

En estos momentos tenemos un artículo en revisión en la revista PLoS ONE titulado: "MicroRNAs expression in chronic lymphocytic leukemia associated with autoimmune hemolytic anemia" que se incluye a continuación.

MicroRNAs expression in chronic lymphocytic leukemia associated with autoimmune hemolytic anemia

Running title: miRNA and CLL associated with AIHA

by

Gerardo Ferrer (1), Alfons Navarro (2), Kate Hodgson (1), Marta Aymerich(3), Arturo Pereira (4), Tycho Baumann (1), Mariano Monzo (2), Carol Moreno (1, 5), and Emili Montserrat (1).

Institute of Hematology and Oncology (1), Hematopathology Unit (3), Hemotherapy-Hemostasis Department (4), Hospital Clínic; Human Anatomy Unit, Molecular Oncology and Embryology Laboratory, University of Barcelona Medical School (2), IDIBAPS, University of Barcelona; and Department of Hematology, Hospital de la Santa Creu i Sant Pau, Universitat Autònoma de Barcelona (5), Spain.

Correspondence:

Emili Montserrat, MD
Department of Hematology,
Hospital Clínic, Villarroel 170.
08036 Barcelona, Spain.
Phone:+ 34 93 227 54 75.
E-mail: emontse@clinic.ub.es

Abstract word count: 177
Main text word count: 2768
Key-words: chronic lymphocytic leukemia, autoimmunity, autoimmune hemolytic anemia, miRNAs.

ABSTRACT

Chronic lymphocytic leukemia (CLL) is frequently associated with autoimmune hemolytic anemia (AIHA). However, the mechanisms governing the association between CLL and AIHA are poorly understood. miRNAs have been implicated in both CLL and autoimmunity. Although different miRNAs signatures have been associated with several clinico-biological forms of CLL, there are no studies correlating miRNAs expression with CLL likely to develop AIHA. In this study we found that malignant B- cells from CLL that subsequently develops AIHA present six downregulated (i.e., miR-19a, miR-20a, miR-146b-5p, miR-324-3p, miR-340 and miR-660) and one upregulated miRNA (i.e., miR-29c). Interestingly, two of these miRNAs (i.e., miR-20a and miR-146b-5p) are known to be involved in autoimmune phenomena, and one (i.e., miR-146b-5p) in both autoimmunity and CLL. Furthermore, we demonstrate that miR-146b-5p modulates CD80, a molecule implicated in the B-T-cell synapse and in restoring the antigen presenting capacity of CLL cells. This study suggests the presence of a distinct miRNA signature in CLL likely to develop AIHA and should be of help in further studies aimed at deciphering the mechanisms of autoimmune phenomena in CLL.

INTRODUCTION

Chronic lymphocytic leukemia (CLL), the most frequent form of leukemia in adults in Western countries, is characterized by the progressive accumulation of a subtype of CD5+ B lymphocytes with a characteristic immunophenotype (i.e., SmIg ^{dim}, CD5+, CD19+, CD20+, CD23+) in peripheral blood, bone marrow and lymphoid tissues. In addition, CLL is frequently associated with autoimmune cytopenias, particularly autoimmune hemolytic anemia (AIHA) [106, 234, 240].

Whereas in the last few years important progress has been made in the understanding of the neoplastic, accumulative component of CLL [10, 12, 241-243], the mechanisms accounting for autoimmunity in this form of leukemia are still poorly understood. It is known, however, that they involve complex interactions among normal B cells, malignant CLL cells, abnormally functioning T cells, microenvironment, and the innate immune system[244]. The physiopathology of autoimmune cytopenias in CLL is only partially established. Most autoimmune cytopenias (90%) in CLL are mediated by non-malignant B-lymphocytes that produce polyclonal high affinity immunoglobulin G (IgG) via a T-cell-mediated mechanism. IgG-opsonized cells are subsequently destroyed by antibody-dependent cellular cytotoxicity. The implication is that CLL cells exert a yet undefined influence on non-malignant lymphocytes, both T- and B-cells, that results in the development of autoimmunity. Neoplastic B cells from CLL can act as antigen-presenting cells, inducing the formation of both autoreactive T helper cells (through the production of BAFF and APRIL) as well as non-functional T regulatory cells (via CD27-CD70 interaction) [168, 244]. As a corollary, malignant B-cells from CLL prone to be complicated by autoimmunity are likely to present some differential characteristics as compared to those cases in which autoimmunity is not observed. To date, CLL complicated by autoimmune cytopenia has been found to be associated with unmutated *IGVH* genes, high ZAP-70 and CD38 expression, stereotyped BCR, and increased serum B2 microglobulin levels

[144, 145, 155]. These features, however, reflect advanced, high-risk CLL rather than specific AIHA-related abnormalities in malignant B CLL cells.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate mRNA translation and play an important role in most biological processes, including cell development, proliferation, apoptosis, and stem cells self-renewal and differentiation [245-250]. miRNAs allow an accurate classification of tumor subtypes and identify apparently missing links between separated pathological pathways [251, 252]. In fact, in CLL different miRNA signatures have been correlated with many aspects of the disease, including *IGVH* mutated and unmutated forms [17, 86, 253], and different T-cell and B-cells subsets [254]. Also, several miRNAs have been found to be dysregulated in both autoimmune disorders and CLL [255-258]. However, the possibility that CLL likely to develop AIAH presents a distinct miRNA signature has not been investigated.

In this study we demonstrate that malignant B- cells from CLL that subsequently develops AIHA present six downregulated (i.e., miR-19a, miR-20a, miR-146b-5p, miR-324-3p, miR-340 and miR-660) and one upregulated miRNA (i.e., miR-29c), suggesting a distinct miRNA pattern for CLL associated with AIHA. We also show that miR-146b-5p regulates the expression of CD80, a molecule that participates in the B-T-cell synapse[259] and which has been associated with the restoring of the antigen presenting cell (APC) capacity of CLL cells [260-263].

MATERIAL AND METHODS

Patients

The study population comprised 14 CLL patients who developed AIHA over the course of their disease, and 19 sex-, age-, and clinical stage-matched patients with CLL who, after a comparable follow up time, did not develop this complication. These patients were retrieved from 211 cases from our database and for whom cell samples at diagnosis were available, the overall prevalence of AIHA in these patients being 7% [155]. CLL diagnosis and staging was carried out according to NCI and NCI-IWCLL criteria [1, 264]. The main characteristics at diagnosis of the patients included in the study are shown in Table 1. None of these patients had received therapy for either CLL or AIHA before cell samples collection. According to accepted criteria, AIHA was diagnosed based on the presence of an otherwise unexplained hemoglobin level < 100 g/L (< 10 g/dL) or hematocrit < 30% and a positive direct antiglobulin test (DAT) for either IgG or C3 and the presence of ≥ 1 indirect marker of hemolysis: high reticulocyte count, low serum haptoglobins levels, increased serum lactate dehydrogenase or bilirubin levels. In one patient in whom DAT was negative, the diagnosis of AIHA was based on the presence of ≥ 2 of the indirect signs of hemolysis [144]. The median time from the diagnosis of CLL to AIHA detection was 40 months (range, 2-94).

Ethics Statement

This study was approved by the Hospital Clínic of Barcelona Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Informed consent was verbally obtained from all individuals still alive and was registered in their medical records.

Isolation of B-cells and RNA extraction

B-cells were isolated from pretreated cryopreserved peripheral blood mononuclear cells by negative selection using the B-cell isolation Kit (Miltenyi Biotec, Auburn, CA), according to the

manufacturer's procedure. Isolated B-cells from three healthy donors and CLL patients were stained with peridinin-chloropophyllproteins conjugated with Cy5.5 (PerCP-Cy5.5) anti-CD19 and allophycocyanin anti-CD5 (BD, San Jose, CA) and B-cell purity of at least 95% in all samples was confirmed by flow cytometry using a FACS CANTO II (BD). As per normal B cells, 95% corresponded to CD19+ CD5- cells. RNA was extracted using Trizol® (Life technologies, Carlsbad, CA) as manufacturer's procedure.

miRNA profiling using multiplex real-time quantitative RT-PCR

miRNA profiling was performed using TaqMan® Array Human Arrays A v2.0 (Life technologies). This method is based on a multiplexing RT-PCR method for the detection 377 mature human miRNAs. RT reactions of 4.50 µL contained: 0.80 µL of 10x RT buffer (Applied Biosystems), 0.2 µL dNTPs (100 mM each), 1.5 µL MultiScribe Reverse Transcriptase (50 U/µL), 0.10 µL RNase Inhibitor (20 U/µL), 0.80 µL Megaplex RT primers (10x), 0.90 µL of MgCl2 (20U/µL), 500 ng of total RNA. RT reactions were incubated in a PTC-100 thermocycler for 2 minutes at 16°C and 1 minute at 42°C for 40 cycles, one second at 50ºC and 5 minutes at 85°C, and then held at 4°C. Real-time PCR reactions were performed on an ABI 7900 HT Sequence Detection System (Applied Biosystems) and contained 450µL of TaqMan Gene Expression Master Mix (Applied Biosystems), 6µL Megaplex RT product, and 444 µL nuclease free water.

Normalization and filtering

The relative miRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Normalization was performed with RNU48. As calibrator sample, we used the miRNA values of a pool of 3 normal B cells samples. All miRNAs expressed in less than 10% of samples were excluded from further analysis, leaving a working-set of 180 miRNAs.

Renilla luciferase reporter vector

Cloning of the target sequence was performed as previously described [265]. Briefly two 47-bp synthetic oligonucleotides were cloned in the psiCHECK-2 vector (Promega) in the 3'UTR of

Renilla luciferase gene. The 2 oligonucleotides sense (5′-TCGAGGTAAAATATTTATGAGAAAGTTCTCATTTAAAATAGATCTGC -3′) and antisense (5′-GGCCGCAGATCTATTTTAAATGAGAACTTTCTCATAAATATTTTACC -3′) were first annealed with a Tris buffer (100 mM Tris HCl, pH 7.5, 1 M NaCl, 10 mM ethylenediaminetetraacetic acid). The insert was ligated into the 3′ UTR of *Renilla luciferase* in a psiCHECK2 vector (Promega). This vector also contains *Firefly luciferase* gene used to normalize for transfection efficiency. The constructs were sequenced to check for the proper insert.

Pre-miRNA transfection and luciferase assay

The cell lines HD-MY-Z [266] and Ramos [267] (DSMZ - the German Resource Centre for Biological Material) were transfected with 1µg of modified psiCHECK-2 vector, plus 200nM of pre-miR-146b-5p or a pre-miR-negative control (Life Technologies). The Renilla luciferase and Firefly luciferase activity was measured at 24 hours after transfection with the Promega Dual luciferase reporter assay system (Promega) in an Orion II microplate luminometer (Berthold Detection Systems GmbH, Pforzheim, Germany). The transfection efficiency was normalized with the Firefly luciferase.

Measurement of CD80 expression by flow cytometry

In order to examine the effect of miR-146b-5p on HD-MY-Z and Ramos cells lines protein levels, cells were transfected with 200nM pre-miR-146b-5p or negative control, and CD80 levels on cell surface were evaluated with phycoerythrin (PE) conjugated anti-human-CD80 (Biolegend San Diego, CA). CD80 was also evaluated on cryopreserved cells from five patients with CLL associated with AIHA and twelve with CLL not associated with autoimmunity.

Statistical analysis

Data were analyzed TIGR Multiexperiment viewer version 4.0 software (Dana-Farber Cancer Institute, Boston, MA, USA), differential expression of miRNAs ($p<0.001$) were calculated with Wilcoxon, Mann-Whitney nonparametric test; R software version 2.9.0 (The R Foundations for

Statistical Computing, Vienna, Austria). SPSS software (version 15; SPSS Inc, Chicago, IL), different groups were compared using the Mann-Whitney U-test, a two-sided p value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics of patients who develop AIHA

In this study we have evaluated miRNAs expression at diagnosis of 14 patients with CLL who over the course of their disease developed AIHA. The median time from diagnosis to the appearance of AIHA was 40 months (range, 2 to 94). These patients were retrieved from 211 cases from our database and for whom cell samples at diagnosis were available. As shown in Table 1, most of these patients were initially diagnosed in early clinical stage (Binet A, 12/14) and only differed from the control population in terms of blood lymphocyte count and serum B2 microglobulin levels.

CLL associated with AIHA tend to cluster together in the unsupervised analysis

Using an approach of unsupervised ordering, 33 CLL cases were classified by similar expression pattern of miRNAs. The hierarchical cluster analysis tends to group those patients associated with AIHA (Figure1). Other clinical and molecular prognostic markers (i.e., cytogenetics, IGVH mutational status, ZAP-70, CD38) presented a heterogeneous distribution (data not shown).

miRNAs expressed in CLL associated with AIHA

The supervised analysis, using Wilcoxon Mann-Whitney nonparametric test based on permutations, revealed six downregulated miRNAs (i.e., miR-19a, miR-20a, miR-146b-5p, miR-324-3p, miR-340 and miR-660) and one upregulated miRNA (miR-29c) in CLL associated with AIHA as compared to CLL without AIHA patients (Table 2*)*. Interestingly, some of these miRNAs have been previously implicated in different diseases, including CLL and autoimmunity (miR-20a, miR-29c and miR-146b-5p; Table 2).

CD80 is targeted by miR-146b-5p and is upregulated in CLL associated with AIHA

To try to shed light on the mechanism by which miR-146b-5p participates in promoting AIHA, we first analyzed its putative targets using the TargetScan 6.0 database, which predicted 224

conserved targets for miR-146b-5p. Of these targets, we selected CD80 for its relevance in B-T-cell synapse. In the Renilla luciferase assay, we inserted the 3'UTR region of CD80 in the 3'UTR of *Renilla* gene in psiCheck2 vector (modified psicheck2 vector). When we transfected cell lines (HD-MY-Z and Ramos) with either the modified psicheck2 vector and the pre-miRNA or the modified vector and pre-miR-negative control. In this experiment we observed a reduction of 28.7 % and 20.6%, respectively in Renilla activity at 24 hours after transfection (Figure 2A). Although HD-MY-Z cells normally express low levels of CD80, we observed a 3.7% reduction of positive cells in HD-MY-Z cells transfected with pre-miR-146b-5p compared to those transfected with the negative control (14.0% vs. 17.7%, respectively; p=0.015). After transfection with pre-miR-146b-5p, Ramos cells also showed a reduction of CD80+ cells (28.7% vs. 41.3%; p=0.020) (Figure 2B). Finally, patients with CLL associated with AIHA had higher levels of CD80 than those without autoimmunity (13.4% *vs.* 9.3% respectively; p=0.027) (Figure 3A).

DISCUSSION

CLL can be considered as a disease with broadly two major components: i) neoplastic, and ii) immune. Whereas over the last years important advances have been made in the understanding of the neoplastic component of the disease [10, 12, 241-243], little progress has been generated in elucidating the mechanisms behind immune disturbances, particularly autoimmune cytopenias, which are frequently encountered over the course of CLL [106, 143]. miRNAs are important regulators of gene expression in the immune system and human cancer. Certain miRNAs serve in important negative feedback loops in the immune system, whereas others amplify the response of the immune system [250]. Interestingly, different miRNAs profiles have been correlated with several aspects of CLL, including *IGVH* mutated and unmutated forms [17, 86, 253], and T-cell and B-cells subsets [254]. However, there are no studies investigating whether CLL prone to be complicated with AIHA is associated with a miRNAs distinct expression pattern.

In this study we have evaluated 377 mature human miRNAs expression at diagnosis of 14 patients who developed AIHA during their clinical evolution. Most of these patients were diagnosed in early phases of their disease, which is consistent with the fact that in most instances this form of leukemia is detected in asymptomatic phase [144, 155, 268]. The increased serum B2 microglobulin in these patients is in keeping with the known correlation between this serum marker and the risk of developing AIHA [144, 155].

We have found that neoplastic B-cells from patients with CLL who subsequently develop AIHA present at diagnosis a miRNA pattern composed of six downregulated (i.e., miR-19a, miR-20a, miR-146b-5p, miR-324-3p, miR-340 and miR-660) and one upregulated miRNAs (i.e.,miR-29c). Interestingly these seven miRNAs have already been shown to be related to other forms of leukemia and solid tumors [269, 270] and, even more importantly, two of them have been

previously related to CLL (i.e., miR-29c and miR-146b-5p) [86, 253] and two to autoimmunity (i.e., miR-20a and miR-146b) [257, 271].

As per the role of the miRNAs related to CLL associated with AIHA, miR-29 facilitates the upregulation of Tcl-1, an important co-activator of Akt [272, 273], as well as that of Bcl-2 protein family members, thereby contributing to antiapoptotic signaling [66, 67]. Moreover, miR-29c has been shown to be involved in the modulation of the B7-H3 molecule, which suppresses the immune escape in solid tumors [274]. In turn, upregulation of miR-146b has been associated with some prognostic markers, including trisomy 12 [86, 253]. In this regard, in our series miR-146b-5p expression was significantly higher in CLL not associated with AIHA than in those cases presenting this complication, a finding not related to an imbalance in trisomy 12 cases in the two groups (data not shown).

Regarding autoimmunity, miR-146b and, more frequently miR-146a, has been reported to be abnormally expressed in autoimmune disorders [257, 258, 275-277]. The levels of these two miRNAs, which only differ in two bases and function redundantly in many systems, seem to be disease-related. Thus, whereas in rheumatoid arthritis and Sjögren's syndrome miR-146b and miR-146a are elevated [275, 277], in systemic lupus erythematosus and osteoarthritis these two miRNAs are downregulated [258, 276, 278]. miR-146 downregulation has been related to the activation of IRAK1, TRAF6, STAT-1 and IRF-5, all of which are important for the IFN pathway [257, 258]. Downregulation of miR-146 contributes to the over-expression of type I IFN and disease activity [258, 278]. Likewise, miR-20a, a member of the cluster 17-92, participates in T cell activation and has been found to be downregulated, along with miR-17, in multiple sclerosis [271]. Finally, in our study no differences were found between the expression of miR-155 in CLL with and without AIHA, this most likely being due to the known overexpression of miR-155 in both autoimmunity and CLL [269, 270].

We have also found that miR-146b-5p regulates the expression of CD80, a molecule that participates in the B-T-cell synapse [259] and that has been associated with the restoring of the APC capacity CLL cells [260-263]. Interestingly, the link between miR-146b-5p and CD80 identified in our study is supported by other investigations in non-hemic autoimmune diseases [258, 276, 279-284]. Thus, whereas in systemic lupus erythematosus CD80 is upregulated [279, 281] and miR-146b is downregulated [258, 276], in rheumatoid arthritis whereas CD80 is almost undetectable [282, 283] miR-146 is upregulated [284]. Finally, it can be speculated that the higher expression of CD80 on neoplastic B-CLL cells could allow to these cells to act more efficiently as APC, thereby contributing to the physiopathology of AIHA in CLL.

This work has a number of limitations to be commented. First, one of the difficulties in investigating biological aspects of autoimmune cytopenias in CLL is the low prevalence of this complication and the difficulty in studying untreated patients. This is reflected in our study in which although the actual number of patients analyzed was 14, the study "denominator" (or number of patients from our database screened) was 211. Second, some prognostic markers were not available in all patients because they were either unknown or not routinely applied at the time of diagnosis, the time from diagnosis to AIHA development ranging from 2 to 94 months. Third, the possibility that clonal evolution, and thus changes in miRNA pattern, occurs in the interval between CLL diagnosis and AIHA appearance cannot be excluded. This, however, does not invalidate the relevance of findings at diagnosis, as demonstrated in other miRNAs studies [86, 251, 253].

In conclusion, this study shows for the first time a correlation between several miRNAs and CLL likely to develop AIHA. In addition, our results indicate that there is a complex connection among CLL, CLL associated with AIHA, and autoimmunity at miRNA level (Figure 4). Finally, additional studies are needed to validate these observations and to further elucidate the

mechanisms connecting CLL and AIHA, including the role of CD80 and miR-146b-5p in CLL prone to develop AIHA.

ACKNOWLEDGMENTS

This work has been supported thanks in part to Red Temàtica de Investigación Cooperativa en Cáncer grant RT 06/0020/002051 Spanish Ministry of Science. Instituto Carlos III FISS PI080304 and PI040698, Generalitat de Catalunya 2009SGR1008, and CLL Global Foundation. GF is a recipient of a grant from Instituto de Salud Carlos III (PFIS). EM is a recipient of the August Pi i Sunyer research award. GF, KH, CM, TB and EM are members of the European Research Initiative on CLL (ERIC). Thanks are also due to Dr. Pablo Engle, Dr. Ricardo Bastos and their group for their support.

AUTHORSHIP AND DISCLOSURES

Contribution: GF designed the study, performed the experiments, analyzed the data, and wrote the paper; AN supervised the experiments, analyzed the data and wrote part of the paper; KH and CM designed the study and provided advice. MA, AP, TB and MM reviewed the paper and provided advice, and EM designed the study along with GF, CM and KH, and wrote the paper. All the authors approved the final version.

Conflict-of-interest disclosure: The authors declare no competing financial interests. Data presented in part at the International Workshop on CLL 2011 (Abstract 2.36) and American Society of Hematology Annual Meeting 2011 (Abstract 3897).

FIGURES

Figure 1: **Unsupervised analysis**. Hierarchical cluster analysis (Euclidian distance) of all analyzed samples.

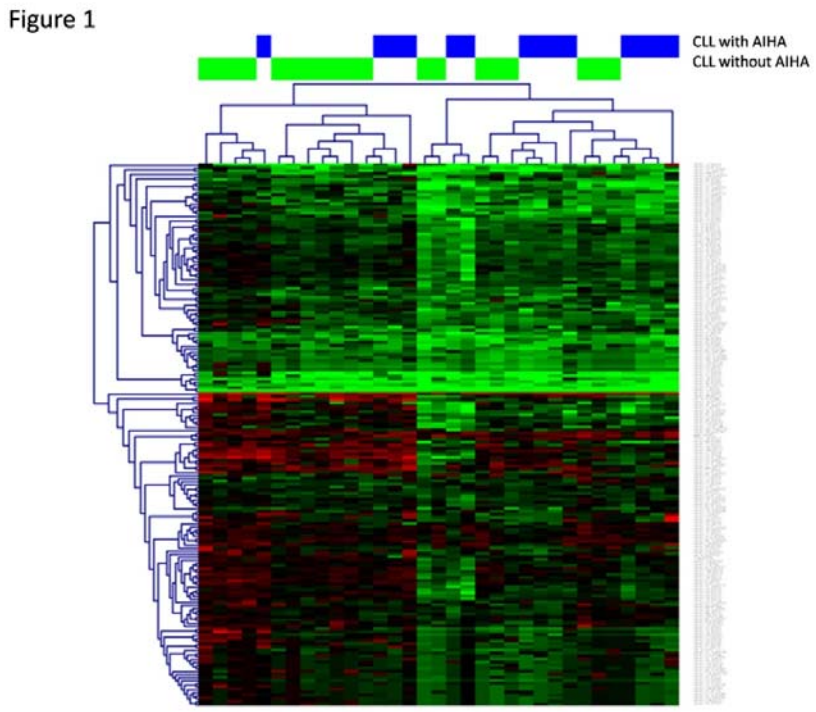


Figure 2: **Validation of CD80 as target of miR-146b-5p.** A) **Renilla/luciferase assay at 24 hours after transfection with pre-miR-146b-5p and modified psiCHECK-2 vector.** B) Percentage of CD80-positive cells, analyzed by flow cytometry 24 hours after transfection with pre-miR-146b-5p.

Figure 2

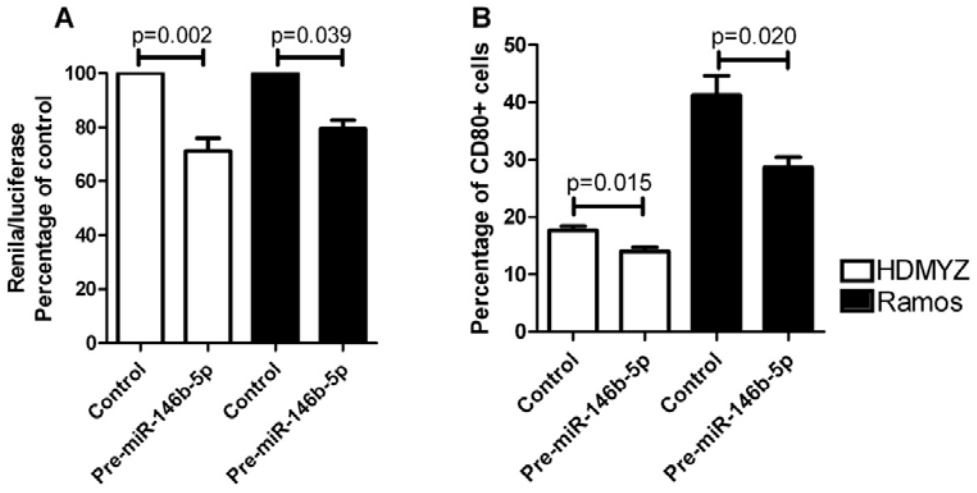


Figure 3: **CD80 and miR-146b-5p expression on CLL cells:** A) Protein levels were analyzed in cryopreserved CLL cells from patients with (n=5) and without (n=12) associated AIHA. B) Expression of miR-146b-5p in purified CD19+ CD5+ CLL cells.

Figure 3

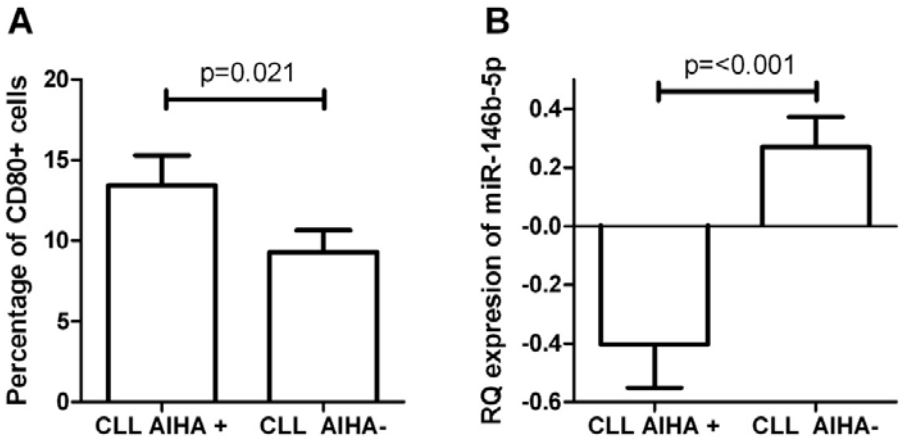
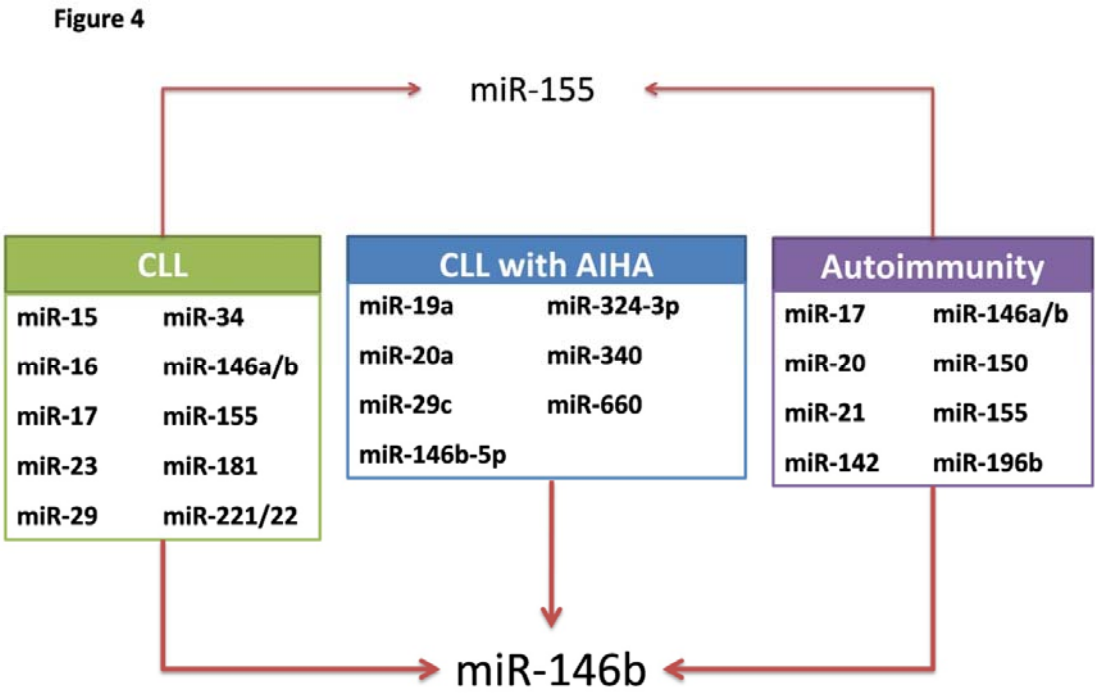


Figure 4: **Schematic representation of miRNAs implicated in autoimmunity, CLL associated with AIHA, and CLL.** miR-146b is present in autoimmune phenomena, CLL, and CLL associated with AIHA. On its turn, miR-155 links autoimmunity with CLL but its expression does not differ between CLL and CLL associated with AIHA.



TABLES

Table 1. **Patients clinical characteristics at CLL diagnosis**

	CLL with AIHA (n=14)	CLL without AIHA (n=19)	P value
Age, years	68	68	ns
Male gender (%)	57	50	ns
Binet stage, n			
A	12	17	
B	-	2	ns
C	2	-	
Blood lymphocyte count (10^6/ml)	12.7 [5.0-87.3]	70.0 [50.0-92.0]	P<0.05
LDH (U/L)	349 [271-617]	332 [256-900]	ns
Beta-2 microglobulin (mg/L)	2.6 [1.2-4.5]	2.0 [1.0-5.7]	P<0.05
Unmutated *IGVH*	1/4	1/6	ns
ZAP-70 ≥ 20%	4/12	4/17	ns
CD38 ≥ 30%	4/11	3/13	ns
High-risk cytogenetics[a]	2/10	5/14	ns
Follow-up (months)	66 [21-138]	76 [35-169]	ns
DAT+, n	2	0	
Number of patients that required CLL therapy	6/14	9/19	ns

[a] 17p-, 11q-, trisomy 12 or 3 or more cytogenetic abnormalities
ns: non-significant

Table 2. **Main characteristics of the miRNAS related to CLL associated with AIHA**

miRNA	ΔRQ	Chromosome localization	Validated targets	Related diseases

miR-19a	-0.1022	13q31.3	PTEN, MECP2, HOXA5	Malignant lymphoma and Cowden syndrome
miR-20a	-0.0470	13q31.3	TGFBR2,	Autoimmunity, B cell lymphoma, T-cell lymphoblastic leukemia.
miR-29c	0.0415	1q32.2	Mcl1, DNMT3B, DNMT3A	CLL, lung cancer, nasopharyngeal carcinomas, neuroblastoma, sarcomas, brain tumors
miR-146b-5p	-0.5890	10q24.32	IRAK1, TRAFF-6, STAT-1 and IRF-5	Autoimmunity,thyroid tumors, breast cancer, glioma, lung cancer
miR-324-3p	-0.1648	17p13.1		Cervical cancer, Kaposi's sarcoma
miR-340	-0.0923	5q35.3		Breast cancer
miR-660	-0.1327	Xp11.23		Prostate cancer

ΔRQ: Differential of the relative quantification ($\log10(2^{-\Delta\Delta cT})$) between samples of CLL associated with AIHA and those not associated with AIHA.

Information obtained from PubMed and the following databases: TarBase v.5c,[285] miRBase[286] and OMIM (in the National Center for Biotechnology Information) (accessed 1st March 2012).

Publicaciones no relacionadas durante el período doctoral

- Fernandez de Larrea C, Navarro A, Tejero R, Tovar N, Diaz T, Cibeira MT, Rosinol L, Rovira M, Ferrer G, Rozman M, Monzo M, Blade J. Impact of miRSNPs on survival and progression in patients with multiple myeloma undergoing autologous stem cell transplantation. Clin Cancer Res. 2012 [Epub ahead of print]

- Diaz T, Navarro A, Ferrer G, Gel B, Gaya A, Artells R, Bellosillo B, Garcia-Garcia M, Serrano S, Martínez A, Monzo M. Lestaurtinib Inhibition of the JAK/STAT Signaling Pathway in Hodgkin Lymphoma Inhibits Proliferation and Induces Apoptosis. *PLoS One* 6(4):e18856, 2011.

- Navarro A, Diaz T, Martinez A, Gaya A, Pons A, Gel B, Codony C, Ferrer G, Martinez C, Montserrat E, Monzo M: Regulation of JAK2 by miR-135a: prognostic impact in classical Hodgkin lymphoma. *Blood* 114:2945-51, 2009.

- Monzo M, Navarro A, Ferrer G, Artells,R: Pharmacogenomics: a tool for improving cancer chemotherapy. *Clin Transl Oncol* 10:628-637, 2008