Tesis Doctorado

Programa de Desarrollo de Ciencias Básicas

Caracterización de una sub población tumoral proliferante en la Leucemia Linfoide Crónica: Articulando la modulación del microambiente inmunológico con la progresión de la leucemia.

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TABLA DE CONTENIDO

RESUMEN

La Leucemia Linfoide Crónica (LLC) se define como un tumor de células B $CD5^+$, cuyas células tumorales han encontrado previamente el antígeno, escapan de la muerte celular y permanecen en reposo, fase G1 del ciclo celular. Evidencias recientes sugieren que el diálogo con células accesorias en microambientes especializados, favorece la progresión de la enfermedad y la refractariedad al tratamiento a través del mantenimiento de una sub-población proliferante de células B. Los linfocitos circulantes en reposo se diferencian de las células proliferantes en relación a la variación de expresión de proteínas asociadas con la proliferación y apoptosis (Calissano et al, 2011; Damle et al, 2007; Palacios et al, 2010; Zucchetto et al, 2009). Por esta razón, aislar y caracterizar la sub-población tumoral recientemente estimulada en el compartimento proliferante podría ser importante para comprender la patogénesis de la enfermedad. Diferentes grupos han estudiado sub-poblaciones proliferantes como las que expresan el marcador CD38 (Damle et al, 2007), o la enzima "activation induced cytidine deaminase ", AID (Palacios et al, 2010), molécula clave en los procesos de hipermutación somática (HS) y cambio de clase (CC), o expresando CD5/CXCR4 (Calissano et al, 2011).

Previamente Oppezzo demostró que AID se encuentra expresada anormalmente en células B de sangre periférica (SP) de pacientes de LLC que presentan un perfil de genes IgVH no mutado (NM), con un proceso activo CC (Oppezzo et al, 2003) y un peor pronóstico clínico (Oppezzo et al, 2005a). AID juega un rol en central en la respuesta inmune adaptativa y es requerida para el CC y la HS. Debido a sus funciones mutagénicas, una desregulación de la enzima ha sido asociada a desórdenes linfoproliferativos. Dado que la expresión de AID resulta de la interacción con un microambiente activado nos preguntamos si el pequeño subgrupo de células B con un proceso activo de CC es responsable de los altos niveles de expresión de AID y podría estar dado por este microambiente particular. En este trabajo identificamos y aislamos esa pequeña sub-población de células tumorales de SP de pacientes NM de LLC. Determinamos que dicho subgrupo presenta altos niveles de expresión de moléculas proliferativas (Ki-67, c-myc), anti-apoptóticas (Bcl-2) y asociadas con un microambiente activado (CD49d, CCL3 y CCL4) así como una baja expresión del inhibidor de la entrada del ciclo celular p27kip1 cuando lo comparamos con la fracción quiescente del mismo paciente. Finalmente, confirmamos que los pacientes NM que presentan este subset proliferante tienen menos sobrevida y refractoriedad al tratamiento (Palacios et al, 2010).

Debido a que las células proliferantes parecen ser calves en la agresividad de la enfermedad realizamos estudios de expresión génica a nivel de ARNm y microRNA de la sub-población proliferante (SPP) y de la quiescente (QS) con el propósito de detectar diferentes vías de señalización que favorezcan la progresión de la enfermedad. Los resultados sugieren que el comportamiento proliferante de la SPP en estos pacientes podría estar originado por la sobre-expresión del microRNA-22 (miR-22). Encontramos que el miR-22 regula negativamente al supresor de tumor PTEN lo cual permite la activación de la vía PI3K/AKT en la SPP. La activación de ésta vía está asociada con la alta capacidad de progresión del ciclo celular mediante la inhibición de la expresión de p27Kip1, la translocación al citoplasma del factor de transcripción FOXO1 y la expresión de survivina en la SPP. Ensayos de transfecciones *in vitro* en células de LLC confirmaron estos resultados. A su vez, observamos que señales del microambiente tumoral (CD40/IL4) son capaces de inducir la expresión del miR-22 *in vitro*. Finalmente, determinamos que células B $Ki67^{pos}$ de nódulos linfáticos de pacientes de LLC NM progresores presentan la misma vía de señalización (PI3K/AKT) activada sugiriendo que lo descrito para SP podría estar ocurriendo en los nódulos de dichos pacientes.

Estos resultados en conjunto, proponen un modelo de crecimiento y proliferación de las células B de LLC y provee evidencias concluyentes de la importancia de las interacciones del microambiente en la inducción de la sobrevida de la población proliferante en LLC. Específicamente, estos resultados enfatizan la importancia de la SPP en pacientes de LLC NM con mal pronóstico y propone un nuevo miRNA (miR-22) como un importante modulador de la vía de señalización PI3K/AKT. Finalmente, estos datos resaltan la importancia fisiológica de mantener una regulación estricta de las diferentes señales en cascada como PI3K/AKT, y sugieren que la sobre-expresión de survivina y la baja expresión de p27kip1 podrían tener un papel clave en la proliferación LLC.

INTRODUCCIÓN

1.0 La Leucemia Linfoide Crónica

1.1 Generalidades

La Leucemia Linfoide Crónica (LLC) es la más frecuente de las leucemias entre las poblaciones adultas de origen caucásico. Su evolución, sigue un curso sumamente variable con pacientes que presentan una sobrevida que oscila entre meses y décadas. Un tercio de los pacientes no requieren tratamiento, presentan una vida prolongada y su muerte, en general, no se encuentra relacionada con la leucemia. Otro tercio de los pacientes comienzan con una fase indolente seguida por una progresión de la enfermedad mientras que el resto presentan una enfermedad agresiva que requiere rápidamente de un tratamiento (Vasconcelos et al, 2003). Los tratamientos disponibles pueden inducir a la remisión de la enfermedad, pero la mayoría de los pacientes indefectiblemente recaen, por lo que esta patología al día de hoy es considerada incurable (Burger et al, 2009a).

1.2 Epidemiología de LLC

La incidencia de esta enfermedad puede variar con la edad y con el sexo. En países occidentales se estima que la incidencia oscila entre 4,1 - 6,5 casos por cada 100.000 habitantes por año. En particular, datos provistos por la agencia nacional de investigación de Estados Unidos ("National Cancer Research, Survilliance Epidemilology and End Results") muestran que para el período 2006-2010 la incidencia fue de 4,3 por cada 100.000 al año (Howlader, 2013). A su vez, se observa que los americanos blancos presentan mayor incidencia que los afro-americanos (Weiss, 1979). Los valores observados en Estados Unidos son similares a los observados en Inglaterra, sin embargo en países asiáticos la incidencia es casi cinco veces menor en comparación con los países occidentales (Tamura et al, 2001). A pesar de estas observaciones, no existen evidencias consistentes capaces de relacionar la predisposición a la enfermedad con el medioambiente (Dighiero & Hamblin, 2008).

Por otro lado, se ha establecido que existe una asociación entre los factores genéticos hereditarios y la etiologíade la LLC (Sellick et al, 2006). A pesar de que la forma de herencia aún se desconoce, se ha visto que esta enfermedad puede presentarse en la familia de los pacientes de LLC de manera poco frecuente. Se ha observado que parientes cercanos de pacientes con LLC tienen tres veces más probabilidad de padecer LLC que otras neoplasias linfoides comparado al resto de la población. En este sentido Rawstron y col. describieron que un 3,5% de los individuos normales por encima de la edad de los 40 años, presentan una población de linfocitos en sangre monoclonales con características similares a la de los pacientes con LLC. Lo más interesante es que la prevalencia de estos linfocitos en parientes cercanos con LLC familiar es de entre 13,5 a 18%. Debido a estos datos, la asociación entre la subclínica de la LLC y la propia enfermedad es un tema de intensa investigación (revisado en (Dighiero & Hamblin, 2008).

Estudios recientes realizados en Uruguay muestran que a semejanza del resto de Europa occidental la incidencia de la LLC es de 5,3 por cada 100.000 habitantes por año (Moro et al, 2009). La edad media para el diagnóstico varía entre 70 años para el hombre y 74 para la mujer en un rango de entre 35 a 90 años, mientras que la edad media de muerte es de 76 y 81 años respectivamente.

1.3 Diagnóstico y clasificación anatomo-clínica de la LLC

1.3-*A* Diagnóstico de la LLC

La mayoría de los pacientes con LLC se diagnostican en ausencia de sintomatología a causa de un hemograma de rutina. El diagnósticose realiza tomando en cuenta el recuento linfocitario en sangre periférica (SP) y el estudio del inmunofenotipo. El diagnóstico clínico de LLC se define por: ***1*- una linfocitosis absoluta de al menos $5x10^9$/L (5000/μl de sangre) de linfocitos maduros en sangre**; ***2*- un inmunofenotipo característico del linfocito B**. En la LLC, la célula B presenta un patrón diferencial de moléculas específicas que permiten su diferenciación de otros tipos de linfomas (Figura 1).

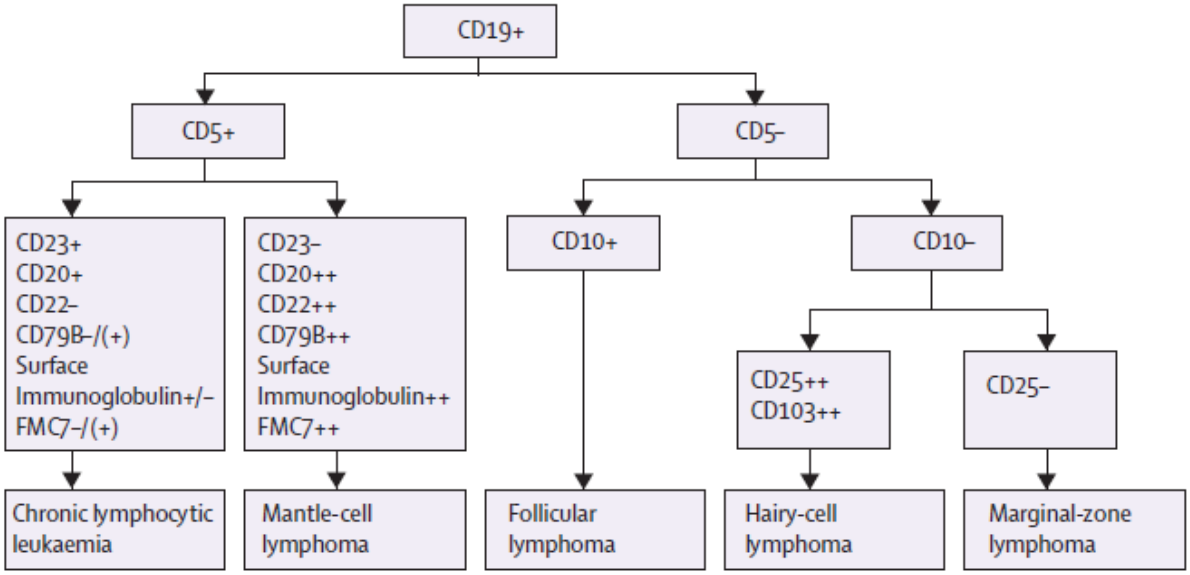

Figura1: Diagnóstico diferencial de linfocitosis CD19+ (Dighiero & Hamblin, 2008)

En cuanto al estudio del inmunofenotipo para el diagnóstico de LLC, es importante destacar que las células de LLC co-expresan el Ag de células T CD5 y el Ag de superficie de las células B CD19, CD20 y CD23. Los niveles de inmunoglobulinas de membrana (Igs), CD20 y CD79b son particularmente bajos comparados con aquellos encontrados en células B de donante sanos (Moreau, Matutes et al. 1997; Ginaldi, De Martinis et al. 1998).

Tanto la LLC como el Linfoma de Células del Manto y el Linfoma de Linfocitos Pequeños (Small Lymphocytic Lymphoma -SLL-) se caracterizan porque sus células tumorales expresan los Ags $CD19^{+}CD5^{+}$. En el caso del Linfoma de Células del Manto las células B expresan altos niveles de CD22, CD20 e Igs de membrana ($CD23^{-}CD22^{++}CD20^{++}$) mientras que en la LLC la célula B expresa bajos niveles de CD23, CD22 e Igs de membrana ($CD23^{+}CD22^{+}CD20^{-}$). Por su parte el SLL es un tipo de cáncer que presenta las mismas características inmunofenotípicas que la LLC (revisado en (Hallek et al, 2008)), sin embargo en el SLL, el tumor se encuentra confinado principalmente en los nódulos linfáticos pero no en sangre ni en médula óseacomo ocurre en la LLC. La definición de SLL requiere entonces la presencia de linfoadenopatías y/o esplenomegalias y a su vez el número de linfocitos en SP no debe exceder los $5x10^{9}$/L.

1.3- *B* CLASIFICACIÓN ANATOMO-CLÍNICA DE LA LLC

A fines de los años 70 Binet y col. realizaron una clasificación anatomo-clínica de pacientes con LLC que permitió subdividirlos en tres grupos pronóstico: bueno (estadio A), intermedio (estadio B) y severo (estadio C) (Binet et al, 1977). Esta clasificación se basa en el número de áreas involucradas, definidas por el agrandamiento de los nódulos linfáticos mayor a 1cm en el diámetro u organomegalia y presencia o ausencia de anemia o trombocitopenia.

Las áreas involucradas en la estratificación por Binet son: 1- cabeza y cuello, 2- axilas, 3- ingles (incluyendo femorales superficiales), 4- bazo palpable y 5- hígado palpable. De acuerdo con la clasificación de estas áreas, un paciente de LLC es estadio A cuando presenta por lo menos 100g/L hemoglobina (Hb), un recuento de plaquetas de al menos $100x10^9$/L y más de 2 zonas involucradas. Un paciente es estadio B cuando presenta al menos 100g/L Hb, un recuento de plaquetas de al menos $100x10^9$/L y más de 3 zonas involucradas. Mientras que el estadio C es independiente de la organomegalia, son aquellos pacientes que presentan menos de 100g/L de Hb y un recuento de plaquetas menor a $100x10^9$/L.

Años más tarde Rai y col.proponen una segunda clasificación con 5 estadios (0, I, II, III y IV), (Rai & Han, 1990). La clasificación de Rai permite definir a los pacientes de bajo riesgo como aquellos que presentan linfocitosis con células leucémicas en sangre y/o en médula (linfocitos >30%). Estos son considerados formalmente estadio 0 de Rai. Pacientes con linfocitosis, aumento del tamaño de los nódulos, esplenomegalia y/o hepatomegalia son definidos como pacientes con riesgo intermedio y formalmente considerados como estadio I o II de Rai. La enfermedad de alto riesgo es considerada en los pacientes que presentan además, anemia (Hb <110g/L) con un estadío III de Rai y aquellos con trombocitopenia (baja cantidad de plaquetas, $<100 \times 10^9$/L) son considerados estadío IV de Rai.

La clasificación de Binet y Rai está directamente relacionada con la sobrevida de los pacientes, siendo aquellos con estadio Binet A o Rai 0 los que presentan una sobrevida media de más de 10 años.Los pacientes con estadío Binet B o Rai I/II presentan una sobrevida media de entre 5 a 7 años mientras que los que presentan una menor sobrevida, de aproximadamente 3 años son los de estadio Binet C o Rai III/IV (Hamblin, 2007).

Al día de hoy ambas son usadas en la estratificación clínica de la LLC.La clasificación Binet es usada principalmente en Europa y parte de Latinoamérica mientras que la clasificación de Rai es usada

sobre todo en América del Norte. Las dos procuran un sistema sumamente simple, que no requiere test de laboratorios y que permiten tanto el diseño de estudios clínicos como de estrategias terapéuticas racionales. Sin embargo, ni la clasificación de Rai ni la de Binet, permiten pronosticar con una certeza razonable la evolución de los pacientes, sobre todo de aquellos que se incluyen en los estadios iniciales de la enfermedad donde el tratamiento podría ser más efectivo (Dighiero, 2003).

En lo que respecta a la predicción del proceso evolutivo de la enfermedad, los mayores avances fueron realizados en la década de 1990 donde gracias al desarrollo tecnológico en el área de la biología molecular se empieza a conocer en más detalle la biología de esta leucemia.

Una de los grandes avances en este campo comienza con el trabajo de Schroeder y Dighiero en 1994 (Schroeder & Dighiero, 1994) sugiriendo que la LLC es una enfermedad heterogénea en donde el proceso tumoral puede originarse ya sea en linfocitos B memoria o linfocitos B vírgenes. En este trabajo se muestra que por lo menos el 50% de los pacientes expresaban los genes variables de sus Igs en forma mutada, mientras que el resto presentan los genes V_H en configuración germinal.Años después este concepto de heterogeneidad en la LLC es reafirmada con los trabajos de Hamblin y col. (Hamblin, Davis et al. 1999) y Damle y col. (Damle, Wasil et al. 1999) en 1999. Ambos trabajos demuestran que además de categorizar a la LLC en un origen tumoral pre o post-centro germinal (CG), el perfil mutacional de los genes V_H del clon tumoral en la LLC surge como el principal método pronóstico en esta leucemia. A partir de entonces es aceptado a nivel clínico que aquellos pacientes con genes V_H mutados (MUT), tienen un mejor pronóstico que los pacientes en los cuales el clon leucémico muestra un gen V_H en su configuración germinal, no mutado (NM).

Los pacientes de LLC MUT fueron originalmente definidos como aquellos que tienen menos del 98% de identidad nucleotídica con respecto a la línea germinal de los genes V_H. A pesar de que otros puntos de corte, como 94% o 95% de identidad fueron propuestos durante varios años, hoy en día la mayoría de las publicaciones han establecido que el mejor punto de corte para discriminar el curso clínico del paciente es 98% (Hamblin, Davis et al. 2008).

Es importante destacar que el clon tumoral presenta un único tipo de rearreglo de las cadenas IGH-VDJ que darán lugar a las Igs de membrana de los linfocitos B tumorales. Se ha determinado que la presencia o ausencia de hipermutación somática (HS) se encuentra asociada con el uso de

determinados segmentos génicos. Pacientes con un rearreglo IGHV1-69 en general presentan un perfil NM, mientras que la mayoría de los pacientes que presentan un rearreglo de la familia IGHV3, tienen un perfil MUT. Al igual que los pacientes de LLC de Europa, Norteamérica, Asia, Argentina (Bezares et al, 2009)y en Uruguay se ha observado que existe un sesgo en el uso del segmento génico de la familia de IGH-VDJ (Bianchi et al. 2010). Los rearreglos de IGVH más frecuentes en los pacientes uruguayos de LLC se parecen más a los europeos del mediterráneo que a los Brasileros. Posiblemente eso se deba a que más del 80% de los Uruguayos y Argentinos presentan ancestros de origen Europeo mientras que en los Brasileros este porcentaje es mucho menor y existe una mayor ascendencia de origen Africano. Esto sugiere que las influencias genéticas podrían ser importantes en el desarrollo de la etiopatogénesis de la LLC, (Bianchi et al, 2010).

1.4 MARCADORES PRONÓSTICO EN LA LLC

En los siguientes años al descubrimiento de genes V_H MUT o NM, numerosos marcadores moleculares han sido propuestos con el objetivo de reemplazar al estudio del perfil mutacional de los genes VH, cuya principal desventaja es la difícil aplicación en la rutina hospitalaria. De acuerdo a Moreno and Montserrat (Moreno & Montserrat, 2008) los marcadores pronósticos se pueden dividir en dos grandes grupos: los clásicos, y los biológicos.

Los **marcadores pronóstico clásicos** son: **i- la estratificación clínica** (Clasificación Binet - Rai)**, ii- recuento linfocitario, iii- morfología de linfocitos de SP, iv- tiempo de duplicación linfocitaria y v- grado de infiltración en médula.**

Tanto el recuento de linfocitos como la morfología de los mismos son estudiados durante el diagnóstico para la clasificación de estadio Binet o Rai. Mientras que el tiempo de duplicación linfocitaria ("Lymphocyte Doubling Time", LDT) está definido como el número de meses que le lleva a los linfocitos doblar su número absoluto. Lo que quiere decir que si el valor de LDT es bajo, en poco tiempo los linfocitos se duplican, por esta razón es que pacientes que presentan un LDT menor a 6 meses se los asocia con una alta proliferación y una enfermedad más agresiva.

Por otro lado, el grado de infiltración medular se determina mediante histología de la médula ósea (Binet 1999; Montserrat 2002). Cuando hay una infiltración medular difusa se ha observado una supervivencia entre 2 a 4 años, mientras que en los casos con un patrón no difuso (nodular, intersticial, mixto), esta ha variado de 8 a 11 años. El patrón difuso generalmente se correlaciona con un estadio clínico avanzado y cuando se observa en un estadio clínico precoz, se considera un indicador de rápida progresión (Catovsky & Murphy, 1995).

Los **marcadores pronósticos biológicos**se pueden subdividir entres grandes grupos: ***i*- marcadores genéticos** (perfil mutacional de los genes VH y aberraciones cromosómicas) ***ii*- expresión anómala de genes en linfocitos leucémicos** (a nivel de ARNm, microRNAs y proteínas), ***iii*- proteínas presentes en el suero.**

i-Marcadores genéticos pronósticos

A pesar de que varios trabajos sugieren que la LLC podría tener un componente genético importante, la naturaleza de esta predisposición no está aún bien dilucidada. Dado que ninguna de las aberraciones cromosómicas descritas se observan en todos los pacientes de LLC, es difícil de asegurar si alguna de las anomalías es un evento inicial en la transformación maligna o bien ocurren durante la evolución de la enfermedad. Trabajos de Döhner y col. demuestran la presencia de aberraciones cromosómicas en un 82% de las LLC estudiadas, sugiriendo la importancia de estas alteraciones en la enfermedad (Dohner et al, 2000b).

La deleción 13q ha sido identificada en un 55% de los pacientes, la trisomía 12 en un 18%, la deleción 11q en un 16%, mientras que la deleción menos frecuente es la 17p, sólo se encuentra en un 7% de pacientes. Resultados recientes sugieren la posibilidad de que una región cromosómica presente un sitio potencial de genes candidatos a sufrir mutaciones relacionadas con la enfermedad (sitio 13q21.33-q22.2). En todas estas deleciones se encuentran genes de importancia en la regulación del ciclo celular y la apoptosis como el gen que codifica a la proteína linfoma2 de célula B ("B-cell lymphoma 2", Bcl-2), p53, la proteína nuclear ataxia telangeiectasia (NPAT), cullina 5 ("cullin5", CUL5) y fosfatasa 2, subunidad regulatoria A beta (PPP2R1B) los cuales podrían estar implicados en el origen y/o progresión de la enfermedad (Dohner, Stilgenbauer et al. 2000; Zenz, Mertens et al. 2009; Gunnarsson, Mansouri et al. 2013; Rossi, Rasi et al. 2013).

La deleción 13q se encuentra a menudo en pacientes MUT y de buen pronóstico, estando directamente relacionada a la expresión del gen BCL-2 (Hallek et al, 2008). Importantes trabajos del grupo de Croce han demostrado que la ausencia de este fragmento de ADN lleva a la falta de expresión de los microRNAs 15 y 16 que son a su vez los responsables de controlar la expresión de genes con propiedades anti-apoptóticas. La deleción 13q y ausencia de los microRNAs 15 y 16 hace que se sobre exprese la proteína Bcl2 impidiendo la muerte del clon leucémico (Calin et al, 2004; Cimmino et al, 2005).

En lo que respecta a la trisomía del cromosoma 12 (tri 12) no se ha encontrado un efecto directo sobre ningún gen. Sin embargo, comparaciones entre pacientes de LLC sin anomalías genéticas y portando la tri 12, demuestran una menor sobrevida y peor evolución en estos últimos casos (Dohner et al, 2000b). Recientemente, se ha observado también que pacientes con la tri 12 y mutaciones en el gen NOTCH1 presentan una menor sobrevida global (Balatti et al, 2012), que está asociada a una mayor resistencia a la muerte celular del linfocito leucémico (Balatti et al, 2012; Fabbri et al, 2011; Puente et al, 2011).

Por su parte, las deleción 11q22-23 afectando genes como Ataxia telangiectasia (ATM) y/o Radixin (RDX) y la deleción 17p afectando genes como TP53 presentan indefectiblemente una evolución clínica desfavorable (Stilgenbauer et al, 2002). Estos pacientes son en general NM, lo cual es consistente con el mal pronóstico (Hamblin et al, 1999). Los pacientes con la deleción 11q22-23 presentan adenopatías voluminosas y una evolución clínica agresiva con una sobrevida corta (Dohner et al, 2000a). Tanto el gen ATM como el gen TP53 (deleción 17p) son esenciales en la reparación del ADN y detención del ciclo celular frente al daño de la célula. La ausencia de estos genes contribuye a una evolución clonal agresiva en donde la adquisición de nuevas variantes genómicas llevan al origen de un clon leucémico refractario al tratamiento (revisado en (Chiorazzi, 2012)).

La evaluación de las alteraciones genéticas antes mencionadas son realizadas por técnicas citogenéticas que presentan limitaciones, solamente pueden detectar lesiones que hayan sido previamente descritas en otros pacientes. Por esta razón, técnicas más sensibles capaces de reconocer nuevas alteraciones genéticas son de gran importancia para el estudio de esta y otras enfermedades. En este sentido es que mediante secuenciado masivo ("whole-exome or whole

genome Sequencing") se han logrado identificar, al día de hoy, más de 25 mutaciones genéticas recurrentes que afectan siete vías de traducción de señales diferentes. Donde los genes mayormente estudiados son los siguientes:*notch1* (NOTCH1), *exportina 1*(XPO1), *diferenciación mieloide gen 88 de respuesta primaria* (MYD88), *"Kelch-like 6"*(KLH6), TP53, *transglutaminasa7* (TGM), *baculovirus IAP conteniendo el repetido3* (BIRC3), *"pleckstrin homology domain containing, family G"* (PLEKHG5), ATM, *factor 3 de splicing, unidad B1*(SF3B1)(Fabbri et al, 2011; Puente et al, 2011; Quesada et al, 2012; Rossi et al, 2012; Wang et al, 2011).

De todos estos genes presentando mutaciones en diferentes casos de LLC uno de los que más atención ha despertado en la biología de esta leucemia es el gen, NOTCH1. Este gen juega un rol importante en la diferenciación de linfocitos B a células plasmáticas productoras de anticuerpo (Santos et al, 2007). La activación de la vía de señalización, en la que se encuentra involucrado NOTCH1, es fundamental en la sobrevida e inhibición de la apoptosis de las células de LLC. Estas mutaciones generan un codón stop prematuro con una proteína más pequeña que se encuentra constitutivamente activa, lo que contribuye a la sobrevida del clon tumoral (Figura 2) (Puente et al, 2011).

Otro de los genes que ha recibido gran atención desde el punto de vista biológico de la LLC luego de los análisis de secuenciado masivo es aquel que codifica para la proteína SF3B1. Esta proteína forma parte de la maquinaria del splicesoma la cual es de gran importancia para el procesamiento del ARNm. SF3B1 cumple funciones importantes en el proceso de "splicing" alternativo generando diferentes proteínas algunas de las cuales son esenciales para la vida celular. Dos estudios recientes han detectado mutaciones de este gen entre el 10-15% de los casos de LLC (Quesada et al, 2012; Wang et al, 2011) y las mismas han sido asociadas a una menor sobrevida de los pacientes de LLC.

Finalmente, el gen BIRC3, ha sido identificado como otro gen mutado en un alto porcentaje de pacientes con LLC. Este gen codifica para una proteína miembro de la familia de inhibidores de la apoptosis. Se ha observado que tanto sólo o junto con el receptor TNF asociado al factor 2 o factor 3 (TRAF2 o TRAF3) coopera con un complejo proteico para regular negativamente la serina-treonina-quinasa MAP3K14, activador central de la vía de señalización del factor nuclear kappa B (NFkB) (Zarnegar et al, 2008). Mutaciones de BIRC3 generan que la vía NFKB se encuentre continuamente activada. La ausencia de las funciones de BIRC3 podrían explicar la activación constitutiva de NFkB en pacientes de LLC previamente documentada (Herishanu et al, 2011). Más

recientemente se ha evidenciado que pacientes que tienen mutaciones en BIRC3 no responden bien a ciertos tipos de terapia, lo que estaría sugiriendo que este es un marcador capaz de ayudar en la predicción evolutiva del paciente (Rossi et al, 2012).

***ii*-Expresión anómala de genes en linfocitos leucémicos** (a nivel de ARNm, microRNAs y proteínas)

El estudio del nivel de expresión de proteínas de ZAP70 (Rassenti et al, 2008), CD38 y de la integrina CD49d (Damle et al, 1999) han demostrado ser buenos predictores de la evolución clínica de pacientes con LLC. La proteína más utilizada como marcador pronóstico es ZAP70. Esta molécula es una tirosina-quinasa de 70 kDa que se expresa generalmente en células "natural killers" y en linfocitos T. Juega un rol central en la vía de señalización del TCR, en migración y apoptosis (Chan et al, 1992). En los linfocitos B, ZAP70 se expresa en células normales y tumorales en diferentes etapas del desarrollo (Scielzo et al, 2006). Luego de una estimulación antigénica, ZAP70es reclutada al complejo proteico de la vía de señalización del receptor de la célula B (BCR) de manera similar a las proteínas con dominios SYK. Un estudio de expresión diferencial entre pacientes NM y MUT determinó que ZAP70 se sobre-expresa en individuos NM de mal pronóstico (Rosenwald et al, 2001). Más recientemente se ha observado que ZAP70 prolonga la vía de señalización del BCR (Chen et al, 2008) y que promueve la migración celular (Richardson et al, 2006). En particular, Calpe y col. observaron que luego de estimular el BCR en células ZAP70 positivas había un incremento del receptor de quimioquina CCR7 que se une a la proteína CCL21 e induce la migración celular hacia los órganos linfoides secundarios (Calpe et al, 2011). Dado que la expresión de ZAP70 en pacientes de LLC se correlaciona con el perfil mutacional de genes VH y con la expresión de CD38, el estudio de los niveles de expresión de ZAP70 se han propuesto como un marcador independiente de la evolución clínica (Figura 2) (Crespo et al, 2003).

Por su parte, CD38 es una glicoproteína de membrana que se expresa en células con origen hematopoyético. Las células B y T activadas expresan CD38, así como las células "natural killers" y las células dendríticas. La proteína CD38 funciona como un receptor de membrana interaccionando con CD31 y llevando a la activación del linfocito B a través de la regulación de los niveles de calcio celular (Malavasi et al, 2008). El porcentaje de células CD38 positivas dentro del clon tumoral es un indicador de la activación celular del clon. Aquellos que presentan un porcentaje elevado responden de mejor manera a las señales de activación (Chiorazzi, 2012). Debido a esto, la expresión de CD38 es una medida de división celular y reflejo del crecimiento *in vivo*. A pesar de

estas características la expresión de CD38 como marcador pronóstico presenta ciertas desventajas: i- la expresión de CD38 varía durante el curso de la enfermedad, ii- CD38 no es exclusiva de linfocitos B tumorales sino que presenta heterogeneidad de la expresión en las muestras de sangre y iii- ausencia de un valor definido de cut-off que permita decir cuando un paciente es o no positivo para CD38. Al día de hoy, los valores sugeridos de puntos de corte varían desde 5-30% (Ghia et al, 2003; Hamblin et al, 2002). Por esta razón es que se ha propuesto a CD38 como un marcador complementario al perfil mutacional de genes VH (Figura 2).

Finalmente, CD49d ha sido propuesto como marcador pronóstico de LLC debido a que pacientes de LLC conmás de 30% de células positivas para CD49d presentan una menor sobrevida (Gattei et al, 2008; Rossi et al, 2008). CD49d es una integrina ($\alpha 1/\beta 4$) que juega un papel importante en la migración de los linfocitos regulando la adhesión de las células a los tejidos. Su unión a fibronectina y a la molécula de adhesión celular vascular ("vascular cell adhesion molecule 1", VCAM1) desencadena señales que contribuyen con la sobrevida celular. Esta integrina previene la apoptosis espontánea o inducida por drogas de células normales y neoplásicas. Su expresión se encuentra asociada a CD38 y por lo tanto a mal pronóstico (Zucchetto et al, 2006).

Con el advenimiento de los análisis genómicos por microarreglos, diferentes marcadores pronósticos implicando la expresión de ARN mensajeros han sido descritos. Dentro de los principales marcadores descritos al momento encontramos el ARNm de la lipoprotein lipasa (LPL) (Oppezzo et al, 2005b) y de CLLU1 (Buhl et al, 2006).

La enzima LPL se encuentra altamente expresada en el musculo, tejido adiposo y glándulas mamarias. Presenta un rol central en el metabolismo lipídico, hidrolizando triacilglicéridos circulantes en ácidos grasos libres y monoacilgliceroles (Wang et al, 1992). Una alta expresión del ARNm del gen LPL en células B de pacientes de LLC ha sido correlacionada con una mala evolución clínica (Oppezzo et al, 2005b). La expresión anómala de esta enzima se encuentra asociada a los marcadores pronósticos CD38, ZAP70, LDT y alto riesgo de aberraciones genéticas (Heintel et al, 2005; Oppezzo et al, 2005b). Estudios recientes de Kaderi y col. proponen a LPL como el mejor marcador pronóstico a nivel de ARN debido a que, a diferencia del resto de los marcadores, éste puede ser medido directamente a partir de muestras de SP sin un requerimiento de aislar los linfocitos B previo a su análisis (Van Bockstaele et al, 2007). Debido a esto, los autores consideran al marcador pronóstico LPL como prometedor para un futuro uso en la clínica (Kaderi et al, 2011).

El otro marcador de importancia propuesto es CLLU1 descrito por Buhl y col. en el año 2006 (Buhl et al, 2006).CLLU1 se encuentra sobre–expresado en pacientes de LLC (CLL upregulated gene 1, CLLU1) que presentan mal pronóstico. Su expresión se encuentra asociada a pacientes NM, con alta expresión de ZAP70 y CD38 (Buhl et al, 2006).

Otro marcador recientemente propuesto que cabe remarcar en el contexto de este trabajo de tesis es la detección del ARNm de la fosfatasa y tensin homóloga deletado en el cromosoma 10 (PTEN). Si bien su relevancia en el área clínica es menor, nos detendremos en él ya que la biología de esta molécula y sus funciones forman parte importante del trabajo de esta tesis.En particular Zhi-Jian Zou y col. observaron que los pacientes de LLC expresan menos PTEN que los donantes sanos y a su vez, que pacientes de mal pronóstico estadio Binet C y ZAP70^{pos} expresaban menos cantidad de PTEN que aquellos con estadios más favorables. Debido a que PTEN regula negativamente la actividad de la serina-treonina quinasa AKT/PKB (relacionada con la sobrevida y proliferación celular) y queuna baja expresión de PTEN está asociada a una menor sobrevida, los autores proponen que el estudio de expresión de ARNm de PTEN podría servir como un nuevo marcador pronóstico para la LLC (Zou et al, 2013).

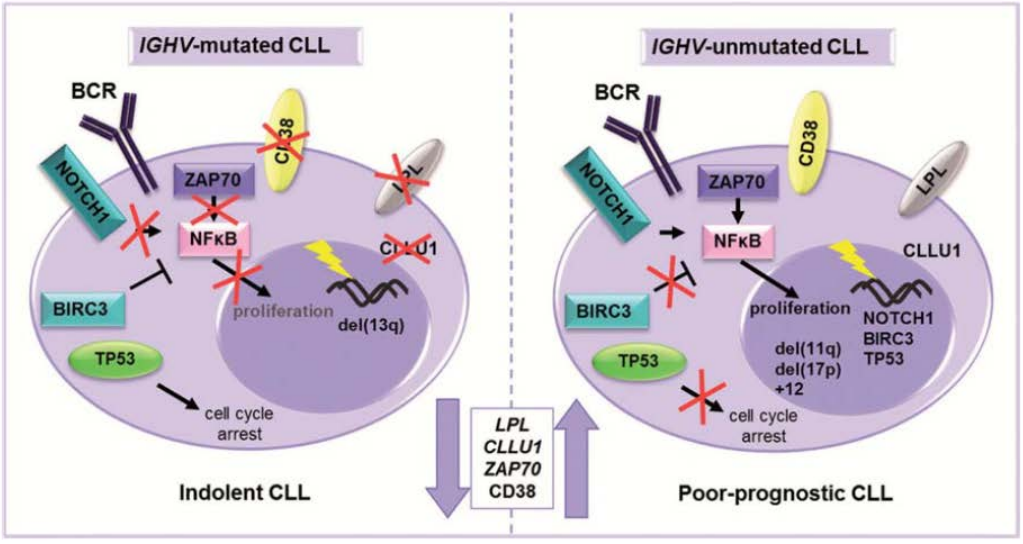

Figura 2: **Ilustración de los marcadores pronósticos genéticos más relevantes relacionados con el perfil mutacional de los genes V_H.** Mutaciones en NOTCH1 así como en BIRC3 pueden potenciar la vía de señalización NFkB e inducir la proliferación de las células B de pacientes de LLC NM. ZAP70 se encuentra involucrado en la vía de señalización del BCR y su sobre-expresión potencia la proliferación por NFkB. La disrupción de TP53 por deleción o mutación es más común en los NM y lleva a un aumento de la sobrevida celular. A pesar de que no se conocen las razones por las cuales existe gran expresión de LPL y CLLU1 en pacientes de mal pronóstico, en los últimos años han demostrado ser buenos marcadores pronóstico, debido a que se correlacionan con una mala evolución clínica(Rosenquist et al, 2013).

Con el propósito de caracterizar a los pacientes de buen y mal pronóstico se han realizado también estudios de expresión diferencial de microRNAs (miRNAs) entre los diferentes perfiles evolutivos de LLC (Calin et al, 2005; Fulci et al, 2007). En particular, el trabajo de Calin y col. determinaron la presencia de 13 miRNAs diferencialmente expresados entre pacientes $ZAP70^{pos}$, NM de un mal pronóstico y $ZAP70^{neg}$, MUT con una enfermedad indolente (Calin et al, 2005). A partir de este trabajo numerosos esfuerzos se han realizado con el propósito de identificar nuevos miRNAs. Al presente, el miR-21, miR-29, miR-34a, miR-181b y miR-223 han sido propuestos como marcadores pronósticos de utilidad en la LLC (Marton et al, 2008; Pekarsky et al, 2006; Rossi et al, 2010; Stamatopoulos et al, 2009; Zenz et al, 2009).

La sobre-expresión del miR-21 se ha observado en pacientes con la deleción 17p (Rossi et al, 2010), mientras que la baja expresión del miR-181b se encuentra asociada a la refractoriedad en la terapia (Marton et al, 2008). El miR-29 junto con el miR-181b regulan la expresión de TCL1, gen implicado en el ciclo celular cuya sobre-expresión por transgénesis en los linfocitos B lleva a la generación de uno de los modelos murinos más representativos de la LLC (Pekarsky et al, 2006). Otro de los miRNAs observados en pacientes con la deleción 17p, es el miR-34 el cual se encuentra sub–expresado en linfocitos B leucémicos (Zenz et al, 2009).

ii-Proteínas del suero como marcadores pronóstico de la LLC

La mayoría de los marcadores disponibles del suero pueden ser fácilmente medibles y proveen de una información útil para el pronóstico, aunque en algunos casos no son específicos de LLC. Tanto la enzima lactato deshidrogenasa (LDH), β2-microglobulina y la timidina quinasa, son proteínas que se han visto incrementadas en el suero de pacientes de LLC con estadios clínicos agresivos. Estas proteínas evaluadas a partir del suero de pacientes son útiles como marcadores pronósticos (Fasola et al, 1984; Hallek et al, 1996).

Si bien con el correr de los años siguen surgiendo candidatos propuestos como marcadores, los más utilizados al momento siguen siendo: la expresión anómala en el linfocito B de ZAP 70, (Crespo et al, 2003) CD38 (Lanham et al, 2003), LPL (Oppezzo et al, 2005b) y CLLU1 (Buhl et al, 2006) así como el estudio de ciertas alteraciones cromosómicas asociadas a la progresión y refractoriedad en el tratamiento como las deleciones 11q y 17p.

En este sentido, un trabajo publicado el 2013 por Rossi y col. proponen que un análisis citogenético y mutacional integrado puede ayudar a interpretar mejor la evolución clínica de los pacientes de LLC. En este trabajo y partir de 1274 muestras de LLC se pudieron agrupar a los pacientes en cuatro categorías de alto a bajo riesgo. Los pacientes con mutaciones en TP53 y/o BIRC3 son de alto riesgo, mientras que los pacientes que presentan mutaciones en NOTCH1 y/o SF3B1 y/o del 11q son de riesgo intermedio. Por su parte los pacientes de LLC que presentan la tri12 y/o del 13q o directamente no tienen alteraciones genéticas son de riesgo bajo a muy bajo y presentan una mayor sobrevida (Rossi et al, 2013).

A modo de resumen podemos decir que al día de hoy en la LLC se considera que no hay un único marcador de progresión sino que la determinación de varios de ellos es el método más seguro para aumentar la posibilidad de obtener una predicción evolutiva lo más acertada posible del paciente.

1.5 Tratamiento en la LLC

Es importante destacar que los marcadores pronósticos mencionados anteriormente son importantes para predecir el curso de la evolución clínica, sin embargo la decisión de tratar o no tratar a un paciente depende básicamente de las manifestaciones clínicas y el grado de avance de la enfermedad del paciente durante el transcurso del tiempo. Los parámetros considerados son el estadio de la enfermedad, la salud general del paciente, marcadores genéticos de alto riesgo y el tipo de terapia recibido durante el transcurso de la enfermedad (primera vs segunda línea de tratamiento, respuesta vs no respuesta de la última terapia (Hallek, 2013).

En general, pacientes asintomáticos que debutan con estadio A de Binet o 0 de Rai, son monitoreados sin terapia. En particular se observó que el tratamiento con agentes alquilantes en pacientes con estadios tempranos de la enfermedad (Estadio A de Binet o 0 de Rai)no promueven la sobrevida de dichos pacientes (Dighiero et al, 1998). Por esta razón, el beneficio potencial de tratar a estos pacientes con drogas anti-leucémicas aún debe ser probado(Hallek, 2013). Por otro lado, pacientes con estadios intermedios y de alto riesgo (B y C de Binet o III o IV de Rai) generalmente se ven beneficiados con el tratamiento. Algunos de los pacientes con estadios intermedios son

monitoreados sin terapia hasta que manifiestan síntomas o una progresiva o activa enfermedad definida por la guía del iwCLL (Hallek et al, 2008).

Los tratamientos disponibles es pueden dividir en dos grandes grupos, i- agentes simples y ii- anticuerpos monoclonales.

i-Agentes simples

La monoterapia con agentes alquilantes ha servido como la primera línea para la terapia para pacientes de LLC. El clorambucil ha sido considerado el "gold standard" por varias décadas (CLL Trialists' Collaborative Group, 1999). A nivel celular reacciona con las bases nitrogenadas del ADN y da lugar a la formación de puentes inter e intracatenarios, alterando las funciones normales del ADN y llevando a la muerte celular (Begleiter et al, 1996). Las ventajas del uso del clorambucil son su baja toxicidad y bajo costo, mientras que las mayores desventajas son su casi inexistente remisión completa y algunos efectos que ocurren cuando se usa por períodos prolongados como citopenias, mielodisplasia y hasta un posible desarrollo de leucemias aguda (Hallek, 2013).

Los análogos de purinas como fludarabina, pentostatina o el cladribina también son usados para tratamiento de la LLC. La fludarabina induce más remisiones (7-40%) que otras quimioterapias convencionales como CHOP (ciclofosfamida doxorrubicina, vincristina y prednisona) CAP (ciclofosfamida doxorrubicina y prednisona) o clorambucil (Johnson et al, 1996; Leporrier et al, 2001; Rai et al, 2000). El efecto citotóxico de la fludarabina resulta en una completa inactivación de la síntesis del ADN seguido de la activación del programa de muerte celular (Ricci et al, 2009).

La bendamustina también ha sido utilizada para el tratamiento de LLC. Es un agente alquilante que también manifiesta propiedades similares a los análogos de purinas. A pesar de que al día de hoy poco se sabe sobre su mecanismo de acción, la bendamustina ha sido asociada con el daño en el ADN, inhibición de los puntos de control mitóticos e ineficiencia en reparar el ADN lo que lleva a la apoptosis celular (Leoni & Hartley, 2011). Un estudio en fase III mostró que tratamiento con Bendamustina ofrece una eficacia significativamente mayor que el clorambucil, y un perfil de toxicidad manejable, cuando se utiliza como terapia de primera línea en pacientes con una LLC avanzada (Knauf et al, 2009).

ii-**Anticuerpos monoclonales**

Con el propósito de eliminar el clon tumoral se han desarrollado anticuerpos específicos contra moléculas claves como el Ag de linfocitos B CD20. La proteína CD20 es una glico-fosfoproteína que se expresa en la superficie de células pre B, linfocitos B maduros normales y tumorales. No se conoce su ligando pero se sospecha que actúa como un canal de calcio en la membrana celular. Debido a que se expresa en la mayoría de los tumores de células B, se han diseñado numerosos anticuerpos recombinantes anti-CD20 (Hagemeister, 2010; Walshe et al, 2008).Al presente los anticuerpos recombinantes más utilizados en la clínica hematológica son el Rituximab, el Ofatumumab y el Obinutuzumab.

El Rituximab es un anticuerpo quimérico de isotipo IgG1 y ha sido uno de los primeros en ser probados en ensayos clínicos en LLC. Luego de este trabajo se ha visto que la utilización del mismo es más eficiente en combinación con quimioterapia que cuando se lo utiliza sólo. Además, se ha evidenciado que ciertos pacientes de LLC tratados con Rituximab junto con ciclofosfamida y fludarabina (FCR) presentan remisiones completas con una mayor sobrevida (Bryan & Borthakur, 2011). Debido a estos resultados la FDA de Estados Unidos aprobó el uso de FCR como el tratamiento de primera línea y la técnica "gold standart" en pacientes con LLC.

El otro anticuerpo anti-CD20 en el mercado es el Ofatumumab. En este caso la molécula es un anticuerpo monoclonal humanizado que se une a un epítope diferente al del Rituximab (Teeling et al, 2006). Se ha demostrado *in vitro* que induce la muerte de los linfocitos B mediante activación de la vía clásica del complemento y mediante citotoxicidad celular dependiente de anticuerpos ("Antibody dependent cellular citotoxicity", ADCC) (Beum et al, 2008). El Ofatumumab permite una mayor unión del complejo C1q del complemento incrementando su actividad, incluso con una baja expresión del Ag CD20. Se postula que este aumento en citotoxicidad de los anticuerpos se debe a que la unión Ig-CD20 hace que el anticuerpo quede más cerca de la superficie y de esta manera podría exponer los sitios de unión al complejo C1q con mayor eficiencia (Beum et al, 2008; Pawluczkowycz et al, 2009; Teeling et al, 2004). Estos cambios estructurales permiten que el anticuerpo se mantenga más tiempo unido alAg lo que potencia las funciones efectoras de los anticuerpos. La FDA Americana y la EMA Europea recientemente aceptaron el uso de este anticuerpo como monoterapia en pacientes que son refractarios a fludarabina y alemtuzumab.

El último anticuerpo anti-CD20 por lanzarse al mercado es llamado Obinutuzumab (GA-101). En este caso es un anticuerpo derivado del Rituximab, que se encuentra completamente humanizado y que además presenta una modificación en la cadena de azúcares en los dominios constantes de la Ig. Esta modificación contribuyea una mayorafinidad de uniónpor los receptoreshumanosFcγRIIIen comparación conanticuerpos no modificados, lo que resulta en una mayor ADCC y apoptosisindependiente de caspasas (Goede et al, 2012).

Estudios en fase I y II con el anticuerpo GA101 en pacientes que sufrieron recaídas o refractarios al tratamiento han mostrado muy buenos resultados (Morschhauser et al, 2013). Debido a esto, un estudio internacional en fase III fue iniciado en el 2010 (CLL11/BO21004) comparando el tratamiento con GA101 más clorambucil con rituximab más clorambucil o el clorambucil sólo en pacientes de LLC previamente no tratados con una comorbilidad en crecimiento. A pesar de que los resultados finales del estudio se esperan para finales del 2013 y no han sido publicados los resultados preliminares son muy prometedores (Hallek, 2013).

Por otro lado, para el tratamiento de LLC también se han usado anticuerpos monoclonales contra otros Ags, como por ejemplo el Alemtuzumab (Camphath-1H) el cual reconoce el Ag CD52 (Hale et al, 1990). CD52 es una glico-fosfoproteína que se encuentra en la superficie de linfocitos B y T normales así como en los tumorales. La función de la proteína al día de hoy no se conoce pero se cree que juega un rol importante en la regulación de la homeostasis del calcio celular y que se encuentra involucrada en la migración y coestimulacion de linfocitos T (Rowan et al, 1995; Watanabe et al, 2006). A pesar de que elAlemtuzumab es comúnmente usado como tratamiento de primera línea para la LLC, se han evidenciado en ciertos pacientes efectos adversos importantes.La monoterapia con este anticuerpoha producido tasas de respuesta de 33 -53%, con una duración media de la respuesta que van desde 9 de 15meses, en pacientes con LLC avanzada tratados previamente con agentes alquilantes y fludarabina (Keating et al, 2002; Rai et al, 2002). Debido a todo esto el uso del Alemtuzumab es considerado una opción terapéutica para pacientes con mal pronóstico que no responden al clásico tratamiento de FCR.

1.4. A NUEVAS DROGAS EN EL TRATAMIENTO DE LA LLC

El BCR forma parte de la vía de traducción de señales esenciales para la sobrevida y proliferación de linfocitos B maduros. Dada su importancia, el diseño de nuevas drogas ha estado enfocado en inhibir la vía de señalización del BCR con el propósito de inducir la muerte del clon tumoral. El Fostamatinib ha sido diseñado en esta línea de pensamiento y está dirigido hacia la inhibición de la tirosina-quinasa del bazo (spleen tyrosine kinase, Syk). Esta proteína se encuentra sobre-expresada en células de pacientes LLC y su inhibición induce apoptosis por disrupción de la vía de señalización del BCR (revisado en (Hallek, 2013)).

Por su parte otra de las drogas que se están evaluando en LLC es el Ibrutinib, un inhibidor específico de la Bruton tirosin-quinasa (Btk) (Figura 3). La Btk permite la activación las vías NFkB y MAPK vía la familia de proteínas quinasas Src estimulando el desarrollo, sobrevida y migración celular (Herman et al, 2011). Al día de hoy existen varias drogas capaces de inhibir la quinasa Btk, sin embargo hasta el momento sólo el Ibrutinib ha pasado los estudios en fase III mostrando que tiene una alta tolerabilidad para pacientes con linfomas de células B. En particular, la terapia con Ibrutinib ha mostrado buenos resultados para los pacientes con deleción 17p y recientemente ha sido aprobado su uso para pacientes con LLC en Estados Unidos (Aalipour & Advani, 2013).

Finalmente uno de los inhibidores más estudiados es el Idelalisib o CAL-101 (class 1 fosfatidil inositol 3- quinasa) inhibe la isoforma δ de la PI3K (Figura 3). La PI3K integra y trasmite señales de diferentes moléculas de superficie, como el BCR (Srinivasan et al, 2009), receptores de quimioquinas y moléculas de adhesión, de esta manera regula las funciones celulares como el crecimiento celular, la sobrevida y migración (Okkenhaug & Vanhaesebroeck, 2003). Las quinasas PI3K se encuentran divididas en tres clases (I-III). Las quinasas de clase I presentan 4 isoformas designadas como PI3Kα, β, γ y δ. Mientras que la PI3K α y β se encuentran expresadas en forma ubicua, la PI3Kγ presenta un rol particular en la activación del linfocito T. La expresión de la isoforma δ se encuentra restringida a células hematopoyéticas donde juega un rol importante en la homeostasis sobrevida y proliferación del linfocito B. En LLC la quinasa PI3K se encuentra activada constitutivamente (Ringshausen et al, 2002). Más aún, señales de sobrevida o crecimiento del microambiente de células estromales, activación de CXCR4 y BCR promueve la activación de PI3K en células B (revisado en (Burger, 2013). El CAL 101 es un potente y altamente selectivo inhibidor de la PI3Kδ, promoviendo apoptosis en células Bde pacientes con diferentes malignidades. Pacientes que

reciben la terapia presentan una re distribución de las células de LLC de los tejidos a la sangre, con una reducción del tamaño del nódulo linfático. Recientemente se ha reportado que CAL-101 inhibe la quimiotaxis producida por las quimioquinas CXCL12 o CXCL13 (Hoellenriegel et al, 2011). Estos resultados *in vitro* fueron corroborados por datos en la clínica donde se observó una marcada reducción de niveles de quimioquinas CCL3, 4 y CXCL13 circulantes. De esta manera inhibe las señales de sobrevida derivadas del BCR y bloquea la activación de la vía de señalización AKT o MAP/ERK quinasas (Hoellenriegel et al, 2011).

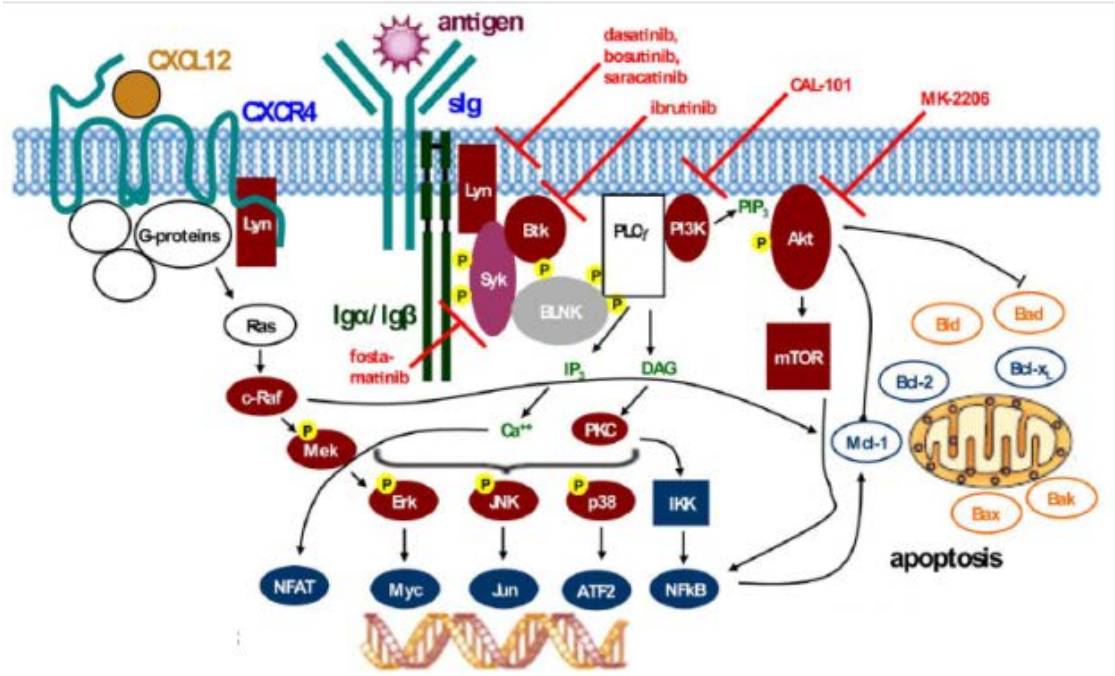

Figura 3: Esquema de las estrategias terapéuticas para LLC tomando como blanco el BCR (Hallek 2013).

Las últimas estrategias de inmunoterapia en la LLC y otros tipos de leucemias y linfomas intentan inmunomodular el sistema inmune en contra del tumor. Este es el caso de los receptores antigénicos quiméricos ("Chimeric Antigen Receptors", CARs technology) (Ho et al, 2003). La estrategia consiste en extraer linfocitos T CD8+ del paciente e *in vitro* transfectar las células con ADN que codifique para un el receptor quimérico de interés y luego re implantar las células modificadas al paciente con el propósito de dirigir los mecanismos efectores de los linfocitos T contra de las células tumorales. En un trabajo de Porter y col. diseñaron el receptor con un dominio extracelular que presenta las regiones variables de las Igs específicas contra CD19, marcador de linfocitos B, junto con un dominio intracelular que corresponde a la cadena Z del receptor del linfocito T. La estrategia consiste en que los linfocitos T modificados, expresen receptores

quiméricos que van a reconocer a las células B CD19 positivas y debido a que presentan moléculas co-estimuladoras, van a desencadenar los mecanismos efectores.

Interesantemente Porter y col. observaron una completa remisión luego de tan solo 10 meses en un paciente con LLC refractaria a los tratamientos clásicos (Porter et al, 2011). Luego de estos resultados, dos pacientes más con LLC avanzada recibieron esta terapia y los tres respondieron al tumor (Kalos et al, 2011). Estos datos resaltan el potencial de esta terapia y provee apoyo para continuar su estudio de células T redirigidas contra CD19 para neoplasias de células B.

A pesar de los grandes avances en el tema, y probablemente debido a la alta heterogeneidad biológica de la LLC una gran cantidad de los pacientes recaen en el correr del tiempo. Incluso, alguno de ellos no generan ningún tipo de respuesta frente al tratamiento. Por esta razón, los investigadores se han centrado en lograr identificar marcadores predictivos frente a determinado tipo de terapia. Predecir grupos de pacientes que responden a una determinada terapia puede ser útil para evitar toxicidad innecesaria dada por el tratamiento.

2.0-LA BIOLOGÍA DEL LINFOCITO B DE LA LLC Y SU MICROAMBIENTE

Los linfocitos de LLC son células pequeñas que presentan un angosto citoplasma y un núcleo denso carente de un nucléolo distinguible con cromatina parcialmente agregada (Hallek et al, 2008). Dichas células tienen la particularidad depresentar una débil expresión del BCR y de los antígenos (Ags) de superficie CD20 y CD22. Expresan de manera constante del marcador CD5 y expresan Igs de superficie IgM e IgD independientemente del estado mutacional de sus genes V_H(Oppezzo et al, 2002). Solo en ciertos casos se encuentran células que hayan realizado el proceso de cambio de clase (Fais et al, 1996; Oppezzo et al, 2002). Finalmente, las células de LLC expresan los Ags HLA de clase I y II y marcadores característicos de la línea B como son CD19, CD20, CD22 y sobre-expresión de CD23. Presentan también frecuentemente CD18, CD27, CD32, CD37, CD39, CD40, CD44, CD45RA y CDw75, mientras que otros Ags característicos del linfocito B como FMC7 y CD10 no están presentes.

2.1 EL RECEPTOR DE LA CÉLULA B EN LA LLC

Una de las principales características del linfocito B leucémicos es la baja expresión de su BCR. Esta molécula es un complejo multimérico formado por la Ig y el heterodímero Igα/Igβ (CD79a/CD79b) anclados en la membrana celular. La unión del Ag al complejo desencadena una activación de la vía de traducción de señales mediada por tirosin-quinasas de la familia Src. La vía del BCR es usada en linfocitos normales para promover la proliferación celular e inducir la producción de anticuerpos. Una vez que la Ig reconoce y se une al Ag específico, el complejo activado recluta quinasas como SYC y LYN las cuales fosforilan dominios de las proteínas Igα/Igβ llamados ITAMS ("immunoreceptor tyrosine – based motifs receptors") (Wang et al, 2013). La fosforilación de estos motivos induce una cascada de señalización rio abajo activando diferentes quinasas como BTK o PI3K (Longo et al, 2007), protein quinasa C, mTOR ("mammalian target of rapamycin") and MAP/ERK(Davids & Brown, 2012). Debido a que la estimulación del BCR promueve la sobrevida y proliferación activando el NFkB (Wang et al, 2013) es que la mayoría de los nuevos blancos terapéuticos están enfocados a inhibir cascadas de señalización celular como las mencionados anteriormente (fosfamatinib, ibrutinib e idelalisib)(Burger, 2013).

En la LLC numerosos trabajos han sido realizados con el objetivo de comprender las causas de la baja expresión del BCR y las implicancias que esto pueda tener en el origen y la evolución del clon tumoral. Thompson y col. sugirieron la presencia de mutaciones puntuales en la molécula Igβ como la principal causa de esta expresión anómala (Thompson et al, 1997). Sin embargo, trabajos posteriores no pudieron confirmar estos resultados. Para el caso de complejos proteicos anclados a la membrana, como el BCR, el correcto plegamiento y ensamblado son necesarios para ser transportados a la superficie celular para poder cumplir con sus funciones. Este proceso se realiza en el retículo endoplasmático, donde las proteínas son modificadas y correctamente plegadas antes de dirigirse al Golgi. Si el proceso de plegamiento y maduración falla, los sistemas de control de calidad celular son capaces de evitar que la proteína siga el curso hacia la membrana y quedan retenidas en el retículo donde las chaperonas colaboran con el plegamiento. En el caso del BCR, varias chaperonas como la calnexina, calreticulina, BiP y GRP94 han sido asociadas al Igα/Igβ (Foy & Matsuuchi, 2001). Payelle-Brogard y col. mostraron que los distintos componentes del BCR (Ig, Igα, e Igβ) presentan una expresión normal tanto a nivel transcripcional como proteico. Sin embargo, resultados de este mismo grupo sugieren que la sub-expresión del BCR en la LLC estaría ligada a un

defecto en el plegamiento y glicosilación de uno de sus componentes. En este trabajo se evidencian defectos en la glicosilación de la cadena μ e Igα lo cual parece determinar un bloqueo en el compartimiento del retículo-endoplásmico, impidiendo el correcto ensamblado y transporte del receptor hacia la membrana (Payelle-Brogard et al, 2002). Estos resultados muestran por primera vez que defectos en el plegamiento y glicosilación de los componentes del BCR pueden ser la causa de su baja expresión en la membrana celular (Payelle-Brogard et al, 2002; Vuillier et al, 2005).

En el mismo sentido, trabajos recientes mostraron que las Igs que llegan a la membrana celular en pacientes de LLC pueden presentar diferentes formas de N-glicosilación en su región constante. Una, la forma madura, similar a las células B normales y una forma inmadura característica de las proteínas del retículo endoplasmatico. En este trabajo se determinó que los pacientes NM presentan en su mayoría la forma inmadura de glicosilación a diferencia de los MUT que se parecen más a los linfocitos B normales con la forma madura de glicosilación. Dado que estas variaciones en el perfil de glicosilación son observadas también en células B normales luego de una estimulación del BCR, se cree que esta glicosilación inmadura podrían estar indicando la presencia de una exposición frente al Ag originando señales de sobrevida y proliferación(Krysov et al, 2010).

2.2 EL LINFOCITO B DE LA LLC PRESENTA INHIBIDA LA APOPTOSIS *IN VIVO* PERO MUERE *IN VITRO*

La célula B de la LLC presenta un defecto en el proceso de muerte celular programada, lo que lleva a la acumulación progresiva de linfocitos B clonales que permanecen bloqueados en las fases iniciales del ciclo celular (G0/G1). La mayoría de los pacientes con LLC presentan elevados niveles de p27kip1 (p27), regulador negativo del ciclo celular(Vrhovac et al, 1998). Dado las funciones claves que tiene p27 en la regulación del ciclo celular, su sobre-expresión podría contribuir a la acumulación de células B en las etapas tempranas del ciclo. Más aún, se ha postulado que la sobrevida de células B de LLC también puede estar dada por la sobre-expresión anómala de la familia de proteínas anti-apoptóticas Bcl2 y Mcl1 (Krajewski et al, 1995). Otros miembros de la familia Bcl2, como BCL-XL y BAG1 también se encuentran sobre-expresados en linfocitos B de LLC mientras que proteínas pro-apoptoticas, como BAX y BCLXS se encuentran reprimidas (Caligaris-Cappio & Hamblin, 1999).

Por otro lado también se ha determinado que los miRNAs pueden contribuira la regulación del ciclo celular reprimiendo genes relacionados con la sobrevida. Los primeros trabajos en esta área llevados a cabo por el grupo de Croce muestran diferencias significativas en la expresión de miRNAs entre células B de LLC y las células normales CD5 positivas. Lo más relevante en el contexto de la resistencia a la apoptosis por el linfocito B leucémico esta dado en los pacientes que presentan la deleción 13q. Las células B que presentan la deleción son incapaces de sintetizar los miRNAs 15 y 16 (Calin et al, 2004). Debido a que en células B normales dichos miRNAs regulan la expresión de Bcl-2, los autores proponen que la sobre-expresión de Bcl2 en pacientes de LLC podría estar dada por la ausencia de los miR-15 y miR-16. Estas observaciones establecen a la familia de proteínas anti-apoptótica Bcl2 como moléculas claves en la sobrevida celular de pacientes de LLC (Cimmino et al, 2005).

En contraste a lo que ocurre *in vivo*, los linfocitos B de LLC rápidamente mueren por apoptosis en cultivos *in vitro* en ausencia de células accesorias, indicando que el microambiente celular juega un rol esencial en la sobrevida de las células de LLC (Caligaris-Cappio, 2003). Estas observaciones hacen pensar que la interacción con el microambiente celular, especialmente con células estromales de la médula ósea, células T activadas ($CD3^{pos}CD4^{pos}$ y $CD40L^{pos}$) y células dendríticas foliculares podrían ser las responsables de mantener el defecto en la apoptosis y de aumentar la sobrevida de las células tumorales (Ghia et al, 2008). Por lo tanto, la exposición de clones malignos a estímulos del microambiente puede resultar en un incremento de la proliferación y la aparición de nuevas aberraciones genéticas que llevan a una enfermedad más agresiva.

Los procesos fisiológicos durante los cuales las células B encuentran el Ag ocurren en microambientes especializados llamados centros germinales (CGs). Los CG son estructuras dinámicas que se forman en los órganos linfoides secundarios capaces de generar microambientes especializados donde las células T junto con diferentes tipos de células adherentes o estromales envían señales de sobrevida a los linfocitos B y forman parte de este ambiente celular.

En LLC el compartimiento proliferativo se encuentra representado en agregados locales de prolinfocitos y parainmunoblastos proliferantes que forman los pseudofolículos o centros proliferantes (CPs)(Lennert et al, 1978). Los pseudo-folículos son estructuras histológicas claves en los nódulos linfáticos, pulpa blanca del bazo y médula ósea donde se encuentran infiltrados de células B de LLC. La interacción de estas células con las células T, células estromales o dendríticas

foliculares activan cascadas de señalización que permiten la expresión marcadores de proliferación (Ki-67) y de progresión de la enfermedad (CD38 y CD49d) (Gattei et al, 2008; Patten et al, 2008).

2.3 ROL DE LAS CÉLULAS T, DE LAS CÉLULAS ESTROMALES Y DE LAS CÉLULAS DENDRÍTICAS DIFERENCIADAS ("NURSE LIKE CELLS") EN LA ACTIVACIÓN DEL CLON LEUCÉMICO

2.3 *A* LAS CITOQUINAS EN EL "HOMING" DEL LINFOCITO B LEUCÉMICO

El patrón de infiltración en los tejidos puede ser variable en los diferentes pacientes de LLC. En general, la mayoría de las células malignas se encuentran en SP y médula ósea, aunque algunos pacientes presentan también los nódulos linfáticos comprometidos. Esta observación clínica resalta la existencia de mecanismos que regulan la migración linfocitaria del clon tumoral. Las quimioquinas, moléculas quimioatrayentes, y sus receptores forman parte del mecanismo de tráfico linfocitaria en el organismo. Recientemente se ha determinado que las células de LLC expresan un grupo específico de quimioquinas y receptores de quimioquinas en respuesta a señales del microambiente (Burger, 2010). Las quimioquinas han sido clasificadas en dos grandes grupos: 1- quimioquinas constitutivas u homeostáticas, regulan la migración y el "homing" bajo condiciones fisiológicas y 2- quimioquinas inducibles, son aquellas que se expresan durante la inflamación en presencia de factores de crecimiento o citoquinas proinflamatorias. Debido a esas funciones, las quimioquinas han sido asociadas a una variedad de procesos patológicos. En particular, durante la tumorogénesis, las quimioquinas contribuyen a la infiltración de células hematopoyéticas a los tejidos así como a la neovascularización, contribuyendo con la distribución del tumor (Burger, 2010).

El tráfico de linfocitos entre la sangre y los órganos linfoides secundarios se encuentra organizado mediante la expresión específica quimioquinas y sus receptores. La distribución de las células B en los nódulos linfáticos estádeterminada por la expresión de CXCR4 y CXCR5 receptores de las quimioquinas CXCL12 y CXCL13 expresadas por las células estromales. A su vez, las células estromales de la médula ósea secretan CXCL9, 10, 11, 12 y 13 y CCL19 y 21 que se unen a los receptores diferentemente expresados CXCR3, CXCR4 y CXCR5 (Davids & Burger, 2012). Las

quimioquinas entonces juegan un rol importante en lo que respecta a la sobrevida dado que favorece la migración de los linfocitos de sangre a los tejidos linfoides permitiendo que reciban las señales de sobrevida y proliferación.

La importancia de las quimioquinas en LLC en relación a la sobrevida del clon tumoral también se ha determinado en el trabajo de Zucchetto y col. donde realizaron un estudio del perfil de expresión diferencial entre células B de LLC $CD38^{pos}$/$CD49d^{pos}$y $CD38^{neg}$/$CD49d^{neg}$. Los resultados mostraron sobre-expresión de las quimioquinas CCL3 y CCL4 en células leucémicas $CD38^{pos}$/$CD49d^{pos}$ (Zucchetto, Benedetti et al. 2009). Los receptores CXCR1 y CXCR5, receptores de CCL3 y CCL4 respectivamente, se encontraron altamente expresados en monocitos derivados de pacientes de LLC. En este trabajo determinaron que CCL3 induce la migración de los monocitos e infiltración de los macrófagos en médula ósea de pacientes de LLC $CD38^{pos}$/$CD49d^{pos}$. A partir de estos resultados los autores propusieron que las quimioquinas CCL3 y CCL4 producidas por células B de LLC son reconocidas por receptores de monocitos/macrófagos y reclutados al microambiente tumoral (Zucchetto et al, 2009). Resaltando la importancia de estas quimioquinas en la progresión tumoral, Sivina y col. han propuesto a CCL3 como un nuevo marcador pronóstico, sugiriendo que su evaluación podría ser útil para conocer la evolución clínica de pacientes de LLC (Sivina et al, 2011).

En las siguientes secciones analizaremos el rol de las células que interaccionan con el clon tumoral leucémico llevando a la progresión de la enfermedad.

2.3 *B* ROL DE LAS CÉLULAS T EN EL MICROAMBIENTE LEUCÉMICO

Es importante destacar que las células T juegan un rol importante en la inhibición de la apoptosis y progresión de la enfermedad de las células B malignas (Ghia et al, 2008). Dichos trabajos sugieren la existencia de un diálogo bidireccional entre las células B de LLC y las células T $CD4^{pos}$ mediado por moléculas de adhesión y quimioquinas que inducen la expresión de varias citoquinas en ambos tipos de células (revisado en (Caligaris-Cappio, 2003)). Las células T expresan interleuquina 4 (IL4), interferón γ (INFγ) e interleuquina 2 (IL2) capas de inhibir la apoptosis de las células B de LLC induciendo la expresión de la proteína anti-apoptótica Bcl2 (Caligaris-Cappio, 2003).

La unión de CD40-CD40L desencadena una vía de traducción de señales que permite la activación de factores de transcripción NFkB/Rel, así como ciertas vías de señalización JAK-STAT formadas por

quinasas Janus (JAK3) y las proteínas traductores de señales y activadores de la transcripción (STAT3). La vía JAK3-STAT3 induce la expresión de altos niveles de proteínas anti-apoptóticas como BCL-XL y MCL1(de Totero et al, 2006), mientras que la activación de NFkB permite la activación de TP63 que a través de la proteína CD49d facilita la migración de las células B leucémicas (Shachar & Haran, 2011). Además de la activación de estas vías, la interacción CD40L-CD40 puede inducir la expresión de proteínas inhibidoras de la apoptosis como es la survivina, un miembro de la familia de proteínas inhibidoras de la apoptosis (IAP). Esta proteína es la única IAP cuya expresión se encuentra regulada por la vía CD40-CD40L. Un trabajo de Granziero y col. mostró que células B de LLC estimuladas con CD40L *in vitro* eran capaces de inducir la expresión de survivina, mostrando que las células survivina positivas también son Bcl2 positivas y presentan un incremento en la sobrevida celular (Granziero et al, 2001).

A pesar de la existencia de datos mostrando la importancia de las señales recibidas a través de la vía CD40L en el clon leucémico, resultados del grupo de Gribben sugieren la existencia de linfocitos T defectuosos en pacientes con leucemia. Análisis de la expresión génica de linfocitos T de pacientes con LLC muestran importantes defectos funcionales a pesar de que se ha evidenciado un aumento del número absoluto de linfocitos T en sangre. La células T de pacientes con LLC presentan una activación crónica dada por la sobre-expresión de CD69, HLA-DR, CD57, una baja expresión de CD28 y CD62L y una expansión oligoclonal que en principio está restringida a la población activada $CD57^{pos}$ (Burger & Gribben, 2013). Además, presentan alteraciones en varios genes relacionados a la formación del citoesqueleto que pueden interferir directamente con la formación de la sinapsis inmunológica (Gorgun et al, 2005). Un dato de interés a resaltar es que dichos cambios han sido observados en células T de donantes sanos co-cultivadas con células de LLC, lo cual indica que las modificaciones estarían inducidas por las propias células leucémicas.

2.3 *C* ROL DE LAS CÉLULAS ESTROMALES EN EL MICROAMBIENTE LEUCÉMICO

Las células T no son las únicas responsables de colaborar con la sobrevida de los linfocitos B leucémicos, sino que señales de distintos tipos de células adherentes accesorias ayudan a la sobrevida del clon. Se ha demostrado que un contacto directo entre las células estromales de la médula ósea y las células leucémicas aumenta la sobrevida de las células de LLC(Lagneaux et al,

1998). Mediante estudios *in vitro* se determinó que las células B de LLC se encuentran en contacto con las células estromales de la médula ósea vía integrinas (β1 y β2) (Lagneaux et al, 1998). Esta unión permite el rescate de la apoptosis y extiende la vida media de las células B, sugiriendo que un mecanismo potencial de sobrevida y acumulación de células B *in vivo* podría ser mediado por esta interacción.

Además de ello las células adherentes de tipo ¨nurse like cells¨ (NLC) de los pacientes de LLC pueden enviar señales de sobrevida a las células leucémicas. Se ha determinado como a partir de células de SP de pacientes con LLC pueden diferenciarse *in vitro* en células NLC que presentan la capacidad de secretar el factor 1 derivado de células estromales (SDF1 o CXCL12) y proteger a las células de una apoptosis espontánea (Burger et al, 2000). Además de ello las células NLC son capaces de secretar el factor de necrosis tumoral APRIL ("a proliferation inducing ligand", APRIL) así como también BAFF ("B-cell activation factor", BAFF). Ambos ligandos tienen la capacidad de proteger al clon leucémico de la apoptosis (Cols et al, 2012). Las células estromales y las NLC tienen una actividad anti-apoptótica y la combinación de estas dos tipos de células generan un ambiente celular que contribuye a la sobrevida de las células leucémicas de LLC (Ramsay & Rodriguez-Justo, 2013).

2.3 *D* OTRAS SEÑALES RECIBIDAS POR EL CLON LEUCÉMICO

Estudios recientes muestran que no sólo las señales enviadas por las células estromales y células T parecen ser esenciales para el dialogo entre el microambiente y las células leucémicas. En este sentido, un trabajo de Seiffert y col. mostraron que los monocitos en presencia de células B de LLC incrementan la expresión de CD14 soluble. Esta molécula se encuentra directamente relacionada con la inducción de la activación del NFkB de las células B de LLC favoreciendo la sobrevida y proliferación (Seiffert et al, 2010).

Las células leucémicas no sólo están expuestas a señales de las células accesorias no malignas en los órganos linfoides, sino que son capaces e censar la presencia de patógenos mediante receptores de patrones moleculares asociados a patógenos. Los "Toll like receptors" o TLRs 7 y 9 son capaces de reconocer simple hebra de ARN o ADN respectivamente. En este sentido Decker y col. describieron que estímulos *in vitro* de células B de LLC con análogos de ADN bacteriano (CpG oligonucleótidos)

inducen la expresión de proteínas que regulan el ciclo celular como la ciclina D2, D3 y baja la expresión del regulador negativo del ciclo celular, p27 (Decker et al, 2002).

Toda la información detallada anteriormente propone la presencia de una conexión entre factores de microambiente y el dilema de la proliferación y apoptosis de las células B de LLC. En la actualidad la mayor parte de los trabajos en el área muestran a esta leucemia como una enfermedad hematológica maligna cuya evolución es totalmente dependiente del microambiente. Esta idea va de la mano con un modelo de heterogeneidad de la LLC en donde existe una sobrevida selectiva de ciertos sub-miembros clonales los cuales podrían recibir señales de sobrevida en estos ambientes especializados de los órganos linfoides.

3.0 Poblaciones proliferantes en la LLC y su asociación con la progresión tumoral

3.1 El linfocito B leucémico puede proliferar

La importancia del microambiente inmunológico en generar señales que contribuyen a la sobrevida y proliferación de las células B leucémicas permitió postular una interesante hipótesis en la cual se contempló la existencia de dos tipos de poblaciones dentro del clon tumoral de un paciente con LLC. La primera, es la población mayoritaria, con características de una célula B quiescente y no proliferante que lleva a la acumulación linfocitaria típica de esta leucemia. La segunda, claramente minoritaria (0,1 a 1% aproximadamente) en donde se mantienen interacciones con el microambiente celular que determinan una alta proliferación celular así como la sobre-expresión de numerosos marcadores moleculares de agresividad tumoral (Burger et al, 2009a; Caligaris-Cappio, 2003; Deaglio & Malavasi, 2009).

Esta hipótesis fue confirmada cuando Messmer y col. realizando marcados radioactivos de las células tumorales*in vivo* mostraron que los linfocitos B de LLC eran capaces de multiplicarse y morir. Estos resultados sugieren por primera vez en la historia de la biología de la LLC, que esta leucemia no es solo una enfermedad estática resultante de la acumulación de linfocitos B, sino también una

enfermedad dinámica en donde el equilibrio de estas dos poblaciones pueden definir la evolución tumoral (Messmer et al, 2005). Apoyando esta hipótesis, los resultados muestran que existe una correlación entre la tasa de nacimiento y muerte celular con la actividad y progresión de la enfermedad. Por lo tanto, al presente se propone que estudiar la tasa de división y muerte celular puede ayudar a identificar pacientes con una LLC más agresiva (Chiorazzi et al, 2005).

Al día de hoy, diferentes grupos de investigación han estudiando distintas sub-poblaciones proliferantes relacionadas con la progresión y el mal pronóstico. En el 2007, Damle y col. proponen que el sub-grupo de células B de LLC expresando CD38y el marcador de proliferación Ki-67 es una sub-población proliferante dentro del clon tumoral de LLC y está asociada a un mal pronóstico (Damle et al, 2007). En el marco de esta tesis de Doctorado, se describe una segunda población proliferante correspondiente a un sub-grupo de células B que con un activo proceso de cambio de clase (CC) sobre-expresan la enzima citidina deaminasa inducida por activación (AID, por "Activation Induced Cytidine Deaminase"). Esta sub-población tumoral es encontrada principalmente en pacientes NM, con una alta progresión de la enfermedad (Palacios et al, 2010). Más recientemente, Calissano y col. propone un modelo donde la sub-población tumoral proliferante expresa altos niveles de CD5 y baja expresión del receptor de quimioquinas CXCR4. Esta expresión diferencial colabora con el "homing" de linfocitos B hacia el nódulo linfático y explica como las células proliferantes reciben sus señales de sobrevida (Calissano et al, 2011).

3.2 Distintas poblaciones proliferantes dentro del clon tumoral y su asociación con la progresión de la LLC

3.2 *A* Población proliferante $CD38^{POS}$ en células B de LLC

La expresión de CD38 y su asociación con la evolución de la enfermedad han sido intensamente estudiadas durante el correr de los años. Se ha demostrado que células B $CD38^{POS}$ responden mejor a las señales del BCR y se caracterizan por una mejor migración. Estudios *in vitro* en el mismo sentido muestran que una activación de CD38 lleva a la proliferación y quimiotaxis de células B de LLC a través de ZAP70 y la vía de señalización ERK1/2. La interacción de CD38 con su receptor CD31 *in vivo*, tiene un rol importante en la interacción célula-célula activando señales de sobrevida tanto en células normales como leucémicas (Malavasi et al, 2008). Con el propósito de profundizar en el área Pepper y col. extendieron estas observaciones comparando el perfil de expresión génica de

células B de LLC $CD38^{pos}$ con $CD38^{neg}$ de cada paciente. Los resultados mostraron que existe un perfil de expresión diferencial entre ambas poblaciones $CD38^{pos}$ vs $CD38^{neg}$. Mediante este estudio determinaron que las células $CD38^{pos}$ sobre-expresan el factor de crecimiento endotelial (VEGF), el cual se encuentra directamente asociado con un incremento de la expresión de la proteína anti-apoptótica Mcl1 (Pepper et al, 2007). A su vez, estudios exhaustivos de la población de linfocitos $CD38^{pos}$ mostraron una asociación entre la expresión de CD38 con un incremento del porcentaje de células Ki-67 y ZAP70 positivas. Estos resultados sugirieron que los miembros del clon $CD38^{pos}$ se encuentran más activados y propensos a entrar al ciclo celular en comparación con su contrapartida $CD38^{neg}$ (Pepper et al, 2007).

Sin embargo, estudios del mismo laboratorio fallaron en establecer una fuerte correlación entre el porcentaje de células B de LLC proliferantes $CD38^{pos}$ con la sobrevida y la progresión de la enfermedad (Calissano et al, 2009). Se determinó que el hecho de que existe un gran porcentaje de células $CD38^{pos}$ en pacientes de mal pronóstico NM, indica que las células leucémicas $CD38^{pos}$ constituyen una población heterogénea. Donde una pequeña fracción de estas células $CD38^{pos}$ presentan un incremento en el potencial proliferativo. A su vez, recordando los resultados de Messmer donde demostró que las células leucémicas proliferantes constituyen entre 0,08% a 1,7%del clon tumoral (Messmer et al, 2005), se sugiere que no todas las células $CD38^{pos}$ son las proliferantes.

3.2 *B* Población proliferante AID^{POS} y un proceso activo de CC en células B de LLC

Debido a que parte de esta tesis está enmarcada en la caracterización y descripción de esta población proliferante este ítem será desarrollado e ilustrado más adelante en la sección de Resultados de la tesis.

3.2 *C* Población proliferante $CXCR4^{LOW}CD5^{HIGH}$ en células B de LLC

Para estudiar la complejidad dentro del tumor en LLC, Calissano y col. midieron la cinética de división celular *in vivo* del linfocito B de LLC mediante el uso de marcado de ADN con deuterio ($_2H$) (Calissano et al, 2011). Seleccionaron clones que presentaban diferente densidad de expresión de marcadores de superficie celular CD5 y moléculas relacionadas con señales de células estromales en los órganos linfoides secundarios como el receptor de quimioquina CXCR4. De esta manera definieron la fracción proliferativa a las células $CXCR4^{low}CD5^{high}$ y que contiene más cantidad de

deuterio, indicando que estas células fueron recientemente divididas. Por otro lado, a la fracción $CXCR4^{high}CD5^{low}$ y menor cantidad de $_2H$ la definieron como la fracción quiescente. A partir de ambas poblaciones asiladas estudiaron el perfil de expresión génica de nueve pacientes. Los resultados mostraron que la fracción proliferativa presenta una sobre-expresión de genes relacionados con la proliferación celular, anti-apoptosis y genes involucrados en el daño oxidativo a diferencia de la quiescente.

El estudio dentro del clon tumoral sugiere un modelo sobre la biología celular de la LLC. En este trabajo se propone que el clon leucémico contiene un espectro de células de la fracción proliferante enriquecida con células recientemente divididas que han emigrado del nódulo linfático. Mientras que la fracción quiescente, se encuentra enriquecida por células más viejas y menos vitales que necesitan migrar hacia los nódulos linfáticos para recibir señales de sobrevida o directamente morir (figura3) (Calissano et al, 2011). Este trabajo resalta una vez más la importancia del microambiente celular en mantener la sobrevida del clon tumoral, propone una interesante explicación a la heterogeneidad encontrada en la LLC.

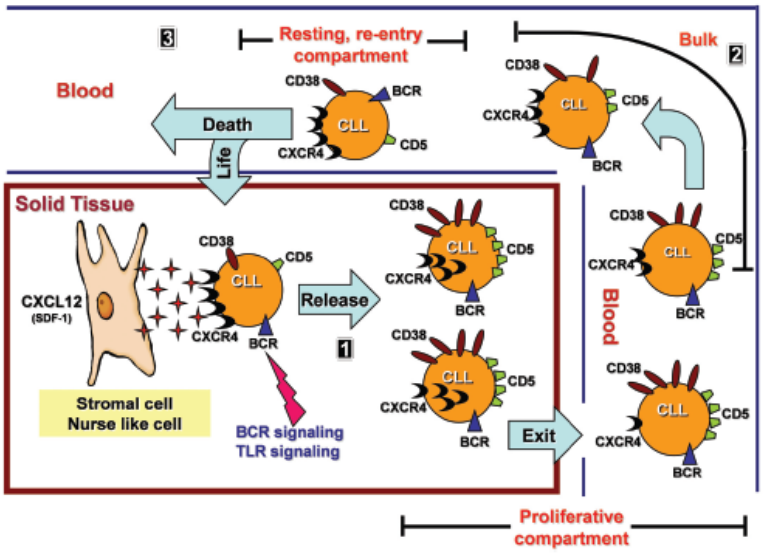

Figura3: Modelo hipotético del ciclo de vida de la célula B de LLC.(1) Las células B de LLC son activadas a través del BCR o TLRs en el microambiente tumoral dentro de los órganos linfoides secundarios (nódulo, bazo). Se dividen y aumentan la expresión de CD5, internalizan CXCR4 y salen del nódulo. La baja expresión de CXCR4 favorece a las células recientemente divididas ($CXCR4^{low}CD5^{high}$) a salir del tejido sólido y alcanzar la SP.**(2)** Las células recientemente divididas alcanzan la sangre como $CXCR4^{low}CD5^{high}$. Conforme pasa el tiempo, posiblemente debido a la ausencia de señales del microambiente inmunogénico, las células comienzan a re expresar el CXCR4 para volver a los órganos linfoides secundarios. Esto hace que podamos identificar un fenotipo con una expresión intermedia de ambos marcadores, $CXCR4^{int}CD5^{int}$ y luego linfocitos B $CXCR4^{high}CD5^{low}$.**(3)** Las células B de LLC que presentan $CXCR4^{high}CD5^{low}$ tienen una alta capacidad de detectar y reconocer un gradiente de CXCL12 y de esta manera re-entrar a los tejidos linfoides y recibir señales de sobrevida. Aquellas células que no son capaces de entrar al tejido linfoide mueren por cansancio (extraído de(Calissano et al, 2011)

4.0 La enzima AID ("Activation Induced Cytidine Deaminase") en el origen y la progresión de las neoplasias linfoides

4.1 Generalidades de la enzima AID

Durante el desarrollo de los linfocitos, ocurren importantes alteraciones a nivel del ADN genómico responsables de los procesos de diversificación génica. Estos procesos son imprescindibles para obtener una respuesta inmune eficaz. El primero de estos eventos es la Recombinación Génica (combinación al azar de los segmentos V-D-J), el mismo ocurre en la médula ósea y es el responsable de generar el repertorio pre-inmune (Sakano et al, 1980). Los otros dos eventos moleculares ocurren sólo en el linfocito B y tienen lugar en los órganos linfoides secundarios. La HS responsable de aumentar la afinidad por el Ag mejorando así su reconocimiento (maduración de la afinidad) y el cambio de clase o la conmutación isotípica (CC o CI), responsable de cambiar la región efectora de las Igs para proceder de esta manera al correcto procesamiento antigénico. Un gran avance en el área inmunológica en la última década unió a ambos procesos a través del descubrimiento de la molécula AID (Muramatsu et al, 1999). Esta enzima aislada de células B luego de un proceso de sustracción entre linfocitos con y sin activación fue descrita como la única molécula indispensable para la iniciación de la HS (Yoshikawa et al, 2002) y de la CC (Okazaki et al, 2002). Dicho descubrimiento permitió postular un origen común para ambos eventos ya que se demostraba por primera vez que la generación de mutaciones puntuales en la hebra de ADN en regiones específicas de los genes de Igs, eran llevados a cabo por la misma molécula. Además del rol mutagénico de AID implicando mutaciones puntuales en la hebra de ADN de genes de Igs, datos recientes proponen otras 2 funciones para esta enzima a saber: **1)** AID estaría implicada en la regulación génica a través de la desmetilación de islas CpG.(Agarwal & Daley, 2010; Popp et al, 2010), **2)** AID es requerida para el control de células B autorreactivas (Kuraoka et al, 2009; Meyers et al, 2011) jugando un papel fundamental en la tolerancia central y periférica (Peron et al, 2012).

La enzima AID se expresa en linfocitos B en CGs(Muramatsu et al, 1999) de los órganos linfoides secundarios, luego de una estimulación antigénica y colaboración de linfocitos T. Durante la reacción en los CG, los linfocitos B ciclan entre dos estados claramente distinguibles: centroblastos, sufren los procesos de HS y expresan grandes cantidades de AID, y los centrocitos en los cuales la expresiónAID es menor (Zan & Casali, 2013). Aunque la expresión de esta enzima en SP se

encuentra reprimida (Crouch et al, 2007), algunas células AID^{pos} pueden ser detectables fuera de los CGs, mayormente en linfocitos B interfoliculares (Moldenhauer et al, 2006).

Las señales que estimulan la expresión de AID en los linfocitos B gatillan la activación de factores de transcripción a nivel molecular como NFkB and STAT6 (Dedeoglu et al, 2004), HoxC4 (Park et al, 2009), Pax5 (Gonda et al, 2003; Oppezzo et al, 2005a), BCL6 and IRF8 (Basso et al, 2012).

La expresión de una enzima con capacidad mutagénica, como AID, debe estar sumamente regulada en la célula y esta regulación debe darse a distintos niveles de complejidad. En el caso de AID ha sido demostrado que existe una regulación negativa a nivel génico del linfocito B a través de los factores de transcripción IRF4, Blimp1 e ID2 (Lee-Theilen & Chaudhuri, 2010). Además de ello existen mecanismos de regulación a nivel transcripcional regulando la cantidad de ARNm de AID. En este caso los miRNA-155 y 181b son responsables de degradar el ARNm de esta enzima (de Yebenes et al, 2008; Teng et al, 2008). Finalmente un mecanismo post-traduccional involucrando la degradación de AID a través de la vía ubiquitina proteasoma ha sido descrita por Aoufouchi y col. Los autores muestran que la poli-ubiquitinación de la proteína en el núcleo marca a AID para su degradación por el proteasoma constituyendo un nivel adicional de regulación (Aoufouchi et al, 2008).

Otra manera de controlar el posible daño de AID sobre el genoma celular es el de la expresión de co-factores proteicos que dirijan a esta mutasa solo a sitios específicos sobre los genes de Igs. Los resultados recopilados al presente sugieren que la presencia de distintos co-factores serian los responsables de otorgarle a la AID esta especificidad. Al presente se han evidenciado varios de estos "partners" de AID, necesarios para dirigir específicamente esta enzima sobre el ADN que debe ser mutado. El primero de ellos es la proteína de replicación A (RPA), un cofactor de AID capaz de otorgar esta especificidad tanto hacia la región "pre-switch" como al locus variable de las Igs (Rada, 2009). Recientemente y además de RPA, el factor de "splicing" SRSF1 parece ser también necesario para dirigir el proceso especifico de HS(Kanehiro et al, 2012). Finalmente un reciente trabajo de Hasler et al., propone al factor de elongación eEF1A como esencial para mantener a la enzima AID en el citoplasma, evitando así una mutación general sobre el ADN (Hasler et al, 2011).

En este sentido es también importante establecer que la localización sub-celular de AID constituye otro de los mecanismos de regulación de la función de esta enzima. En dondeuna buena estrategia para limitar su actividad es regulando su presencia en el núcleo. En este sentido se han

determinado que el pasaje de AID del citoplasma al núcleo, del núcleo al citoplasma junto con la retención de AID en el citoplasma y la desestabilización de la enzima dentro del núcleo constituyen mecanismos de la regulación de la funcionalidad de AID (Aoufouchi et al, 2008; Patenaude et al, 2009). En particular se ha determinado que AID presenta una región de exportación nuclear ("nuclear export signal", NES) que es reconocida por un receptor soluble llamado "receptor chromosome region maintenance/exportin 1" (CRM1), el cual colabora con el transporte de AID desde el núcleo al citoplasma. La inhibición del receptor mostró una acumulación de AID en el núcleo demostrando de esta manera AID es una proteína que presenta un transporte dinámico de ida y vuelta entre el núcleo y el citoplasma (McBride et al, 2004).Por otro lado también y a pesar de ser una proteína pequeña, más pequeña que el poro nuclear, se observó que el pasaje de de AID desde el citoplasma hacia el núcleo requiere de un transporte activo (McBride et al, 2004).Más aún, Hasler y col. sugieren que la difusión pasiva se encuentra al menos en parte limitada por el factor eEF1A. En este trabajo se determinó que eEF1A forma un complejo con AID citoplásmica y podría de esta manera favorecer la retención de AID en el citoplasma, a pesar de que esto aún no ha sido del todo bien demostrado (Hasler et al, 2011).A su vez, en relación a la retención de AID en el citoplasma, Orthwein y col. mostraron que existe una interacción física y funcional entre AID y la chaperona HSP90 ("heat shock protein 90 kD"). Se cree que su unión colabora con la estabilidad y modula su función. De hecho su interacción previene la degradación proteosomal y por lo tanto determina niveles estables de AID funcional (Orthwein et al, 2010).

La perdida de algunos o varios de estos mecanismos de control en cualquiera de los diferentes niveles de expresión de AID, podrían en un determinado ambiente celular, convertir a esta enzima en un agente mutágeno activo (Okazaki et al, 2003). En consecuencia con esta hipótesis la enzima AID ha sido relacionada con el origen de muchos síndromes linfoproliferativos (Okazaki et al, 2003; Perez-Duran et al, 2007; Ramiro et al, 2006).

4.2 La acción mutagénica de la enzima AID y su asociación con el desarrollo neoplásico.

La mayor parte de las neoplasias linfocitarias son originadas a partir de translocaciones cromosómicas que pueden activar distintos oncogenes, reuniendo uno de los loci de Igs con un proto-oncogen, como es el caso de c-myc/IgH en los linfomas de Burkitt, BCL2/IgH en linfomas no Hodkin´s o BCL6 en los linfomas difusos de células B (Perez-Duran et al, 2007). Debido a la acción mutagénica de AID, una expresión aberrante ha sido asociada a múltiples enfermedades, como alergia, inflamación, autoinmunidad (Zan & Casali, 2013) y a la generación de síndromes linfoproliferativos (Okazaki et al, 2003; Perez-Duran et al, 2007; Ramiro et al, 2006). La sobreexpresión de AID ha sido implicada directamente con la generación de linfomas a través de modelos transgénicos (Okazaki et al, 2003), mientras que se la ha relacionando también con la presencia de translocaciones especificas (Duquette et al, 2005).

Datos *invivo* en un modelo de hyperplasia linfocitaria, muestran que en ausencia de AID, no existen translocacion del proto-oncogen c-myc (Duquette et al, 2005). El mecanismo molecular por el cual AID lleva a la transformación maligna no está del todo claro. Sin embargo, recientes trabajos del grupo de M. Nussenzweig y de F. Alt, utilizando la identificación masiva de translocaciones en el genoma, muestran que además de las mutaciones puntuales, AID es capaz de generar cortes dobles en la hebra de ADN sobre otros genes que no son de Igs, con la consecuente translocación cromosómica (Chiarle et al, 2011; Klein et al, 2011).

4.3 Implicancias de AID en la LLC

A diferencia de lo que ocurre con los linfocitos B normales, donde la expresión de AID se encuentra restringida en los órganos linfoides secundarios luego de una estimulación antigénica, nuestro grupo describió la expresión de AID en muestras de SP de pacientes NM de mal pronóstico (Oppezzo et al, 2005a; Oppezzo et al, 2003).

La caracterización clínica y molecular de estos pacientes permitió observar la presencia de un clon tumoral minoritario con un activo proceso de CC a IgG y/o IgA y correlacionar la presencia de este proceso con la expresión anómala de AID en SP. Estudios adicionales mostraron que esta expresión de AID anormal, era funcional ya que se encontraron mutaciones típicas del proceso de deaminación por AID en el ADN de la región pre-switch de estas células. Además de ello la activación *in vitro* de estas células con CD40L + IL4 resultó también en la presencia de mutaciones puntuales en la región pre-switch Sμ, junto con el correspondiente CC a IgG, a pesar de que el proceso de HS seguía bloqueado. A partir de estos resultados se propone que AID podría actuar en forma diferencial para ambos procesos probablemente a través de la presencia de co-factores específicos, aún desconocidos para ambos eventos (Oppezzo et al, 2003). Estudios adicionales fueron dirigidos a entender la regulación de la expresión de AID en pacientes de LLC que sobre-expresan la proteína. Estos estudios permitieron describir uno de los posibles mecanismos de control en la expresión donde se demostró la presencia de un "splicing" alternativo del factor de trascripción Pax-5, cuya traducción genera una proteína capaz de unirse al promotor de AID y que posiblemente, a través de un mecanismo de competición con la molécula entera del gen Pax-5 regula negativamente la expresión de AID (Oppezzo et al, 2005a).

La evidencia de la expresión anómala de AID en pacientes NM de mal pronóstico fue fortalecida por los estudios de Heintel y col., donde los autores determinaron que existe una relación entre la expresión de AID con la progresión de la enfermedad y por esta razón es que han propuesto el estudio de la expresión de AID como marcador pronóstico para la LLC (Heintel et al, 2004).

Por otro lado, Leuenberger y col. observaron una asociación significativa entre la expresión de AID con un pronóstico clínico desfavorable y la presencia de translocaciones cromosómicas involucrando las deleción p53 y ATM en LLC y SLL (Leuenberger et al, 2010). Estas observaciones también apoyan la hipótesis de que la expresión de AID en LLC está fuertemente relacionada con a un curso agresivo de la enfermedad. Tomando en cuenta que ATM y p53 juegan un papel clave en la reparación del daño en el ADN, incluso del daño del ADN iniciado por AID, la ausencia de estas moléculas podrían favorecer la evolución clonal dirigida por AID. De hecho, se ha sugerido que mutaciones en TP53 podrían estar relacionadas con la expresión de AID en LLC (Malcikova et al, 2008).

Más recientemente nuevas evidencias han sido descritas en relación a la expresión de AID y su asociación con las aberraciones cromosómicas en células B de LLC (Patten et al, 2012). En particular,

Patten y col. determinaron que la expresión de AID se encuentra restringida a un subgrupo de células que se están dividiendo en pacientes con LLC. A su vez, observaron que la enzima AID expresada en células B activadas con CD40L+IL4 es completamente funcional. Por lo tanto, dado que los pacientes que expresan más AID presentan más alteraciones cromosómicas y una peor evolución clínica, este trabajo refuerza la idea de que la producción de AID en células B de pacientes de LLC podría estar implicada en la evolución clonal de la enfermedad (Patten et al, 2012).

En este momento más estudios son necesarios para directamente correlacionar AID con algún rol etiológico en las aberraciones genómicas de la LLC. Si estas presunciones son verdaderas, la expresión constitutiva de AID en la historia del clon leucémico podría ser un evento clave en la progresión de la enfermedad de esta leucemia y un blanco potencial para prevenir la progresión de la enfermedad.

Los aportes de esta tesis de Doctorado, están centrados en aquellos pacientes de LLC NM que presentan la existencia de una sub-población clonal leucémica con un proceso activo de CC. Como fue descrito que estos pacientes expresan altos niveles de la enzima AID en SP (Oppezzo et al, 2003), que la sobre-expresión de AID podría ser responsable de la progresión tumoral (Perez-Duran et al, 2007), y que la expresión de AID es típica de un linfocito B activado en el CG, este trabajo plantea e intenta responder dos preguntas fundamentales en el área

¿La presencia de una sub-población tumoral con un proceso activo de CC en SP es un ejemplo de sub-poblaciones proliferantes en el seno del clon leucémico?

¿La expresión de la enzima AID en SP de pacientes NM es un indicio de progresión leucémica y evolución clonal?

OBJETIVOS

Hoy en día se encuentra aceptado entre los investigadores que el dialogo entre las células accesorias especializadas y los linfocitos B de LLC leucémicos favorece la progresión de la enfermedad promoviendo el crecimiento de células B malignas (Burger et al, 2009a). Por lo tanto

comprender ese diálogo nos puede dar nuevas claves sobre la biología celular y molecular de la LLC con el propósito de diseñar nuevas estrategias terapéuticas. En este sentido aislar el subgrupo de células B tumorales que han recibido recientemente señales del microambiente activado en los compartimentos proliferantes de los pacientes de LLC progresores es importante para entender la patogénesis de la LLC.

El caso particular de estos pacientes NM de LLC sobre-expresando la enzima mutagénica AID, nos ha llevado a postular diferentes objetivos que intentan por una parte, obtener un mayor conocimiento en el área de la biología del linfocito B leucémico, y por otra recolectar información sobre las interacciones que la célula tumoral es capaz de mantener en un microambiente especializado y que le otorga características de proliferación y progresión tumoral.

En este sentido es que nos planteamos el siguiente objetivo general:

Caracterización molecular de una población proliferante dentro del clon tumoral leucémico y su asociación con la progresión clínica en pacientes con Leucemia Linfoide Crónica

OBJETIVOS ESPECÍFICOS

1. **Estudios moleculares de una sub-población leucémica con un proceso activo de conmutación isotipica.**
2. **Aislamiento y caracterización molecular de la sub-población leucémica encontrada en pacientes con mala evolución tumoral.**
3. **Estudios genómicos identificando el perfil de expresión de ARNs mensajeros de diferentes sub-poblaciones leucémicas**
4. **Estudios genómicos identificando el perfil de expresión de microARNs de diferentes sub-poblaciones leucémicas**
5. **Análisis bioinformáticos de los estudios genómicos y selección de posibles blancos terapéuticos identificados en la población leucémica proliferante**
6. **Análisis moleculares y funcionales de las diferentes moléculas seleccionadas como blancos terapéuticos en la LLC**

RESULTADOS

"High expression of AID and active Class Switch Recombination might accounts for a more aggressive disease in unmutated CLL patients: Link with an activated microenvironment in CLL disease"

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La LLC es una hemopatía definida por la acumulación de linfocitos B clonales en sangre, médula ósea y órganos linfoides. Donde la vida media de las células B se ve prolongada por un defecto en la apoptosis celular que lleva a la acumulación de linfocitos B maduros y de tamaño pequeño en SP. Sin embargo, esta visión tradicional fue puesta en duda cuando trabajos del grupo de Chiorazzi mostraron que los linfocitos B de LLC eran capaces de proliferar y morir, sugiriendo que la LLC es una enfermedad dinámica (Messmer et al, 2005). Lo cierto es que la mayoría de los eventos proliferativos ocurren en los órganos linfoides secundarios, donde las células B pueden estar en estrecho contacto con células del microambiente tumoral recibiendo señales de sobrevida y proliferación (Caligaris-Cappio, 2003). En estos CG los linfocitos B son capaces de activarse mediante señales CD40-CD40L de los linfocitos T e inducir la expresión de AID. Debido a que datos previos del grupo de trabajo sugieren una asociación entre la expresión de AID y la ocurrencia de CC en pacientes con alta progresión leucémica (Oppezzo et al, 2003) nos planteamos estudiar la asociación que podría existir entre la expresión de AID, el proceso de CC y la activación por el microambiente inmunogénico en estos pacientes.

En este trabajo pudimos determinar que la expresión de AID se encuentra mayormente restringida a la sub-población de linfocitos B con un activo proceso de CC ($IgM^{pos}IgG^{pos}$ e IgG^{pos}) en pacientes NM y de mal pronóstico. Interesantemente observamos que la sub-población de linfocitos B AID^{pos} sobre-expresa moléculas relacionadas con la proliferación y anti-apoptosis. En particular determinamos que existe una sobre-expresión del Ag nuclear Ki-67, del oncogen c-myc y de la proteína anti-apoptótica Bcl2, así como una disminución del regulador negativo del ciclo celular p27, comparadas con su contrapartida IgM^{pos}. Estos resultados constituyen una evidencia de que las células presentan características de células activadas proliferantes. Además, observamos que el subset AID^{pos} expresa moléculas relacionadas con la interacción con el microambiente en CPs y migración de linfocitos como CD49d y las quimioquinas CCL3 y CCL4. Como ya se ha referido anteriormente, la integrina CD49d es un marcador de mal pronóstico de la LLC que participa en la interacción con células estromales en el microambiente tumoral desencadenando señales de sobrevida y proliferación. A su vez, la expresión de CCL3/4 por parte de los linfocitos B de LLC permiten el reclutamiento de monocitos a los tejidos linfoides favoreciendo así las señales de sobrevida del clon tumoral (Zucchetto et al, 2009). Finalmente observamos que pacientes que presentan esta sub-población con un activo proceso de CC y una alta expresión de AID se asocia con una evolución más agresiva de la enfermedad.

Éstos resultados nos han llevado a cuestionarnos sobre cuál es el rol de la sub-población AID^{pos} con características célula B activada proliferante en pacientes de LLC. Un dato interesante a destacar es que sibien hemos observado que dichas células presentan inhibida la muerte celular, nosotros esperaríamos que con el correr del tiempo todo el clon tumoral fuera IgG y esto en los hechos no ocurre. Tomando en cuenta que existe una baja frecuencia de pacientes cuyo clon tumoral no es IgM y que estos pacientes en general no presentan mal pronóstico, podríamos sugerir que ésta sub-población de linfocitos B previamente recibió señales de sobrevida en CG, realizaron el CC y migraron a SP donde probablemente si no retornan al CG terminen muriendo con el transcurso del tiempo.

La presencia de un CC activo en SP de pacientes con LLC fue previamente descrita por Fais y col (Fais et al, 1996) pero su asociación con un mal pronóstico fue sugerida por Oppezzo y col. (Oppezzo et al, 2005a) y confirmada en este trabajo (Palacios et al, 2010). Si bien es posible pensar que las células de isotipo IgG ya establecido se dirijan a una vía de apoptosis, la pregunta interesante a responder es ¿porqué la presencia de dicha sub-población celular se encuentra asociada a una progresión leucémica?. Será que, ¿la alta linfocitosis que presentan estos pacientes NM es la causa de encontrar una parte de la sub-población proliferante en SP?. En este caso el estudio de ganglios de dichos pacientes podría ayudar a contestar al menos en parte esta pregunta. Otro de los interrogantes que genera la descripción de esta sub-población tumoral expresando la enzima AID es, si dicha expresión es responsable de la mala evolución leucémica, o si es una consecuencia de la activación antigénica sin efectos importantes sobre la progresión del tumor.

En resumen, este trabajo agrega nuevas evidencias que apoyan la hipótesis de que la LLC no solo es una enfermedad acumulativa de linfocitos B quiescentes (Burger et al, 2009a; Chiorazzi, 2007) sino también una leucemia en la que existen linfocitos tumorales con una alta capacidad proliferante. Donde el equilibrio entre ambos compartimientos celulares, podría ser el responsable de la heterogeneidad clínica observada en esta leucemia así como del proceso evolutivo de la misma. Al día de hoy la demostración de esta hipótesis sigue siendo uno de los temas centrales en la biología de la LLC, no solo con el objetivo de entender la fisiopatología y el origen de la progresión leucémica, sino también con el propósito de identificar nuevos blancos terapéuticos. El poder caracterizar esta población proliferante en el seno del clon tumoralpermitiría actuar sobre posibles moléculas blancos específicas para con el linfocito B proliferante con el objetivo final de lograr una terapia efectiva para esta leucemia (Burger et al, 2009a).

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High expression of AID and active class switch recombination might account for a more aggressive disease in unmutated CLL patients: link with an activated microenvironment in CLL disease

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High expression of AID and active class switch recombination might account for a more aggressive disease in unmutated CLL patients: link with an activated microenvironment in CLL disease

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Interaction of chronic lymphocytic leukemia (CLL) B cells with tissue microenvironment has been suggested to favor disease progression by promoting malignant B-cell growth. Previous work has shown expression in peripheral blood (PB) of CLL B cells of activation-induced cytidine deaminase (AID) among CLL patients with an unmutated (UM) profile of immunoglobulin genes and with ongoing class switch recombination (CSR) process. Because AID expression results from interaction with activated tissue microenvironment, we speculated whether the small subset with ongoing CSR is responsible for high levels of AID expression and could be derived from this particular microenvironment. In this work, we quantified AID expression and ongoing CSR in PB of 50 CLL patients and characterized the expression of different molecules related to microenvironment interaction. Our results show that among UM patients (1) high AID expression is restricted to the subpopulation of tumoral cells ongoing CSR; (2) this small subset expresses high levels of proliferation, antiapoptotic and progression markers (Ki-67, c-*myc*, Bcl-2, CD49d, and CCL3/4 chemokines). Overall, this work outlines the importance of a cellular subset in PB of UM CLL patients with a poor clinical outcome, high AID levels, and ongoing CSR, whose presence might be a hallmark of a recent contact with the microenvironment. (*Blood*. 2010;115(22): 4488-4496)

Introduction

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease following a variable course with survival ranging from months to decades. One-third of patients never require treatment and have a long survival; in another third, an initial indolent phase is followed by disease progression and treatment requirement; the remaining third exhibits an aggressive disease at the onset and needs immediate treatment.[1] The Rai[2] and Binet[3] staging systems provided a foundation for the prognosis and design of therapeutic strategies. An unmutated (UM) profile of immunoglobulin (Ig) VH genes,[4,5] as well as the presence of genetic lesions at chromosome 17p13, or at 11q23,[6] constitute, to date, the poor prognosis indicators.[7] In addition, in the last years, CD38 and ζ-associated protein 70 (ZAP-70) expressions have shown important prognostic information.[8] However, neither Rai/Binet staging systems nor the molecular markers described to date are able to completely predict the progression of disease and/or explain the heterogeneous progression profile in the clinical course of CLL. One of the long-term goals of the hematologic community is to provide a molecular explanation for this marked clinical heterogeneity of CLL highlighted by the differential mutational profile.[9]

The traditional view has been that CLL is a disease deriving from an inherent defect in apoptosis, or programmed cell death in which, slowly proliferating B lymphocytes accumulate because of this diminished cell death. Increased expression of antiapoptotic Bcl-2 protein and blockade of tumoral CLL B cells in a G_0-G_1 phase support this observation. However, recent studies showed that CLL is a dynamic process which results from cells that proliferate and die, often at appreciable levels.[10] These observations have turned the attention toward the occurrence of different subpopulations inside the tumoral clone in which a homeostatic balance exists in patients with stable lymphocyte counts and good clinical course or an imbalance in patients with rising lymphocyte counts and poor outcome. It is clear that most, if not all, proliferative events occur in the tissues where leukemic cells are able to exploit microenvironment interactions to avoid apoptosis and to acquire tumoral growing conditions.[10]

Activation-induced cytidine deaminase (AID), a B cell–restricted enzyme, is principally induced through the contact of T and B cells via CD40-CD40 ligand (CD40L) interactions and is required for somatic hypermutation (HMS) and class switch recombination (CSR) process.[11] The mutational activity of AID identifies this enzyme as the first genome mutator in humans with oncogenic potential.[12] Supporting this view, different works report that constitutive AID expression is associated with a loss in the target specificity and with lymphoproliferative disorders.[12,13] Interestingly, in CLL disease we have described that AID is expressed in a percentage of patients with CLL with UM VH genes and with active CSR.[14] Despite expression of a functional AID as assessed by an active CSR and mutations induced in the preswitch μ region, CLL B cells in these patients did not succeed to achieve the process

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of somatic hypermutation.[14] Although clonal CSR has been described in CLL B cells long ago[15,16] and different works have shown that this process occurs principally in patients with UM disease,[14,17] the origin and the biologic implications of this subpopulation in the physiopathology of CLL remains elusive.

Inhibition of apoptosis may occur in vivo in pseudofollicles observed in the lymph nodes, and in the cell clusters described in the bone marrow. These pseudofollicles include in close contact with proliferating B cells increased numbers of CD4 T cells expressing CD40L, which is necessary for AID expression. These activated CD4 T cells could be recruited by tumor B cells through the expression of T cell–attracting chemokines such as CCL17 and CCL22[18] and/or CCL3 and CCL4.[19] Besides this, the CD38 and CD49d proteins appear to be important additional players interacting with nurselike, stromal, and endothelial cells to complete the activation pathway within the proliferative centers.[20] Overall, these observations favor the view that certain cellular subsets in CLL could receive survival signals in the specific microenvironments, increasing their proliferative potential and consequently associated with a more aggressive disease.

Because AID expression in CLL is associated with ongoing CSR in patients with UM disease, we investigated the relation of AID expression, CSR process, and microenvironment activation in peripheral blood (PB) of patients with CLL with different clinical profiles. In this work we examined whether the small subset of tumoral cells with ongoing CSR is responsible for AID expression and whether this subpopulation could have an increased activated and proliferative potential related to the progression of the disease. Our results show that high expression of AID is almost exclusively restricted to the subpopulation of tumoral B cells having an active CSR process. This subset expresses higher levels of proliferation and antiapoptotic molecules such as Ki-67, c-*myc*, and Bcl-2. In addition, present are high levels of CD49d and CCL3/CCL4 chemokines, as well as a decreased expression of cell cycle inhibitor $p27^{-kip1}$ compared with their quiescent counterpart IgM B cells. Finally, the presence of this subpopulation in patients with UM CLL is closely related to an aggressive course of the disease.

Methods

Patient samples

PB was obtained from 50 patients with a typical diagnosis of B-cell CLL (B-CLL), displaying, respectively an UM profile in 25 and a mutated (MUT) profile in 25 (Table 1). Among the patients with MUT disease with a median follow-up of 36 months (range, 6-240 months), 21 corresponded to stage A and 3 to stage C; 3 required treatment, and 2 among them died. With a median follow-up of 48 months (range, 3-108 months) there were 13 patients in stage A, 8 in stage B, and 4 in stage C in the UM group; 16 required treatment and 5 died. All patients were followed at the Hospital Maciel from Montevideo and provided an informed consent in accordance with the ethical regulations from Uruguay and the Declaration of Helsinki. The study was approved by the Institutional Review Board of Institut Pasteur Montivideo. The diagnosis of B-CLL relied on cytologic features of mature lymphocytes and a characteristic phenotype ($CD5^+$, $CD23^+$, low expression of CD79b, and of surface immunoglobulin).

Phenotypic and functional studies of B cells

Blood collection was carried out in a period close to the diagnostic time for each patient as indicated in Table 1. The peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals) and immediately cryopreserved in liquid nitrogen. Phenotypic analysis of leukemic cells was performed with anti-CD19 phycoerythrin (PE), anti-CD5 fluorescein-isothiocyanate (FITC), anti–human μ chains $F(ab')_2$ conjugated with PE, and anti–human γ chains $F(ab')_2$ conjugated with FITC. Negative controls were performed by incubating the cells with irrelevant $F\text{-}(ab')_2$ antibodies conjugated to PE or FITC. Forward and side scatters were used to gate out contaminating debris and the cells killed during the staining procedure. All antibodies were from Dako SA. Data were acquired, and analysis was performed with the use of a CyAn Flow Cytometer (Beckman Coulter).

Analysis of progression and proliferation markers by flow cytometry

PBMCs (1×10^6) obtained from patients with CLL were incubated for 30 minutes at 4°C with the antibody in phosphate-buffered saline buffer supplemented with 0.5% bovine serum albumin, washed twice, and analyzed by flow cytometry. Anti-CD49d conjugated with allophycocyanin antibody was obtained from BioLegend, and anti-CD38 conjugated with PE–Texas Red was obtained from Invitrogen. For intracellular detection of Bcl-2 and Ki-67 proteins, 1×10^6 PBMCs were fixed in phosphate-buffered saline 4% paraformaldehyde and permeabilized in the same buffer containing 0.5% saponin and 5% fetal bovine serum before the addition of the specific antibody (10 μg/mL). After 45 minutes of incubation at 4°C, with anti–human Ki-67 Alexa Fluor 647 or anti–human Bcl-2 Alexa Fluor 647 the cells were washed twice and analyzed by flow cytometry. Negative isotype controls were performed by incubating the cells with irrelevant antibody in the same experimental conditions. Anti–Ki-67, anti–Bcl-2, and negative isotype control conjugated to Alexa Fluor 647 were obtained from Santa Cruz Biotechnology Inc. In all cases, expression of these CLL progression markers (CD49d and CD38) as well the proliferation and antiapoptotic markers (Ki-67 and Bcl-2) were analyzed in the different CLL B subsets (IgM^+, IgG^+, and IgM^+/IgG^+) with the use of Summit v4.3 from Dako.

Cell-sorting studies

Sorting experiments of B-CLL cells were performed with the use of the MoFlo cell sorter (Beckman Coulter) with the same antibodies described earlier to isolate the following 3 different populations of CLL B cells: (1) IgM^+ subset expressing $CD19^+$, $CD5^+$, and IgM surface markers; (2) IgG^+ subset expressing $CD19^+$, $CD5^+$, and IgG membrane proteins; and (3) IgM^+/IgG^+ subset corresponding to cells $CD19^+$, $CD5^+$ markers and expressing simultaneously IgM and IgG. In all cases, purity of isolated subpopulations was shown to be greater than 98% before flow cytometric evaluation.

Extraction and analysis of RNA transcripts by reverse transcription–PCR

RNA from total CLL B cells was isolated from 1 to 5×10^6 cells, and cDNA synthesis was performed as described.[21] When RNA extraction was performed from isolated IgM^+, IgG^+, and IgM^+/IgG^+ subpopulations before cell sorter assays, the mirVana isolation kit (Applied Biosystems), rRNAsin, RNase inhibitor (Promega), and Superscript II reverse transcriptase (Invitrogen) were used to achieve a maximal performance in the mRNA extraction. Despite this care, the mRNA from the IgM^+/IgG^+ subset was only successful performed in CLL 01, whereas this could not be successfully achieved in the other CLL cases, given the low amounts of cells showing the double marking. Amplifications of circular transcripts (CTs), AID, c-*myc*, $p27^{-Kip1}$, and CCL3 and CCL4 chemokines were performed with an initial denaturation step at 95°C for 4 minutes, followed by 30 cycles of 1 minute at 95°C, 1 minute at 62°C, and 1 minute at 72°C and final elongation step at 72°C for 5 minutes. For gene expression analyses the used primers were as follows: for CTs (forward, 5′-GGC CCT TCC AGA TCT TTG AG-3′, and reverse, 5′-CTC TCA GGA CTG ATG GGA AGC CCC G-3′), for AID (forward, 5′-GAG GCA AGA AGA CAC TCT GG-3′, and reverse, 5′-CTA CTT CTG TGA GGA CCG C-3′), for c-*myc* (forward, 5′-CTT TGT GTG CCC CGC TCC AG-3′, and reverse, 5′-GCG CTC AGA TCC TGC AGG TA-3′), for $p27^{-Kip1}$ (forward, 5′-AGG TGC GAG TGT CTA ACG GG-3′, and reverse, 5′-GCG CAT TGC TCC GCT AAC CC-3′), for CCL3 (forward, 5′-GAC ACT CGA GCC CAC ATT CCG-3′, and reverse, 5′-CCCCTCAGGCACTCAGCTCC-3′), and for

CCL4 (forward, 5′-CCA CCA ATA CCA TGA AGC TCT G-3′, and reverse, 5′-CCT AAT ACA ATA ACA CGG CAC ATA A-3′). In the case of CTs the fragments obtained in polymerase chain reaction (PCR) amplification were transferred and hybridized with specific probes labeled for Cμ and glyceraldehyde phosphate dehydrogenase (GAPDH) α-[32P] deoxycytidine-5′-triphosphate to increase the signal response.

Quantitative real-time PCR

For gene expression analyses of AID, we used Corbette Rotor Gene 6000 Real-Time PCR and the SYBR Green I dye. Primers used in this study were the same that for the reverse transcription (RT)–PCR analysis. Total RNA (1 μg) was isolated from 5×10^6 B cells and retro-transcribed as described.[21] One microliter from a 20-μL cDNA reaction was used for AID and GAPDH amplification in a PCR reaction including 40 cycles of amplification (95°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds). Positive calibrator values were obtained from tonsil samples and from CD40L and interleukin-4 (IL-4)–activated CLL B cells. For this, kinetic studies of AID expression were made to evaluate the higher expression of mRNA before activation. Stimulation of CLL B cells was carried out in vitro by monolayer culturing of 1×10^6/mL fibroblast expressing recombinant soluble CD40L and IL-4 (1000 U/mL; PharMingen).

Statistical analyses

Expression of AID mRNA and Ki-67, Bcl-2, and CD49d proteins were compared between IgM^+, IgM^+/IgG^+, and IgG^+ subsets with the Mann-Whitney test. On finding significant differences across groups, a Bonferroni-like adjusted pairwise comparison was made to determine which groups differed from each another. The Spearman rank correlation coefficient was calculated to determine the strength of association between AID expression, percentage of clonal-related CSR, and progression-free survival (PFS). Variables with *P* values less than .05 were considered to be significant. Overall survival (OS) and PFS were calculated from Table 1 and used to perform the Kaplan-Meier method. All analyses were done with GraphPad Prism, Version 4.0 (GraphPad Software Inc).

Results

Analysis of AID expression in CLL B cells

To obtain a positive calibrator for the quantitative PCR we compared levels of AID expression between tonsil samples, Daudi cell line, and B-CLL stimulated through CD40L and IL-4. Taking into account results from Guikema et al[22] and our observations, we assumed that mRNA AID expression levels after CD40L/IL-4 activation are comparable to those of B cells in an activated microenvironment. In addition, values from PBMCs from 8 healthy donors were used as negative controls. In this context, we considered as having high AID expression ($AID^{+/+}$) those patients with CLL whose levels ranged between 80% and 100% of relative units of AID/GAPDH mRNA transcripts (Figure 1). Patients with an AID expression ranging between 40% and 80% and less than 40% of the positive control were considered as having low AID expression (AID^+) or negative (AID^{neg}), respectively (Figure 1). According to these criteria, we analyzed the mRNA AID expression in 50 patients with CLL (UM = 25; MUT = 25). Results show that 6 of the 25 patients with UM CLL corresponded to group $AID^{+/+}$, 14 to AID^+, and 5 to AID^{neg}. Interestingly, no $AID^{+/+}$ expression could be detected among patients with a MUT profile, 7 patients displayed lower expression of AID (AID^+), whereas no AID transcripts were found in the remaining 18 MUT CLLs. In summary, in our series, 80% of patients with UM disease express AID transcripts, whereas in MUT CLLs this only occurs in 28%. These results are close to those previously reported by Heintel et al.[23]

Analysis of CSR fraction cells in CLL

The in vivo plasticity of CLL is further underscored by reports showing that IgM^+ leukemic cells can give rise to clonally related IgG^+ or IgA^+ elements, possibly by ongoing in vivo CSR process in the proliferating centers.[21,24] However, the implication of this activation and of this tumoral subpopulation in the physiopathology of CLL remains elusive as yet.

To better characterize the tumoral subset with ongoing CSR we studied CLL B cells with specific human antibodies (anti-μ, anti-γ, anti-CD5, and anti-CD19) from 50 patients and analyzed the clonal identity for cells with ongoing CSR. Results show the presence of IgG^+ and IgG^+/IgM^+ subpopulations ranged from 0.5% to 8% in 44 of the 50 patents with CLL by cytometric assays (Table 1). In addition, we performed RT-PCR assays with

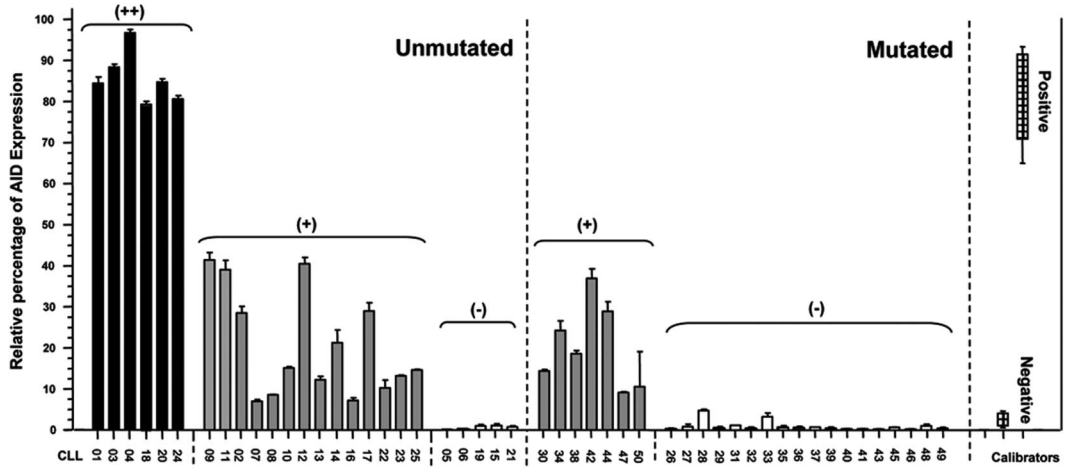

Figure 1. AID mRNA expression levels in patients with mutated and unmutated CLL. Quantitative PCR for AID and GAPDH as the endogenous control was performed on PBMCs from patients with MUT and UM CLL. CLL B cells stimulated with CD40L/IL-4, tonsil samples, and PBMCs from 8 healthy donors were used as positive and negative calibrators, respectively. By subtraction of the mean threshold cycle (Ct) triplicate AID measurements with the mean Ct from triplicate GAPDH measurements, the mean ΔCt was calculated. The ΔΔCt values were calculated with the mean ΔCt of the 8 healthy donors and the 3 independently experiments of CLL B cells activated with CD40L/IL-4 as calibrators. AID negativity was defined by the absence of AID expression in duplicate analysis. The expression factor difference and range were calculated by the following formulas: $2^{-\Delta\Delta Ct}$ (mean factor difference); $2^{-(\Delta\Delta Ct - \Delta Ct\ SD)}$ and $2^{-(\Delta\Delta Ct + \Delta Ct\ SD)}$ (error bars indicate range factor difference). The factor difference conversion of the ΔΔCt is depicted in the graph in relative percentages of AID expression.

Table 1. Clinical and molecular characterization of patients with CLL

CLL no.	Progression status	Binet stage	Lymphocyte count, $\times 10^3/\mu L$	CD38*	LPL	Diagnostic and blood collection	TFT, mo	A/D†	AID (Q-PCR)	Percentage of CSR‡	VDJ clonal identity	Mutational status§
1¶	Progressor	C	128.0	+	+	2000	9	D	+/+	8.2	Yes	UM
2‖	Progressor	B	80.0	+	+	1999	18	D	+	4.1	Yes	UM
3¶	Progressor	C	31.00	+	+	1998	6	D	+/+	5.4	Yes	UM
4¶	Progressor	B	105.00	+	+	2000	8	D	+/+	6.4	Yes	UM
5**	Indolent	A	20.00	+	+	1999	N/T	A	−	1.3	N/A	UM
6**	Progressor	B	32.50	−	+	2001	N/T	A	−	0.3	N/A	UM
7‖	Indolent	B	17.00	+	−	2000	N/T	A	+	2.7	Yes	UM
8‖	Progressor	A	70.00	−	−	2002	42	A	+	1.6	N/A	UM
9‖	Progressor	B	60.00	+	+	2005	12	A	+	4	Yes	UM
10‖	Indolent	A	10.00	+	+	1999	N/T	A	+	0.7	N/A	UM
11‖	Progressor	C	88.00	+	+	2001	15	A	+	3.8	Yes	UM
12‖	Progressor	B	54.00	+	+	2001	6	A	+	4.5	Yes	UM
13‖	Indolent	A	20.00	−	+	2005	54	A	+	1.3	No	UM
14‖	Indolent	A	7.00	+	+	2006	36	A	+	3.3	Yes	UM
15**	Indolent	A	20.40	+	+	2004	N/T	A	−	0.3	No	UM
16‖	Progressor	B	148.00	+	+	2005	3	A	+	1.4	No	UM
17‖	Progressor	A	54.50	−	+	2007	17	A	+	2.5	No	UM
18††	Indolent	A	15.00	+	+	2006	12	A	+/+	5.9	Yes	UM
19**	Indolent	A	11.00	+	+	2005	N/T	A	−	0.6	No	UM
20††	Progressor	C	220.00	+	+	2007	3	D	+/+	7.4	Yes	UM
21**	Indolent	A	8.90	+	−	2008	N/T	A	−	1.1	No	UM
22‖	Progressor	B	17.80	+	+	2008	11	A	+	3.5	Yes	UM
23‖	Indolent	A	28.00	−	+	2008	N/T	A	+	4.1	No	UM
24††	Progressor	A	80.00	+	+	2009	10	A	+/+	5.5	Yes	UM
25‖	Progressor	A	10.50	+	+	2005	N/T	A	+	1	N/A	UM
26**	Progressor	A	17.20	−	−	1999	N/T	A	−	0.9	N/A	Mut
27**	Progressor	A	14.00	−	−	2000	N/T	A	−	0.4	N/A	Mut
28**	Progressor	A	5.00	+	−	1989	N/T	A	−	0.6	No	Mut
29**	Progressor	A	11.60	+	−	2001	N/T	A	−	0.1	N/A	Mut
30‖	Progressor	A	18.00	−	−	1999	N/T	A	+	1.8	Yes	Mut
31**	Progressor	B	38.00	+	−	2000	N/T	D	−	0.5	N/A	Mut
32**	Indolent	A	47.60	−	−	2002	N/T	A	−	2.5	No	Mut
33**	Indolent	A	15.40	−	−	2006	N/T	A	−	0.8	No	Mut
34‖	Indolent	A	15.00	−	+	2007	N/T	A	+	0.5	N/A	Mut
35**	Indolent	A	10.20	−	+	2006	25	A	−	1.2	No	Mut
36**	Indolent	A	27.60	−	+	2007	N/T	A	−	0.4	N/A	Mut
37**	Indolent	A	15.00	−	−	2006	N/T	A	−	0.3	No	Mut
38‖	Indolent	A	12.80	−	+	2004	N/T	A	+	2.1	No	Mut
39**	Indolent	A	15.60	−	+	2006	N/T	A	−	0.5	No	Mut
40**	Indolent	A	12.00	−	+	1997	N/T	A	−	0.1	N/A	Mut
41**	Progressor	C	18.00	−	+	2007	6	D	−	1.0	N/A	Mut
42‖	Indolent	A	3.40	−	−	2008	N/T	A	+	1.7	Yes	Mut
43**	Indolent	A	28.40	−	+	2007	N/T	A	−	2.0	No	Mut
44‖	Indolent	A	8.00	−	−	2006	N/T	A	+	2.2	No	Mut
45**	Progressor	B	105.00	−	+	2007	12	A	−	2.0	No	Mut
46**	Indolent	A	2.47	−	−	2008	N/T	A	−	0.1	N/A	Mut
47‖	Indolent	C	5.00	−	−	2009	N/T	A	+	1.8	No	Mut
48**	Indolent	A	12.60	−	−	2008	N/T	A	−	2.1	No	Mut
49**	Indolent	A	5.80	−	−	2008	N/T	A	−	1.1	N/A	Mut
50‖	Indolent	A	11.50	−	−	2008	N/T	A	+	2.1	Yes	Mut

LPL indicates lipoprotein lipase; TFT, time from initial diagnosis to first treatment for clinical progression; A/D, alive/dead; CSR, class switch recombination; UM, unmutated; Mut, mutated; N/T, no treatment; and N/A, no amplification.

*Obtained results in cytometric assays, using a CD38 cutoff of 30%.

†Related to decease.

‡Percentage of IgG^+ and IgM^+/IgG^+ CLL B cells by cytometric studies.

§Less than or equal to 2% difference from germline gene defined patients with UM disease, ≥ 2% difference defines patients with MUT.

¶High AID expression and CSR ≥ 5%.

‖Low AID expression and CSR ≤ 5%.

**No AID expression and CSR ≤ 2.5%.

specific familial VH genes and Cγ primers and sequenced the obtained products to evaluate the correspondence with the tumoral clone. Amplifications were obtained for 36 of the 44 patients with CLL (72% of the 50 patients with CLL). However, an identical tumoral VH gene sequence was found in only 16 of these 36 CLLs, representing 32% of the total population (6 from the UM $AID^{+/+}$ CLL B cells, 7 from UM AID^+ CLL B cells, and 3 from MUT AID^+ CLL B cells; Table 1). Thus, CSR related to the tumoral clone ranged between 2.5% and 8.5% of the tumoral population among patients with UM disease. In contrast, unrelated tumoral CSR never exceeded 2% and is associated with low or no expression of AID mRNA (Table 1).

AID expression is mainly restricted to the subset of tumoral cells displaying an ongoing CSR

As reported by Cerutti et al[24] B cells with ongoing CSR are absent in normal PB and segregates within founder germinal center (GC) and GC B cells. By contrast, ongoing CSR has been described in the PB of patients with UM disease expressing AID constitutively.[14] Because AID expression in CLL B cells is confined to a small subset[25] and results from signals received through interaction with microenvironment and CD4 T cells, we examined whether its expression is restricted to tumoral cells with ongoing CSR. To substantiate this possibility, we isolated by cell sorting cytometry the subsets expressing the different immunoglobulin isotypes within the tumoral clone in the 3 AID$^{+/+}$ UM CLLs. Tumoral B cells expressing clonal membrane IgG (IgG$^+$) or intracellular IgM and IgG (B cells in transition state, IgM$^+$/IgG$^+$) and the typical surface IgM (IgM$^+$) were isolated with a greater than 98% purity as assessed by cytometric assays. A representative patient with CLL is shown in Figure 2A. Afterward, the mRNA was extracted, and RT-PCR was performed to determine CSR transcripts (Figure 2E), CTs, and AID expression in these different subsets. Because CTs are rapidly degraded by nucleases, it constitutes the specific molecular markers of ongoing CSR in B cells. Results from semiquantitative RT-PCR indicate that both subpopulations IgG$^+$ and IgG$^+$/IgM$^+$ express CTs, confirming that ongoing CSR occurs in both subpopulations (Figure 2F). Finally, we found that AID expression is mainly restricted to the subpopulations IgG$^+$ and IgG$^+$/IgM$^+$ (Figure 2G).

The subpopulation with high levels of AID and ongoing CSR displays an increased proliferative potential

Because AID is up-regulated after antigen activation and is the principal enzyme implicated in CSR, which occurs in GCs, we determined the relationship between AID expression with different proliferation, antiapoptotic, and activation markers such as Ki-67, Bcl-2, and c-*myc*, respectively, in the 3 tumoral subpopulations (IgM$^+$, IgG$^+$, and IgM$^+$/IgG$^+$).

The cytometric assays from one representative patient for Ki-67 expression are depicted in Figure 3A. In addition, in the 6 patients studied, results show significantly increased expression of Ki-67 in the IgG$^+$ and IgM$^+$/IgG$^+$ subsets compared with the IgM$^+$ subpopulation (comparing IgM$^+$ [mean = 92.25] with IgM$^+$/IgG$^+$ [mean = 150.8]; $P < .01$, Mann-Whitney test, and comparing IgM$^+$ [mean = 92.25] with IgG$^+$ [mean = 145.25]; $P \leq .01$, Mann-Whitney test; Figure 3A).

Expression of Bcl-2 protein is shown in Figure 3B in the same representative patient among the 6 studied. Significantly higher expressions were found comparing IgM$^+$ (mean = 31.3) with IgM$^+$/IgG$^+$ (mean = 47.62) ($P \leq .01$, Mann-Whitney test). A more significant difference was found in the case of IgM$^+$ with IgG$^+$ subpopulations (IgM$^+$: mean = 31.3; IgG$^+$: mean = 49.75; $P < .01$, Mann-Whitney test), suggesting a more increased antiapoptotic profile for both IgG$^+$ and IgM$^+$/IgG$^+$ than in the typically CLL IgM B cell (Figure 3B).

Because c-*myc* protein was observed in the highest amount at the proliferative B-lymphoblast stage and undetectable in plasma cells,[26] we evaluated at the RNA level c-*myc* expression in the 3 subpopulations. Results show higher expression of c-*myc* RNA in the IgG$^+$ and IgM$^+$/IgG$^+$ subsets compared with the IgM$^+$ tumoral counterpart (Figure 3C).

The cyclin-dependent kinase inhibitor p27^{-kip1} promotes exit to cell cycle and is overexpressed in CLL B cells.[27] Therefore, we examined its expression at the mRNA level in the 3 isolated subpopulations. Semiquantitative RT-PCR results show a high

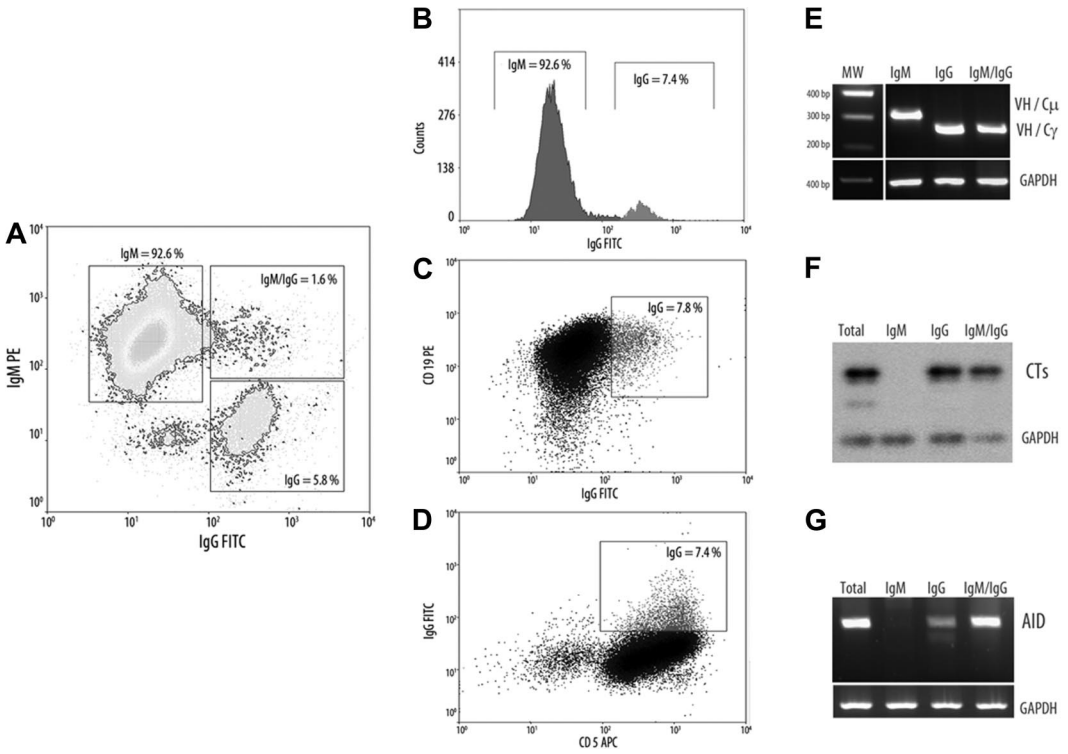

Figure 2. Characterization of CLL B cells with ongoing IgG CSR. (A-D) Representative flow cytometric profile from a patient with UM AID^{++} CLL. The 3 CLL B-cell subsets as well as the surface expression of CD5 and CD19 markers are depicted (IgM$^+$, IgM$^+$/IgG$^+$, and IgG$^+$). (E-G) Semiquantitative RT-PCR from the 3 cell sorter–isolated subpopulations. (E) Clonal isotype switch transcripts with tumor-related VH and Cμ or Cγ primers. (F) Amplification of CTs and subsequently hybridization with Cμ probe encompassing 1-180 nt of the first Cμ exon. (D) AID amplification from isolated CLL subsets. For all PCR analysis putative B-cell contamination between isolated subsets, moreover to cytometric purity analysis, was estimated by amplifying tumor-related VH-Cμ inside the IgG$^+$ subset and with VH-Cγ inside the IgM$^+$ subset. GAPDH was amplified in all cases as internal control.

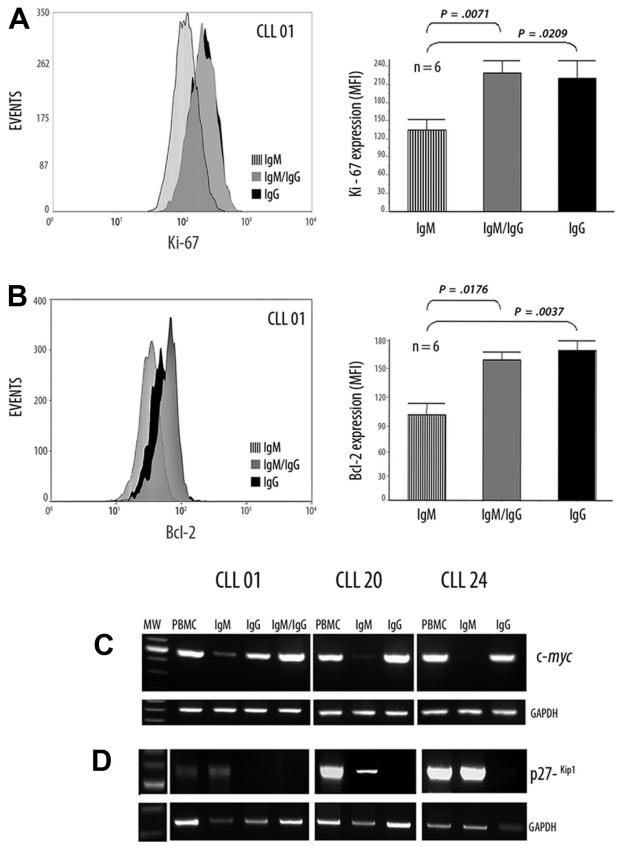

Figure 3. Differential expression of Ki-67, Bcl-2, c-*myc*, and p27^{-kip1} in CLL B cells with ongoing IgG CSR and in their IgM counterpart. (A-B) Flow cytometric analysis, in a representative patient with UM CLL, showing the Ki-67 and Bcl-2 protein expressions, respectively. Values are mean ± SE of mean fluorescence intensity (MFI) of, respectively, Ki-67 and Bcl-2 expression from 6 patients with UM CLL with high AID expression levels. (C-D) Semiquantitative RT-PCR from the cell sorter–isolated subpopulations with c-*myc*–specific primers (C) and with p27^{-kip1}-specific primers (D) are depicted. GAPDH was amplified in all cases as internal semiquantitative control.

expression of p27^{-Kip1} in the IgM$^+$ isolated subset; whereas no, or very weak, expression was found in both IgG$^+$ and IgM$^+$/IgG$^+$ isolated subpopulations (Figure 3D). Taken together, these results outline the idea that IgG$^+$ and IgM$^+$/IgG$^+$ subsets exhibit an activated phenotypic profile containing CLL B cells with increased proliferative and antiapoptotic potential.

AID$^{+/+}$ subpopulation with ongoing CSR cells displays expression molecules resulting from proliferation center contact

Expression of CD49d/α4-integrin has been associated with high levels of CD38 glycoprotein and expression of CCL3/CCL4 chemokines in progressive disease.[20] Because all these molecules appear to be important players in the survival of CLL B cells, we compared in the IgM$^+$ and the IgG$^+$ subpopulations the levels of protein expression for CD49d and CD38 markers. Results show that CLL B-cell IgG$^+$ express increased membrane CD49d levels compared with its IgM$^+$ counterpart (IgM$^+$: mean = 115.8; IgG$^+$: mean = 239.2; $P < .01$, Mann-Whitney test; Figure 4A). Interestingly, when the IgG$^+$ subset was studied for CD38 expression in the 3 representative patients, only a fraction (< 50%) of this subpopulation was competent to express CD38 (Figure 4C). Finally, we evaluated CCL3 and CCL4 chemokine mRNA expression profiles in both subpopulations in the same 3 patients. Results show that transcripts for CCL3 are exclusively observed within the IgG$^+$ subset, whereas higher levels of CCL4 transcripts were observed in this same subpopulation compared with its IgM$^+$ counterpart. Overall, these results favor the view that the tumoral subset with ongoing CSR and high AID expression constitutes an activated subset expressing molecules associated with the progressive disease and that may result from a recent contact with proliferative centers.

High expression of AID and high percentage of clonal CSR in PBMCs delineate a subgroup with poor prognosis among patients with UM CLL

Significant differences concerning AID expression in the 3 subgroups (AID$^{+/+}$: mean = 1.58; AID$^+$: mean = 1.40; AID^{neg}: mean = 1.22) were observed in patients with UM CLL after quantitative PCR analysis ($P < .001$, Mann-Whitney test; Figure 5A). As for the clonally related CSR process, the same 3 subgroups were also identified displaying significant differences between high clonally related CSR (mean = 6.36), low clonally related CSR (mean = 3.75), and not clonally related CSR (mean 0.72,) processes ($P < .001$, Mann-Whitney test; Figure 5B). Because our results suggest that CLL B cells with high expression of AID and high percentage of ongoing CSR correspond to an activated and proliferative subpopulation (see Figures 3-4), we speculated whether this subset could be associated with the disease progression. Thus, we segregated patients with UM CLL into 3 subgroups: subgroup I, AID$^{+/+}$ with higher percentage of clonally related CSR (≥ 5%); subgroup II, AID$^+$ and lower percentage of clonally related CSR; and subgroup III, AID^{neg} without clonally related CSR. Results show that among the 6 patients from subgroup I, all required treatment at 3, 6, 8, 9, 10, and 12 months. Four of these 6 patients died of causes related to disease at 6, 24, 72, and 84 months after diagnosis. Interestingly, all these patients displayed high values of AID expression and tumoral-related CSR with values higher than 5% (Table 1; Figure 5A-B). Subgroup II

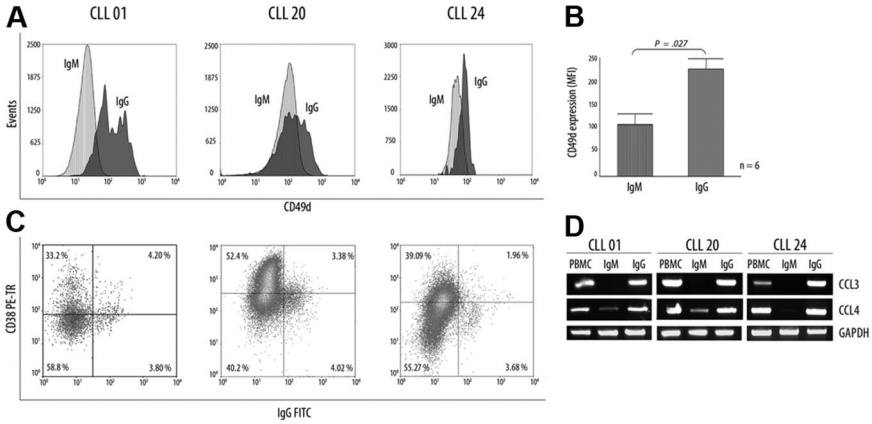

Figure 4. Differential expression of CD49d, CCL3, and CCL4 chemokines in CLL B cells with ongoing IgG CSR and in their IgM counterpart. (A) Flow cytometric analysis from CD49d protein expression in 3 representative patients. Although increased levels of CD49d are found in the UM CLLs, expression values for this protein were always higher in the IgG$^+$ subset that in their counterpart IgM$^+$ subset. (B) Values are mean (± SE) for MFI of CD49d expression in the 6 patients with CLL with high AID expression levels. (C) Flow cytometric profiles of CD38 expression inside the IgG subset from 3 representative patients with CLL. Numbers in quadrants indicate percentages of cells. (D) CCL3 and CCL4 mRNA profile expressions in the different isolated subsets (IgM$^+$ and IgG$^+$) from the 3 representative patients with UM CLL.

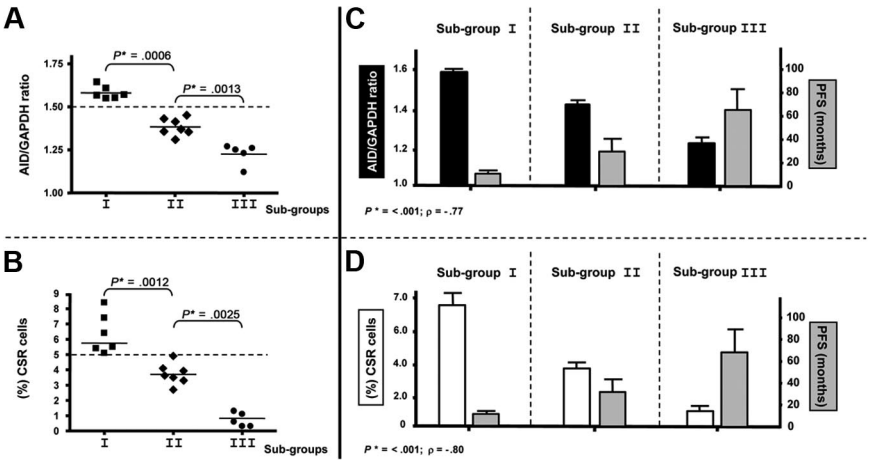

Figure 5. AID expression levels and high CSR segregate patients with UM CLL into 3 subgroups with different clinical progression. (A-B) Significant differences in *P* values in mRNA AID expression (A) and percentage of CLL B cells with clonally related CSR (B) allow us to segregate patients with UM disease into 3 different groups: subgroup I (6 patients) identified as $AID^{+/+}$ and high clonal CSR ($\geq$ 5%), subgroup II (7 patients) identified as AID^{+} and low clonal CSR, and subgroup III (5 patients) identified as AID^{neg} without clonal CSR. Significant *P* values are shown (*) ($P \leq .001$, Mann-Whitney test). (C-D) Correlations of AID expression and PFS (C) and clonal CSR and PFS (D) for the 3 subgroups are plotted. Both analyses indicate a significant negative correlation; $P \leq .001$ considered significant (*) by Spearman rank test. $P < .001$ (Spearman rank coefficient $\rho = -0.77$) for AID expression correlated to PFS and $P < .001$ (Spearman rank coefficient $\rho = -0.80$) for clonal CSR linked to PFS.

contained 7 patients. Six of the 7 patients received treatment at 6, 11, 12, 15, 18, and 36 months, and 1 of them died at 70 months from disease-related causes. In subgroup III, the 5 patients failing to express AID, no tumoral CSR was found, and none of them received treatment and remain alive to date. To link a clinical poor outcome with high AID expression and high clonally related CSR, a Spearman rank correlation test was carried out. A significant negative correlation comparing the 3 subgroups was found between AID expression and PFS ($P < .001$; Spearman rank coefficient $\rho = -0.77$), as well as between CSR percentage and PFS ($P < .001$; Spearman rank coefficient $\rho = -0.80$; Figure 5C and 5D, respectively). Despite the low number of patients with CLL with clonally related CSR in the UM group, PFS and OS analyses were performed. Results showed significant differences for PFS analysis ($P = .007$) and low but still significant difference for OS analysis ($P = .021$; Figure 6A and 6B, respectively).

Among the patients with MUT disease, none of the 6 patients expressing low levels of AID required treatment, and all patients are alive with a mean time of follow-up of 36 months. Among the 19 patients with MUT disease failing to express AID transcripts, 3 required treatment at 6, 12, and 25 months, and 1 died of causes unrelated to CLL at 120 months without receiving previous treatment. Overall, these results outline the importance of the subset with ongoing high clonally related CSR and expressing high levels of AID among patients with UM disease and suggest that the analysis of this subpopulation could be important to identify a more aggressive form in CLL disease.

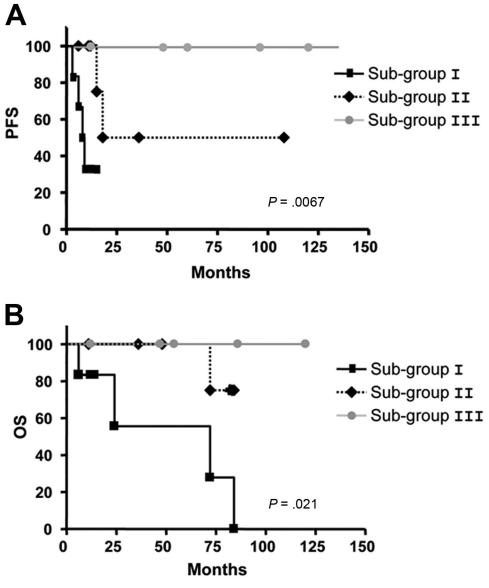

Figure 6. Kaplan-Meier curves based on AID and CSR expression in the 3 subgroups in patients with UM CLL comparing the PFS and OS. The Kaplan-Meier method was used to construct survival curves for PFS (A) and OS (B), and results were compared with the log-rank test Spearman. *P* values refer to the log-rank test. Subgroup I corresponds to UM CLL $AID^{+/+}$ and CSR $\geq$ 5%; subgroup II to UM CLLs AID^{+} and low clonally related CSR, and subgroup III to UM CLL AID^{neg} and CSR not related to the tumoral clone.

Discussion

Important progress resulting in high levels of clinical and even molecular remissions has been recently achieved in CLL treatment. However, CLL remains an incurable disease. Recently, compelling evidence suggests that crosstalk with accessory cells in specialized tissue microenvironments, such as the bone marrow and secondary lymphoid organs, favors disease progression by promoting malignant B-cell growth and drug resistance. Therefore, understanding the crosstalk between malignant B cells and their milieu could give us new keys into the cellular and molecular biology of CLL that can finally lead to novel strategies in the treatment of this disease. Nevertheless, we need a more proper knowledge about the signals received and/or transmitted by CLL B lymphocytes, interacting with T lymphocytes, and/or with stromal, endothelial, dendritic, and nurselike cells in the particular CLL microenvironment. The simple observation that CLL B cells progressively accumulate in vivo, but undergo apoptosis when cultured in vitro, draws attention to the microenvironment and its ability to deliver signals that may ensure the survival of malignant cells. Further, it is self-evident that the accumulated CLL B cells in the PB are constantly nourished by an upstream proliferation cell compartment. It is reasonable to assume that the balance between the 2 compartments may be at the basis of the highly variable clinical course of CLL, which may behave as a stable and indolent monoclonal lymphocytosis or as an aggressive disease.

In this study we attempted to provide additional evidence related to the importance of the microenvironment signals in the clinical course of CLL. For this, we analyzed a particular tumoral subpopulation displaying ongoing CSR[17,28] and high AID expression in the PB of patients with UM CLL. Ongoing CSR process[24] and high expression of AID[29] are physiologically confined to the GC, a highly specialized and activated microenvironment originated during an immune response. However, in previous work, we demonstrated expression of AID in the PB of patients with UM CLL, which additionally displayed a subpopulation of tumoral cells with ongoing CSR.[14] Because a subsequent work has shown that AID expression is restricted to a small proportion of the tumoral CLL clone,[30] the patients with UM disease with this subpopulation and high

expression of a potential mutator such as AID are interesting to study. Given this, we hypothesized that the subset with ongoing CSR could be responsible for AID expression and might correspond to a CLL in which an activated tumor microenvironment is present, being that this is responsible for a poorer clinical outcome. We speculated that this continuous activation might lead to a highly proliferative disease whose hallmark is the presence of this particular subset in the PB of this UM CLL.

Our results show that almost all mRNA AID expression is restricted to the subpopulation of tumoral cells with ongoing CSR. Furthermore, this subpopulation is clonally related to the tumoral IgM counterpart but expresses higher levels of Ki-67, c-*myc*, Bcl-2, CD49d, and CCL3/4 chemokines as well as lower levels of the cell-cycle inhibitory protein $p27^{-kip1}$ and, more importantly, is associated with disease progression. Active CSR process is currently measured by the presence of switch circle or their transcribed chimeric I-Cμ product referred as the CTs. Because they are rapidly degraded by nucleases, they constitute specific molecular markers of ongoing CSR. Our results show the presence of CTs in the tumoral subset IgM^+/IgG^+, which is a minor pool of CLL B cells in a transitional state of CSR. Interestingly, in the established IgG^+ CLL B cells CTs were also identified, suggesting that at least some of them come out of an active CSR process. High expression of AID was observed in the IgM^+/IgG^+ and IgG^+ subpopulations but not in their IgM^+ counterpart. These results suggest that the subset with ongoing CSR is responsible for almost all AID expression and that this activation might have recently been triggered, in an activated CLL microenvironment. To clarify the importance of the microenvironment interactions with the different clinical progression of these patients with UM CLL, we asked, which is the stimulus for this constitutive expression and where does it occur? These interesting questions remain to be elucidated.

In this context, we have evaluated the expression of different key molecules implicated in cell-cycle regulation, proliferation, and tumoral progression. Higher expression of Ki-67, c-*myc*, Bcl-2, as well as the underexpression of the cell-cycle inhibitor $p27^{-kip1}$, was found among the tumoral subsets with ongoing CSR and high AID expression, compared with their IgM counterpart. The Ki-67 is a nuclear protein up-regulated in the G_1, S, G_2, and M phases of the cell cycle but absent in resting cells (G_0 phase).[31] In addition, c-*myc* oncogene has been proposed to be expressed at a stage of differentiation in B cells. Studies of Larsson et al[26] show that CLL B cells could be induced to proliferate and differentiate, after in vitro incubation with 12-*O*-tetradecanoyl-phorpol-13-acetate. In this case the CLL B cells change from G_0 to G_1, and this process is accompanied by a dramatic increase in the expression of c-*myc*.[26] In our studies, both subpopulations (IgM^+/IgG^+ and IgG^+) depicted significantly higher levels of Ki-67 and Bcl-2 protein and the c-*myc* mRNA, suggesting an activated state for these particular subsets of CLL B cells. Because some reports indicated that AID expression could vary during evolution of CLL disease,[23,25] we have studied 2 patients (CLL 18 and 20) at 2 time points with a 2-year interval. Results showed no significant changes in AID, CSR, and Ki-67 expression, suggesting that the presence of IgM^+/IgG^+ and IgG^+ subsets as well as AID expression are maintained during the course of the disease (data not shown).

Recent reports suggest that CD38 and CD49d molecules are negative prognostic factors in CLL and could be implicated in the molecular crosstalk between malignant B cells and their microenvironment.[20,32] In addition,CCL/3 and CCL/4 chemokines have been identified to contribute to the recruitment of cells from the monocyte macrophage lineage in these important interactions with CLL B cells and stromal cells.[19,20] Interestingly, our results in the IgG^+ subsets show higher expression of CD49d protein and CCL3/4 mRNA than in the IgM^+ tumoral subpopulation. These results are in agreement with previous reports showing that these molecules are important players in the activation of CLL B cells interacting with their microenvironment.

With the use of a nonradioactive, stable isotopic labeling method to measure CLL kinetics, Messmer et al[33] demonstrated that B-CLL is not a static disease that results simply from accumulation of long-lived lymphocytes, but it is a dynamic process in which cells proliferate and die. Markers such as CD38 have been proposed as helping to identify the proliferative pool.[34] However, the fact that CD38 is expressed in a high percentage of tumoral cells in patients with UM disease and recent results from Calissano et al[35] fail to establish a strong correlation between the percentage of $CD38^+$ proliferating cells in CLL clones and survival and disease progression indicate that $CD38^+$ tumoral cells constitute a heterogeneous population, including a small fraction of cells with an increased proliferative potential. Results from Messmer et al[33] indicating that proliferating rates of CLL cells range from 0.08% to 1.7% suggest that, as in the case of $CD38^+$ subset, not all the $AID^{+/+}$ subpopulation with ongoing CSR correspond to cells in a state of active proliferation. Our results showing that only a fraction of tumoral cells displaying ongoing CSR coexpress CD38 support this last view. To our knowledge only one work has correlated AID and CD38 expression in CLL B cells.[36] Because a positive correlation was found, we can presume that this small $CD38^+$ subset within the IgG^+ B cells could be the same that express high levels of AID enzyme. Further studies isolating the tumoral IgG^+ subset coexpressing CD38 from IgG^+CD38^- cells are warranted.

Intriguingly, it is difficult to determine the precise role of these highly proliferating activated tumoral B cells. Because the presence of this subset is clearly associated with poor prognosis, it might have an adjuvant role in the maintenance of the CLL proliferative pool. However, given their increased proliferative potential, they should normally outnumber the IgM^+ cells, but this is not the case. Thus, we could assume that these cells should undergo apoptosis once leaving the pseudofollicles. A recent work suggesting a link between AID expression and B-cell apoptosis in the GC favors this view.[37] In these conditions, the IgG^+ subset could reflect the existence of an active microenvironment leading to permanent stimulation of the IgM^+ pool, which would turn on the CSR machinery maintaining this IgG^+ subset in the PB. If true, the hypothesis that in the UM subgroup stimulation of BCR takes place by an unknown autoantigen[38-40] and that this is responsible for consecutive stimulations and is able to sustain survival/expansion signals in the tumoral clone results in an interesting issue highlighted by these results.

Finally, our results indicate that among UM CLLs exists a subgroup of patients identified by high levels of AID expression and ongoing clonally related CSR, which displays an even worse prognosis. These findings have been obtained in a retrospective series of patients with a short follow-up. Prospective studies in larger series of patients are needed to raise conclusive evidence to our results and to determine whether expression of the different C_H isotypes in tumoral cells should be routinely included in flow cytometric studies to better assess prognosis among patients with UM CLL.

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Authorship

Contribution: F.P. and P. Moreno performed experiments and wrote the paper; P. Morande, C.A., A.C., and V.P. performed experiments and collected CLL samples; A.I.L. and R.G. performed clinical activities and data collection of patients with CLL; M.G., G.D., and O.P. contributed to scientific design and revised the paper; and P.O. designed research, coordinated the study and data analysis, and wrote the paper.

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"ACTIVATION OF THE PI3K/AKT PATHWAY BY MICRORNA-22 RESULTS IN B-CLL CELL PROLIFERATION"

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La LLC es la más frecuente de las leucemias de los países occidentales y se caracteriza por una expansión de linfocitos B monoclonales en sangre, médula ósea y órganos linfoides secundarios (Chiorazzi et al, 2005).

Han surgido evidencias, en la última década que relacionan el rol del microambiente inmunológico con la progresión de la enfermedad, donde la interacción de las células B de LLC con células accesorias como, linfocitos T, células estromales, desencadenan activación de vías de traducción de señales que favorecen la sobrevida y proliferación del clon tumoral (Burger, 2013). Como ha sido previamente descrito la LLC es una enfermedad heterogénea (Calissano et al, 2011; Damle et al, 2007; Messmer et al, 2005) donde la gran mayoría de las células B se encuentran en SP en G0/G1 del ciclo celular y constituyen al compartimento quiescente o células en reposo, mientras que los prolinfocitos y parainmunoblastos en los CPs de la médula ósea, nódulos linfáticos y otros tejidos linfoides constituyen el compartimento proliferante (Caligaris-Cappio, 2003). Estas células se encuentran en estrecho contacto con las células del microambiente tumoral recibiendo señales que favorecen la sobrevida, la proliferación y posiblemente la refractoriedad al tratamientodel clon tumoral.

Las células proliferantes, aunque forman parte del mismo clon leucémico, difieren de las células en reposo en cuanto al perfil de expresión de moléculas relacionadas con el ciclo celular y apoptosis como CD38, Mcl1 (Pepper et al, 2007), survivina (Granziero et al, 2001) con la migración linfocitaria CCL22 (Ghia et al, 2002), CCL3/4 (Zucchetto et al, 2009) y genes de proliferación como Ki-67 y c-myc (Palacios et al, 2010). Otra diferencia interesante es la expresión diferencial de p27, regulador negativo del ciclo celular que se encuentra altamente expresada en células de SP y ausente en los CPs (Sanchez-Beato et al, 1997).

Varios estímulos han sido descritos capaces de activar la vía anti-apoptótica de la fosfatidil-inositol 3-quinasa/AKT (PI3K/AKT) en el microambiente tumoral. La vía de señalización se encuentra involucrada en una variedad de procesos incluyendo, migración celular, síntesis y metabolismo de proteínas, muerte y sobrevida celular. PI3Ks se encuentran comúnmente activadas en cánceres humanos (Bunney & Katan, 2010), debido a mutaciones de la proteína, o mediante la activación gatillada por receptores de superficie. En particular, en leucemias y linfomas esta vía se encuentra constitutivamente activada, presumiblemente debido a señales del microambiente. Debido al rol importante que juega la vía PI3K/AKT en la sobrevida del clon tumoral es que diferentes estrategias de inhibición de quinasas se están estudiando como posibles terapias (Burger, 2013).

La PI3K cataliza la fosforilación del segundo mensajero lipidico fosfatidil-insitol (4, 5) bifosfato (PIP_2) en fosfatidil-inositol (3, 4, 5) trifosfato (PIP_3), el cual recluta y activa la quinasa dependiente de fosfatidil-inositol 1 (PDK1). La PDK1 es capaz de fosforilar y activar la serina-treonina quinasa AKT o proteín quinasa B (AKT/PKB) directamente relacionada con la sobrevida y proliferación celular. AKT activada es responsable de inhibir el factor de transcripción FOXO1 (Huang & Tindall, 2011), quien regula positivamente la expresión del regulador negativo del ciclo celular p27 (Zhang et al, 2011), lo cual resulta en proliferación celular y sobrevida. AKT activada puede inducir la expresión del inhibidor de la apoptosis survivina mediante la activación de la vía NFkB y favorecer la proliferación del clon tumoral (Hideshima et al, 2007).

Las funciones de la PI3K se encuentran reguladas por el supresor de tumor PTEN (fosfatasa homologo de tensina y deletado en el cromosoma 10) quien cataliza la formación de PIP_2 a partir de la desfosforilación de la PIP_3 (Stambolic et al, 1998). La vía PTEN/PI3K/AKT es una de las vías de traducción de señales más comúnmente alteradas en tumores humanos (Cully et al, 2006). Recientemente Shehata y col. resaltan la importancia de las funciones de PTEN en células de LLC. En este trabajo determinan que un aumento de las funciones de PTEN en células B de LLC pueden inhibir la vía de señalización PI3K/AKT y de esta manera inducir la muerte del clon tumoral (Shehata et al, 2010). En este sentido es que los autores proponen el diseño nuevas terapias que favorezcan las funciones de PTEN para el tratamiento de pacientes de LLC.

La regulación de la activación de la vía PTEN/PI3K/AKT puede estar dada también, a nivel del ARNm por miRNAs. En particular, los miRNAs son pequeñas moléculas de ARN no codificante que regulan la expresión de proteínas blanco mediante su unión al extremo 3'UTR (Bartel, 2004). Interesantemente debido al rol que cumplen en la regulación de la expresión génica, una desrregulacion del los miRNAspueden favorecer o inhibir el desarrollo del cáncer y de esta manera es que al día de hoy, se los considera como supresores de tumores u oncogenes (Lawrie, 2007). Recientemente Bar y col. muestran que el miR-22inhibe la expresión de PTENy activa la vía AKT directamente relacionada con la progresión del ciclo celular y la sobrevida en varios tipos de tumores celulares (Bar & Dikstein, 2010). De esta manera el miR-22 puede actuar como un regulador de la vía PTEN/AKT/FOXO1.

Nuestro grupo ha descritouna sub-población de linfocitos B de LLC, derivados del compartimento proliferante recientemente en contacto con células de microambiente tumoral, a partir de SP de

pacientes de LLC progresores de mal pronóstico (Palacios et al, 2010). Estas células presentan no sólo un proceso activo de CC y expresión anómala de la enzima AID sino también un comportamiento proliferativo basado en la alta expresión de BCL2 y c-myc, baja expresión de p27 a nivel de ARNm comparado con su contrapartida de célula en reposo.

Tomando en cuenta que el diálogo entre las células B con el microambiente inmunológico favorece la progresión de la enfermedad, el bloqueo de estas señales podría ser una buena estrategia para la terapia de pacientes progresores o refractarios al tratamiento (Burger et al, 2009a). Con el propósito de identificar blancos terapéuticos capaces de romper esta interacción y con el objetivo de responder algunas de las preguntas planteadas en el primer artículo de esta Tesis, estudiamos el perfil de expresión génica del sub-grupo de células B proliferantes AID^{pos}y lo comparamos con el perfil de expresión correspondiente a su contraparte quiescente del mismo paciente. Para ello aislamos la fracción quiescente (IgM^{pos}, Ki-67^{low}, AID^{neg}) y la proliferante (IgG^{pos}, Ki-67^{high}, AID^{pos}) para luego proceder a la extracción de ARN total. Con este material se realizan posteriormente estudios por microarreglos ya sea de los ARNm como de los microARNs de ambas poblaciones para un número de 4 pacientes.

Nuestros resultados muestran que los genes (miRNAs y ARNm) diferencialmente expresados se encuentran relacionados con moléculas implicadas en el control del ciclo y la proliferación celular así como también a la progresión tumoral. Estos datos refuerzan el planteo generado en nuestro primer artículo que sugiere que la sub-población IgG^{pos}, Ki-67^{high}, AID^{pos} es un ejemplo de fenotipo de una célula leucémica activada en pacientes de LLC NM progresores.

Por otra parte, los aportes específicos de este trabajo resaltan la expresión del miR-22 como una molécula clave en la activación de la vía de señalización PI3K/AKT lo cual nos brinda una posible explicación para entender el comportamiento proliferante de la sub-población estudiada. En particular, identificamos una menor expresión de PTEN a nivel de ARNm como de proteína y una mayor activación de la proteína AKT fosforilada ($pAKT^{Thr308}$).También determinamos que el factor de transcripción FOXO1 se encuentra inactivado ubicándose mayormente en el citoplasma de dichas células. Observamos una disminución del regulador negativo p27 y un aumento del inhibidor de la apoptosis survivina en la población proliferante en comparación con la quiescente. Estos resultados indican que la vía PTEN/AKT/FOXO se encuentra activada en la población proliferante. Tomando en cuenta que el miR-22 regula negativamente el supresor de PTEN nos preguntamos si el miR-22 es al menos en parte responsable de la activación de AKT en dichas células. Mediante

estudios *in vitro* en células B de pacientes de LLC determinamos que el miR-22 regula negativamente PTEN y está directamente asociado con la proliferación celular.

Previamente describimos que la sub-población proliferante presenta moléculas relacionadas un microambiente inmunológico activado, como ser una alta expresión de AID, CD49d y quimioquinas CCL3/4. Más recientemente identificamos una alta expresión de survivina en dicha población celular, a su vez, la expresión de dicha proteína puede ser regulada por señales de tipo T dependientes en células B de pacientes de LLC (Granziero et al, 2001). Debido a esto, y a que poco se conoce sobre la regulación de la expresión del miR-22, nos preguntamos si el miR-22 puede ser gatillado por señales del microambiente tumoral. Los resultados mostraron que las señales CD40L/IL4 son capaces de inducir la expresión del miR-22 *in vitro*, sugiriendo que la sub-población proliferante recientemente recibió señales del microambiente capaces de inducir la expresión del miR-22 y por consiguiente activar la vía PTEN/AKT/surv favoreciendo sobrevida y proliferación celular.

Dado que todos estos resultados fueron determinados a partir de muestras de células B de pacientes de LLC de SP, nos preguntamos si las células proliferantes en un órgano linfoide secundario de pacientes de LLC presentan características similares a las que observamos anteriormente en sangre. Para ello, aislamos células Ki-67^{pos} de las Ki-67^{neg} a partir de tres nódulos linfáticos de pacientes de LLC progresores, y evaluamos la expresión de PTEN, pAKT$^{-Thr308}$, survivina y p27. Nuestros resultados mostraron que al igual que la población proliferante en SP, en los nódulos observamos que la población Ki-67^{pos} presenta una baja expresión de PTEN, alta expresión de pAKT y survivina y una baja expresión de p27 en comparación con la población Ki-67^{neg}. Estos resultados confirman la importancia de la vía PI3K/AKT en la proliferación celular y sugieren el mismo circuito regulatorio, miR-22/PTEN/AKT/surv descrito como activo en células B de SP AID^{pos} de pacientes NM progresores.

En resumen estos resultados resaltan la importancia del microambiente tumoral en relación a la sobrevida del clon tumoral. En este sentido, proponemos que una sub-población de linfocitos B de pacientes de LLC NM progresores presenta característica de célula recientemente activada en CPs de los órganos linfoides secundarios. Estas señales podrían ser las responsables de la expresión de AID y del miR-22. El miR-22 en células B de LLC inhibe la expresión de PTEN y por consiguiente activa AKT. La quinasa AKT activada inhibe a FOXO y p27 moléculas claves en la regulación del ciclo celular y activa una proteína de la familia de inhibidores de la apoptosis, survivina a través de la

activación del NFkB. Por su parte el NFKB regula la expresión de AID (Dedeoglu et al, 2004) por lo tanto esto sugiere que el miR-22 podría ser al menos en parte responsable de la expresión anómala de AID a través de la vía NFkB/AKT. De todas maneras más estudios serian necesarios para confirmar esta hipótesis.

Mediante este trabajo proponemos al miR-22 como molécula clave en la regulación de la vía de señalización PTEN/AKT mediante la regulación negativa de PTEN en células B de pacientes de LLC y resalta la importancia de las interacciones con el microambiente en sostener el pool proliferante en las LLC progresoras.

Activation of the PI3K/AKT pathway by microRNA-22 results in B-CLL cell proliferation

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INTRODUCTION

Chronic Lymphocytic Leukemia (CLL) is an incurable disease of unknown etiology characterized by progressive accumulation of clonal B lymphocytes with mature morphology and phenotype (Dighiero & Hamblin, 2008). Contrary to its earlier description as a relatively homogeneous disease, ultimate evidences displays that CLL becoming a more heterogeneous disease with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis (Chiorazzi, 2007); (Damle et al, 2007). Most of circulating peripheral blood (PB) CLL cells are in G0/G1 of cell cycle and constitutes what is sometimes referred as the accumulative compartment or quiescent fraction (QF). However, there is also a proliferative fraction (PF) comprised of prolymphocytes and paraimmunoblasts which are part of the pseudofollicular proliferation centers (PCs) in the bone marrow (BM), lymph nodes (LN) and other lymphoid tissues (Caligaris-Cappio, 2011). Thus, evolution of the disease could depend on the relative balance between the quiescent and the proliferative fractions. During recent years, extensive work has been devoted to the study of both fractions which differ in terms of expression of several molecules, including chemokines such as CCL-22 (Ghia et al, 2002), CCL3 or CCL4 (Burger et al, 2009b), activator molecules such as CD38 and Mcl-1 (Pepper et al, 2007), apoptosis-regulators such as Survivin (Granziero et al, 2001) and proliferation related genes such as Ki-67 and/or c-myc (Palacios et al, 2010). Another interesting difference between proliferative and quiescent fractions is the expression of p27-Kip1 (p27), a key regulator of cell cycle, which is high in the QF and virtually absent in the PF (Palacios et al, 2010). In this regard, isolation and analysis of the tumoral subset that is being triggered in the proliferative compartments of progressive CLL cases is an important aim to understand CLL pathogenesis. Different groups have tried to assess this issue by studying different CLL proliferative fractions like that expressing CD38 marker (Damle et al, 2007), or that expressing activation induced cytidine deaminase (AID), the key enzyme implicated in somatic mutation and class switch recombination (Palacios et al, 2010), or CD5/CXCL4 molecules (Calissano et al, 2011).

There is increasing evidence suggesting that crosstalk with accessory cells in specialized tissue microenvironments favours disease progression by promoting the development of malignant subclones with changing dominance over time (Caligaris-Cappio, 2011). Understanding the crosstalk between malignant B-cells and their milieu could give us new keys on the cellular and molecular biology of CLL that can finally lead to novel strategies for disease treatment (Burger et al, 2009a). Several stimuli that are endogenously produced in the tumor microenvironment were shown to activate the antiapoptotic phosphatidyl-inositol 3-kinase (PI3-K)/AKT pathway. PI3-Ks is commonly activated in human cancers(Bunney & Katan, 2010), and specifically in leukemia and/or lymphoma diseases this cascade is now targeted in clinical trials (Burger & Hoellenriegel, 2011). Targeting of PI3-K cascade is characterized by a pattern of response given by a very

significant reduction of the tumor mass which is associated to an important tumoral hyperlymphocytosis in PB. This last phenomenon could be the consequence of a recirculation of tumor cells, which would be forced to egress from their protective niches in the lymphoid organs. When the PF is deprived of this protection, these tumor cells would be doomed to spontaneous apoptosis. Thus, inhibitors of PI3K/AKT pathway could constitute a significant advance in CLL treatment.

In our laboratory, we first reported that in contrast to normal circulating B-lymphocytes, which only express AID transcripts following CD40L or LPS stimulation, in most unmutated (UM) CLL cases PB leukemic cells are able to express high levels of an active AID enzyme (Oppezzo et al, 2003). Since AID expression results from signals received in activated lymphoid secondary organs, we subsequently investigated whether AID enzyme is active in the leukemic clone and if its expression is related to CSR process. Our results showed that AID expression is functional and that is mainly restricted to the subpopulation of tumoral cells having a CSR process. More important, the presence of this subpopulation in PB from CLL patients is closely related to an aggressive course of the disease (Oppezzo et al, 2005a; Palacios et al, 2010). This small proliferative clonal subset ongoing CSR has been probably induced by a recent contact with the microenvironment in the PB of the progressive UM cases. This interaction appears to be responsible not only for AID overexpression, but also for elevated levels of proliferation and anti-apoptotic molecules like Ki-67, c-myc and Bcl-2.Additionally, it also expresses high levels of proteins associated to progression such as CD49d and CCL3/CCL4 chemokines, as well as a decreased expression of the cell cycle inhibitor p27 when compared with its quiescent tumoral counterpart expressing exclusively IgM (Palacios et al, 2010).

In this work, we have characterized the genomic expression profile of this proliferative subset. With the aim to obtain putative targets able to interfere with the microenvironment proactive role on CLL B-cells, we isolated clonally related subsets from the same patients as follows: the QF (IgM+, Ki-67low, AIDneg) and the PF (IgG+, Ki-67+, AID+), we analyzed global mRNA and microRNAs profiles followed by a bioinformatic analysis in order to obtain the most enriched gene pathways characteristic of this proliferative CLL subset.Our results suggest that the proliferative behaviour of this subpopulation expressing AID enzyme in progressive CLL patients mainly depends on expression of microRNA-22. Up-regulation of this microRNA in the proliferative subset results in lower expression of the PTEN suppressor gene which in turn appears to switch on the antiapoptotic PI3-K/AKT pathway. Activation of this cascade is associated to cytosolic translocation of FOXO1 transcription factor which in turn leads to downregulation of p27 protein, and Survivin expression resulting altogether, in a higher cell cycle progression activity. Finally, we could confirm through transfection experiments with miR-22 and its respective antagomir, the key role of this microRNA in CLL proliferation and we propose that a similar regulatory loop could be present in the leukemic clone in PCs of some CLL patients.

MATERIAL AND METHODS

Patient samples

PB was obtained from 22 patients with a typical diagnosis of B-CLL, 12 displaying an UM and 10 a Mut IgVH profile (Table 1). With a median follow-up of 48 months (range 3-108 months) there were 3 Binet stages A, 6 stages B and 3 stages C in the UM group, 9 required treatment and 7 deceased. LN samples were obtained from 3 UM CLL patients (number 07, 08 and 12 in table 1). All patients were followed at the Hospital Maciel

from Montevideo and provided an informed consent according to the ethical regulations from Uruguay and the Helsinki Declaration. The diagnosis of B-CLL relied on cytological features of mature lymphocytes and a characteristic phenotype ($CD5^+$, $CD23^+$, low expression of CD79b and of surface Ig).

Phenotypic and functional studies of B cells

Blood collection was carried out in a period close to diagnostic time for each patient as indicated in Table 1. Mononuclear cells from PB (PBMC) and manually disaggregated LN with diffuse infiltration of B-CLL cells were isolated by centrifugation on Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) and immediately cryo-preserved in liquid N_2.Phenotypic analysis of leukemic cells was performed with phycoerythrin (PE) labelled anti-CD19, fluorescein-isothiocyanate (FITC) labelled anti-CD5, PE labelled anti-human μ chains $F(ab')_2$, and FITC labelled anti-human γ chains $F(ab')_2$ antibodies. Negative controls were performed by incubating the cells with irrelevant PE or FITC conjugated F-$(ab')_2$ antibodies. Forward and side scatters were used to gate out contaminating debris and the cells killed during the staining procedure. All antibodies were from Dako France SAS. Data were acquired and analysis performed using a CyAn Flow Cytometer (Beckman, Coulter).

Cell sorting studies

Sorting experiments of B-CLL cells were performed with a MoFlo cell sorter (Beckman Coulter). For the separation of cells from PB the followings antibodies were used: Alexa Fluor 647 conjugated anti-Ki67 (Santa Cruz Biotechnology, Inc), PE conjugated anti-human μ chains $F(ab')_2$ and FITC conjugated anti-human γ chains $F(ab')_2$ (Dako France SAS). We isolate the following CLL B-cells populations: **1) $Ki67^{neg}$ IgM^{pos}** subset expressing $CD19^+$, $CD5^+$ and IgM surface markers; **2) $Ki67^{pos}$ IgG^{pos}** subset expressing $CD19^+$, $CD5^+$ and IgG membrane proteins. For cell sorting of cells from LN, FITC conjugated anti-Ki-67 from Dako, Inc (F0788) and PE conjugated anti-human CD19 from Dako, Inc. (R0808) were used. $CD19^{pos}$ $Ki\text{-}67^{neg}$ and $CD19^{pos}$ $Ki\text{-}67^{pos}$ populations were sorted. In all cases, purity of isolated sub-populations was shown to be >96 % before flow cytometry or fluorescent microscopy evaluation.

MicroRNA and mRNA Array analysis

Microarray procedures

Total RNA from CLL B-cells was isolated from 1-5x10^6 cells using mirVana isolation kit (Applied Biosystems, Ambion, U.S.). Total RNA was used to perform the array for mRNA and microRNA. RNA concentration and integrity were determined using a NanoDrop-1000 Spectrophotometer (NanoDrop Technologies) and a 2100 Bioanalyzer (Agilent) with an RNA 6000 Nano LabChip Kit, using the Eukaryote Total RNA Nano assay according to the manufacturer's instructions. RNAs with an RNA integrity number (RIN) greater than 8 were used. Microarray for mRNA analysis was performed using a 4x44K Whole Human Genome Oligo Microarray (G4112F, Agilent), in a two-color design. The Low RNA Input Linear Amplification Kit (Agilent) was used to generate fluorescent complementary RNA (cRNA) for the microarray hybridizations. Briefly, we amplified and labeled 500ng of total RNA using the Cy5-CTP or Cy3-CTP dyes. Equal amounts of labeled samples (quiescent and proliferative) were hybridized to the arrays at 65°C for 17 hours in a rotating oven (Agilent). Arrays were washed with the wash buffers 1 and 2 and the stabilization and drying solutions from Agilent.

Slides were scanned on an Agilent DNA microarray scanner, and microarray data were extracted with Agilent's Feature Extraction software (v9.5). Four biological dye swap replicates were performed.
Microarray for miRNA analysis was performed using a 8x15K Human miRNA Microarray (G4470B, Agilent), one color platform containing probes for 470 human miRNAs according to the manufacturer's guidelines. Data were extracted by Feature Extraction software (Agilent Technologies).

Microarray data analysis

Data analysis was accomplished using 'R' (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org), mainly through packages in the Bioconductor suite (Gentleman et al., 2004). Probes were flagged for filtering considering saturation, signal above background and uniformity. For microRNA analysis probes that had more than one replicate flagged where eliminated. After probe filtering, arrays were background corrected using normexp methodology. Signal intensity was standardized across arrays via quantile normalization algorithm. For mRNA expression analysis probes that had any of the replicates flagged were eliminated. After probe filtering normalization was performed within and between arrays using loess and Aquantile methods, respectively. Differential expression was assayed using the limma software package (Smyth et al, 2005)and the ontology analysis conducted with GOHyperGAll function (Horan et al., 2008).

Analysis of RNA transcripts by reverse transcription PCR (RT-PCR) and quantitative PCR (Q-PCR)

Total RNA were isolated from 5-20x10^5 B-cells and retro-transcribed using SuperScript™ II Reverse Transcriptase Kit (Invitrogen) and RNase inhibitor (rRNAsin, Promega) was used to achieve a maximal performance. For gene expression analysis of Survivin also called baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5), Bcl-2-related protein A1 (BCLA1), cyclin D2 (CCND2), paxillin (PXN), proto-oncogene tyrosine-protein kinase Fyn (FYN), mitogen-activated protein kinase 1 (MAPK1), c-myc, forkhead box O1 (FOXO1), cyclin-dependent kinase inhibitor 1B (p27), phosphatase and tensin homolog (PTEN) and beta 2 microglobulin (B2M) we used one µl from a 1/10 dilution of cDNA reaction for amplification in a PCR reaction including 10 min. at 95°C and 40 cycles of amplification (95°C 15 sec; 60°C 30 sec; 72°C 30 sec.). Primers details are provided in the supplementary table 3. We used Corbette Rotor Gene 6000 Real-Time PCR and the SYBR Green I dye (Green PCR Master Mix and SYBR ® Green RT-PCR Reagents Kit- Applied Biosystems by Life technology). Amplifications of AID were performed with an initial denaturation step at 95°C for 4 min, followed by 30 cycles of 1 min. at 95°C, 1 min. at 62°C, and 1 min. at 72°C and a final elongation step at 72°C for 5 minutes. The primers used were: Forward 5'- GAG GCA AGA AGA CACTCT GG-3' and Reverse 5'- CTA CTT CTG TGA GGA CCG C-3'.

MicroRNA expression analysis by quantitative Q-PCR

Total RNA from CLL B-cells was isolated using mirVana isolation kit (Applied Biosystems, Ambion, U.S.) and a particular cDNA for each microRNA was synthesized using stem loop retro transcription method (Vasconcelos et al, 2005). As for the expression analysis of mRNA we used Corbette Rotor Gene 6000 RT PCR and the SYBR Green I dye for the quantitative PCR reaction. One µl from 10µl miR specific cDNA reaction was used for amplification in a PCR reaction including 10 min. 95°C hold and 40 cycles of amplification with (95 °C 15 sec; 58°C 30 sec; 72°C 30 sec.). The primers for retro-transcription and for

expression analysis of miR-22, miR-107, miR-15b, miR-26, miR-29 and miR-150 are described on supplementary table 3.

Flow cytometric analysis of total AKT, phospho-AKT and PTEN protein expression

For intracellular detection of AKT, phospho-AKT and PTEN proteins, $5x10^5$ PBMC or LN cells were fixed in PBS 4% paraformaldheyde**and permeabilized** *in* the same buffer containing 0.5% saponin and 5% of fetal bovine serum (FBS) before the addition of the specific antibody. For the analysis of total AKT we used anti-AKT rabbit mAb (#4685, Cell Signaling, Houston, U.S.) and a secondary FITC labelled goat anti-rabbit IgG (Cell Signalling, Boston, U.S.). For assessing the phosphorylation of AKT we used anti-phospho-AKT ($pAKT^{-Thr308}$) conjugated with Alexa 647 (#3375,Thr 308 Cell Signalling, Boston, U.S.). For PTEN detection we used mouse anti-human PTEN (Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.) and a secondary allophycocyanin (APC) labeled goat anti-mouse IgG antibody (Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.). After 45 minutes of antibody incubation at 4ºC, cells were washed twice and analyzed by flow cytometry. Negative isotype controls were performed by incubating the cells with irrelevant antibody in the same experimental conditions. In all cases, expression of these molecules were analyzed in the different CLL B-subsets (quiescent and proliferative cells) using Summit v4.3, from Dako (Colorado, Inc.).

Confocal Microscopy

4% PFA fixated cells were attached to poly-L-lysine covered slides as described elsewhere (Mehta et al., 1983) and washed 3 times with PBS. Unspecific site blocking was performed by incubating the cells with 3% BSA/4% FBS blocking solution containing 0.5% saponin as permeabilization agent for 30 minutes at 37 ºC. Cells were incubated for 1 hour at room temperature with mouse anti-human PTEN (sc-7974, Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.) 1:20; rabbit anti-FOXO1a (sc-67140, Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.) 1:100; goat anti-Survivin (sc-8807, Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.) 1:100 or mouse anti-p27 (#3698, Cell Signaling, Boston, U.S.) 1:1600, all of them diluted in blocking solution containing saponin. All washes were performed with blocking solution containing saponin and then incubated with anti-mouse Alexa 488 (A11029, Invitrogen, U.S.) 1:2000, anti-rabbit Alexa 546 1:1000 (A11012, Invitrogen, U.S.), anti-goat Alexa 633 1:1000 (A21082, Invitrogen, U.S.) and anti-mouse PE (P8547, Invitrogen, U.S.) respectively, for 1 hour at room temperature. After washing with blocking solution containing saponin an additional nuclear staining with DAPI or Propidium Iodide (0,1 µg/mL) for 15 min was performed. Cells were imaged using a Leica SP5 or an Olympus FluoView 1000 confocal laser scanning microscope.

microRNA transfections

We performed transfection of PBMC of CLL patients with miR-22, antagomiR-22 or irrelevant miR conjugated to Cy3 fluorophoro using lipofectamine 2000 (Invitrogen, U.S.). Briefly, $2x10^6$ PBMC were resuspended in Opti-MEM I (Invitrogen, U.S.) and transfected with 50nM of microRNA and 5µl/ml of lipofectamine. After 6 hours the cells were washed and cultured for 24hrs in RPMI 1640 media (Invitrogen, U.S.) supplemented with 20% FBSand then analyzed by flow cytometry and confocal microscopy.

CLL B-cells stimulation

Stimulation of PBMCs from CLL patients were carried out on 2,5 x 10^6 cells/ml, cultured in RPMI 1640 media supplemented with 10% FBS, 4mM glutamine, 100U/ml penicillin and 100mg/ml streptomycin. Activation with human soluble CD40L (Preprotech, Mexico, S.A, de C.V) and IL-4 (Preprotech, Mexico, S.A, de C.V) was performed for 4 days at 5μg/ml and 5ng/ml, respectively. Samples were used for RNA extraction, flow cytometry and confocal microscopy analysis. Cell proliferation was evaluated by intracellular detection of Ki-67 protein through anti–human Ki-67 Alexa Fluor 647 (Santa Cruz Biotechnology, Inc. Dallas, Texas, U.S.). The success of CD40L/IL-4 activation was evaluated by positive RT-PCR for AID transcripts.

Statistical analyses

Expression of mRNA of different genes evaluated by Q-PCR were compared between proliferative and quiescent CLL B-cell fractions using either paired Wilcoxon Signed Rank Test or two tailed unpaired Student's t-test. Concerning protein levels evaluation of PTEN, AKT, $pAKT^{-Thr308}$, FOXO1, Survivin, p27 and Ki-67 molecules analysis by paired Wilcoxon Signed Rank Test were performed. Variables with P values of less than 0.05 were considered to be significant. All analyses were done using GraphPad Prism, version 4.0 (GraphPad Software, San Diego, CA).

For microarray, data analysis statistical significance (p,0.05) was calculated using student's t-test followed by Benjamini-Hochberg false discovery rate correction (FDR) on GeneSpring GX11.0.2 software.

RESULTS

1. Isolation of proliferative and quiescent CLL subsets in progressive UM CLL patients

Since, the presence of the proliferative subset ongoing CSR has been correlated with a poor outcome (Palacios et al, 2010), we performed cytometry assays from isolated PB of 80 Um CLL cases in order to determine which was the frequency of this subset in our cohort. We found that 15% (12 out of 80 analysed CLLs) exhibited this proliferative subpopulation characterized by the presence of a small subset among tumoral cells expressing either IgM and IgG, or IgG alone (data not shown and table 1 Um cases). Figure 1A-C shows a representative UM case with these CLL subpopulations. All of them were characterized by intracytoplasmic and surface expression of IgM, IgG, CD5 and a tumoral clone IgVH rearrangement. A full characterization of the genomic expression profile (GEP) from these fractions was carried out in order to gain further insight on this proliferative subpopulation. To accomplish this, we selected four typical UM and progressive CLL cases in order to fully characterize the messenger RNA (mRNA) and microRNAS expression profiles of both subpopulations. Cell sorting experiments allowed to isolate in one hand the quiescent population (IgM^+, $Ki\text{-}67^{low}$, AID^{neg}), marked as R3, and in the other hand the proliferative one characterized by IgG expression, highest Ki-67 and AID expression (IgG^+, $Ki\text{-}67^+$, AID^+), marked as R4. (Figure 1D-E) (so far referred as R3 for the quiescent and R4 for the proliferative one).Clinical and molecular characterization of CLL patients used to perform GEP (01-04) as well as the other CLLs (progressive and indolent cases) included in this study are provided in table 1.

2. Transcriptome and miRNome analysis of the CLL proliferative fraction suggest a role for the PI3K/AKT activated pathway

To gain insight into the mechanism involved in CLL proliferation, we first evaluated the differential microRNAs expression profiles of quiescent and resting fractions isolated from the CLL cases as depicted in table 1. Analysis of microRNAs expression profile was performed through the hybridization platform able to assess the expression levels of 723 human microRNAs. Fold change analysis followed by Students t-test and Benjamini-Hochberg FDR correction, displayed six microRNAs differentially expressed (p-value ≤ 0,05)**.** The most overexpressed microRNAs in the proliferative subset were miR-22, miR-107 and miR-15b, whereas miR-26a, mir-29a and miR-150 were down-regulated (Table 2). These results were confirmed by Q-PCR as depicted in Figure 2A. miR-22 and miR-15b were significantly overexpressed in the proliferative fraction ($p = 0.012$ and $p = 0.032$, respectively, Wilcoxon matched pairs test), but no significant differences was found for the miR-107. Results showed that the microRNA miR-22 is the most differentially overexpressed between the quiescent and the proliferative CLL fractions (Figure 2A). Bioinformatics analysis of target genes for these microRNAs revealed that these differentially expressed microRNAs in the PF regulate genes principally involved in cell cycle activation, cell proliferation and tumor progression (Table 2).

Next, the same isolated fractions were examined to characterize the mRNA GEP of the proliferative CLL subset. Extracted RNAs were hybridized onto a 4×44K Human Genome Oligo Microarray interrogating §41,000 unique human genes. Our initial study showed a significant number of affected genes grouped in a typical immune activated response. In this analysis different clusters such as cell activation, precursor metabolites and energy, response to stimulus and stress and others are highlighted, (figure 2B). For a complete list of up and down-regulated genes with their corresponding *p*-values, see supplementary material Table S1. After stringent quality control steps, we proceeded to perform bioinformatics analysis in order to better define the involved gene pathways. In these conditions, the PI3-K/AKT pathway, in which 22 genes are highlighted, appeared to play a major role in the PF. (Figure 2C) and supplemental data Table S2). Within this pathway, up and down regulated mRNA targets previously linked with tumor progression and cell proliferation, were found to be differentially expressed among quiescent and proliferative fractions. Among the most upregulated genes we found classical cell cycle activators like Cyclin D2 (CCND2) and mitogen-activated protein kinase 1 (MAPK1), key anti-apoptotic molecules like BCL2A1 and Survivin and proteins implicated in proliferation and migration of tumoral cells like FYN, Talin-1 and Paxillin. Additionally, the PF displayed low expression of different tumour suppressor genes such as PTEN and p27 and interestingly this subset also showed low expression levels of the transcription factor FOXO1 (figure 2D). Q-PCR was performed for all these mRNAs and the fold change expression levels were confirmed (Table 3).

Interestingly, recent evidence demonstrated that miR-22 controls the signalling kinetics of PTEN/AKT/FOXO1 pathway in human cell lines and plays an important role in the regulation of this cascade (Bar & Dikstein, 2010). Since, AKT pathway is one of the most promising targets with encouraging clinical results in CLL (Herman et al, 2010), we further investigated the molecular mechanism of miR-22 over expression and its putative consequences on the AKT pathway in the proliferative and the quiescent CLL fractions.

3. High expression of miRNA-22, is associated with a phosphorylated AKT protein status, low PTEN expression andcytoplasmic inactivation of FOXO1

Taking account that GEP of the microRNAs and mRNAs in the proliferative CLL subset underscored AKT signaling we explored whether proliferative behavior of this CLL fraction could be associated to miR-22 overexpression and to PTEN/AKT/FOXO1 pathway. For this, we evaluated at the mRNA and protein levels the PTEN expression, as well as the total AKT expression and its phosphorylated form ($pAKT^{-Thr308}$) which is a hallmark of the PI3K/AKT pathway activation. Finally we also evaluated the nuclear or cytosolic localization of the transcription factor FOXO1, which is considered as a marker of PI3K/AKT activation pathway (Stahl et al, 2002).

Results showed that in the PF miR-22 is overexpressed and PTEN mRNA is downregulated when compared with their quiescent counterpart. As shown in Figure 3A, miR-22 is three fold overexpressed (mean = 2,7 ± 1.2, p = 0.032) in the PF. In contrast, a twofold decrease in mRNA PTEN expression in the PF was found (mean = [-2.5 ± 0,6], p = 0.021) when compared with its resting counterpart. In line with these results, significantly increased PTEN protein staining is visualized on the CLL B-cells corresponding to the QF when compared with the CLL B-cells of the proliferative pool from 6 CLL cases analyzed, (52.8 ± 7.2 vs 41,3 ± 7,0 respectively, p = 0.017, Wilcoxon matched pairs test), figure 3B. A representative picture of PTEN expression in the quiescent and proliferative fractions from CLL 06 is shown in figure 3C.

Activation of PI3-K leads to generation of $pAKT^{-Thr308}$ through PDK1. Since PTEN is a natural negative regulator of PI3-K signaling we investigated the expression of total AKT1 and of its phosphorylated form$pAKT^{-Thr308}$. Results showed a significant increase of the $pAKT^{-Thr308}$ form in the PF (mean = 62,86 ± 10,64) when compared with their quiescent counterpart (mean = 34,29 ± 5,0; p = 0,078, Wilcoxon matched pairs test) from 7 different patients (figure 3D). In contrast, no significant differences were found in both fractions from the same CLL cases when total AKT was analyzed (QF, mean = 51,24 ± 8,13 and PF, mean 53,57 ± 7,28; p = 0,657, Wilcoxon matched pairs test), figure 3E. A representative case (CLL 02) after flow cytometry analysis for mean fluorescence intensities (MFI) of $pAKT^{-Thr308}$ in the PF (black peak), QF (white peak) and isotype control (dashed peak) is depicted in Figure 3F.

Since, AKT activity is associated to nuclear export and cytosolic degradation of FoxO proteins and since FOXO1 was found downregulated at the mRNA level in the proliferative subset (GEP assays figure and table 3), we assumed that high phosphorylation of AKT protein should lead to the inactivation of FOXO1. Statistical analysis showed that cytoplasmic expression of FOXO1 in the PF is significantly increased when compared with its resting counterpart. Cytosolic/nuclear relationship between proliferative and quiescent fractions clearly demonstrated this assumption (mean = 0.070 ± 0,036 vs mean = 0.210 ± 0,056, respectively, p = 0,0156, Wilcoxon matched pairs test, Figure 3G). Whereas quiescent $IgM^{+}/Ki\text{-}67^{low}/AID^{neg}$ CLL B-cells displayed an homogenous nuclear localization, the proliferative $IgG^{+}/Ki\text{-}67^{+}/AID^{+}$CLL B-cells principally exhibited a cytosolic localization of FOXO1 (figure 4H), which is associated with degradation of this transcription factor.

Overall these results suggest that proliferation of this CLL subset, could be associated to the inhibition of PTEN expression and activation of the AKT pathway as is established by the presence of phosphorylated $AKT^{T\text{-}308}$ and cytosolic translocation of FOXO1.

4. The activation of the PTEN/AKT/FOXO1 pathway in proliferative CLL fraction results in downregulation of p27 and in high expression of Survivin molecules.

After downregulation of PTEN and activation of the AKT cascade, FoxO proteins leave the nucleus and turn off gene expression molecules able to promote cell cycle arrest or apoptosis (Greer & Brunet, 2005). To gain more insight on the proliferation origins of this CLL subset, we evaluated protein expression of Survivin and p27 proteins, two key molecules implicated in cell cycle regulation of CLL B-cells. Whereas Survivin is a negative regulator of apoptosis, p27 is an inhibitor of cell cycle progression. Since both proteins are downstream of the PTEN/AKT/FOXO pathway (Chakrabarty et al, 2013; Stahl et al, 2002)and this cascade has been implicated in CLL progression (Longo et al, 2007), we reasoned that expression analysis of these molecules could help explaining the differential behavior between quiescent and proliferative CLL subsets.

After confocal microscopy studies we observed that p27 expression is principally visualized in the accumulative IgM subset from PB of these patients. In contrast lower expression of this cell cycle inhibitor is found in the PF (figure 4A). Consequent with the GEP results, the statistical analysis displayed a lower expression of p27 in the proliferative fraction (mean = 33,08 ± 5,4;) compared with the quiescent subset (mean = 64,44 ± 9,64; p = 0,0313, Wilcoxon matched pairs test) from 6 CLL patients, (figure 4B).

In addition, epifluorescence studies of the Survivin molecule showed a clear expression of this apoptotic inhibitor in the PF extracted from PB of progressive CLL cases, which contrasted with low or absent expression of this molecule in the resting IgM fraction, (Figure 4C). As shown in figure 4D, statistical analysis confirmed this observation in another 6 UM CLL patients (mean = 1145 ± 140,7 for the proliferative fraction vs mean = 399 ± 35,0 for the quiescent counterpart (p = 0,0301, Wilcoxon matched pairs test).

Overall, these results suggest that the proliferative behavior of this CLL fraction could be related with the low expression of p27 protein and the high expression of the Survivin molecule. In agreement with our previous results the differential expression of p27 and the Survivin molecules in these Um CLL patients may result as a consequence of an activated PTEN/AKT/FOXO1 pathway.

5. Transfection experiments in CLL B-cells with miR-22 and antagomir-22 reveal a key role of this microRNA in the regulation of Survivin and p27 proteins through the PTEN/AKT pathway

Expression of miR-22 regulates the signalling kinetics of PTEN/AKT/FOXO1pathway in human cell lines (Bar & Dikstein, 2010). Taking into account that the ttreatment with antagomirs may represent a therapeutic strategy in several neoplasm (Cho, 2010), we explored the consequences of miRNA-22 inhibition in progressive CLL cases with an activated AKT signaling. Given that our previous results suggest the participation of miR-22 as a regulator in CLL B-cells proliferation, we speculated whether inhibition of miR-22 might couldresults in upregulation of PTEN and p27 molecules. To attempt this, we first evaluated in 22 CLL cases (10 indolent and 12 progressive cases) the phosphorylated $AKT^{-Thr308}$ protein status (Figure 5A). Next, we selected the ten most progressive cases with lowest PTEN expression (progressive CLLs in figure 5B) and we performed miR-22 inhibition by antagomir-22 transfection. Our results showed that after antagomir-22 transfection, PTEN protein levels were increased at similar expression values of some indolent CLLs (Figure

5B, indolent CLLs column). Statistical analysis demonstrated significant differences (*p = 0.002)* when comparing CLL B-cells treated with antagomiR-22 (mean = 3.84 ± 0,48), to those transfected with irrelevant miR (mean = 1.67 ± 0,33) and with untrasfected (UT) control (mean = 1.26 ± 0,23). In contrast, no significant differences were found between irrelevant miR and UT cells (*p=0.331,* figure 5B). A representative case of the 10 CLL B-cells cases transfected with antagomir-22 and the irrelevant microRNA control is shown in figure 5C.

As has been described (Vrhovac et al, 1998) upregulation of p27 is higher in mutated and indolent CLLs than in progressive cases. Regarding p27 expression, basal levels of these molecules evaluated in PB from five progressives and five indolent CLLs from our series are shown, (first section of figure 5D). Interestingly, after transfection with antagomiR-22 we observed a significant increase in p27 protein levels compared with UT and with irrelevant miR controls (*p = 0.035,* mean = 93.4 ± 5,8 for antagomir-22, mean = 61.4 ± 8,37 for irrelevant miR and mean = 52.1 ± 2.3, for UT cells), figure 5D. Statistical analysis confirmed that transfection with antagomir-22 resulted in overexpression of p27 protein compared with irrelevant and UT cells control. A typical immunorreactive pattern of p27 protein, similar to those observed for the quiescent fraction previously described in figure 3 and 4, was found. A representative CLL sample transfected with antagomir-22 and with irrelevant controls is depicted by confocal microscopy analysis in figure 5F. These results show that specific inhibition of miR-22 in progressive CLLs cases increases the protein levels of the tumor suppressor PTEN and p27 cell cycle regulator and suggest an important role for this microRNA in CLL.

To deep more insights into the role of this microRNA on the proliferative behaviour of CLL, we evaluated the assumption that miR-22 could be also implicated in the Survivin expression which was previously described in the PF ongoing CSR process (figure 4). For this, we first evaluated Survivin basal expression in the PB of indolent and progressive CLL cases. As has been described (Granziero et al, 2001), only some patients responder to CD40 engagement express Survivin in the PB (first section figure 5E). To confirm the mentioned assumption we transfected CLL B-cells from the progressive cases (CLL 01, 02, 04, 05, 07 and 12, table 1) and analyzed Survivin expression at 48 hs after treatment by confocal microscopy analysis. Interestingly, our results suggest that after transfection with miR-22 Survivin expression is upregulated in 4 of the 6 evaluated patients (CLLs 01, 02, 04, and 07, table 1) at significant levels (*p = 0.030,* mean = 15.3 ± 1,2), compared with those transfected with irrelevant miR (mean = 11.3 ± 1,0) (figure 6E). No significant differences (*p= 0.093*) were found among irrelevant miR and UT cells. In CLL B cells from those patients that upregulate Survivin proteins levels, a clear cytosolic expression after mir-22 transfection was found (figure 5F). In contrast, low expression or non expression is exhibited in these cells after irrelevant miR transfection and UT cells culture. An immunorreactive pattern corresponding to cytosolic localization of this protein (figure 5F) reminiscent to those displayed in the PF isolated from progressive CLL cases from figure 4, was observed.

Altogether, these results suggest that microRNA-22 could play an important role in the proliferative behaviour of CLL B-cells by preventing the expression of suppressor tumor molecules like PTEN and cell cycle inhibitor protein like p27 and switching on the expression of the Survivin protein.

6. CD40 engagement induce miR-22 expression, AKT activation, Survivin expression and CLL proliferation.

To deep insights into the molecular mechanism that could trigger the PI3K/AKT cascade and lead to CLL proliferation, we evaluated the assumption that external signals as CD40L/IL-4 could be at the origin of this pathway and lead to miR-22 overexpression. To attempt this, we selected CD40 responder patients (CLLs 01, 02, 04 and 07) and stimulated them with CD40L/IL-4. Our results show that CD40 and IL-4 receptors engagement results in a specific overexpression of the microRNA-22, whereas no effects were evidenced in another microRNAs control samples like miR1202, previously described in CLL (Marton et al, 2008) or miR15b after the same activation. Interestingly, these *in vitro* studies also showed increased levels of the $pAKT^{-Thr308}$ form and Survivin protein compared with unstimulated (US) samples (figure 6A-C).

To gain more insights into the clinical relevance of miRNA-22 over PTEN/AKT pathway, we investigated whether decreased proliferation in CLL after CD40 engagement could be obtained following in vitro treatment with specific antagomir-22. For this, we selected four indolent responder to CD40 stimulation CLLs (13, 16, 20 and 22 in Table1). Following isolation of CLL B-cells by CD19 and CD5 incubation, we proceeded to stimulate *in vitro* CLL B-cells with CD40L/IL4 and transfected them with:**a)** miR-22 in order to increase the expression of the proliferation marker Ki-67 or **b)** Antagomir-22 in order to downregulate Ki-67 expression. Our results showed that eitherCD40 engagement or either miR-22 transfection, results in an increased Ki-67 expression compared whit US samples. Interestingly, transfection with miR-22 andactivation with CD40L/IL-4 appears to act in a synergistically form resulting in increasedamounts (three to five fold) of Ki-67 protein in some cases (CLLs 13, 16 and 22) (figure 6D). Additionally, this proliferative pattern assessed by Ki-67 expression is downregulated after inhibition of the miR-22 by their specific antagomir, which was previously shown to increase the expression of PTEN and p27 molecules (figure 3 and 6D).

Although preliminary, these results suggest that at least in some patients, CLL proliferation is upregulated by the presence of miR-22, which in turn leads to the PI3K/AKT pathway activation and Survivin expression after CD40 engagement.

7. Proliferative behaviour of CLL B-cells from lymph nodes is associated with absence of PTEN expression, activation of PI3K/AKT pathway, downregulation of p27 and high expression of Survivin molecules.

To confirm the relevance of our results and to demonstrate that this cascade is also active in the PCs of some CLL cases, we examined the *in-vivo* expression of different molecules involved in the PI3K/AKT signalling. Our initial hypothesis was that the proliferative behaviour of the PF results from downregulation of the PTEN molecule and activation of the PI3K/AKT pathway, which in turn induces cell proliferation underlined by downregulation of p27 cell cycle regulator and high expression of Survivin protein. With this in mind, we could study three different LN from UM and progressive CLLs and we isolated Ki-67 positive and Ki-67 negative fractions from them. Next, we evaluated the PTEN expression, the PI3K/AKT activation as assessed by the presence of $pAKT^{-Thr308}$ and finally we tested whether this proliferative behaviour assessed by Ki-67 expression is correlated with high expression of Survivin and p27 downregulation.

Our results showed that between 7-10% of Ki-67 positive B cells are found in the LN of the selected patients. We could corroborate by CD5 and CD19 markers that almost all this Ki-67 subset corresponded to the leukemic B-cells (data not shown). R1 depicts the selected Ki-67 negative fraction and R2 the selected Ki-67

positive cells which were used in the subsequent experiments (Figure 7A). As shown in figure 7B, a PTEN protein expression profile, similar to that of the quiescent CLL IgM of the PB was observed in the Ki-67 negative cells (R1), whereas low or no expression was found in the Ki-67 positive cells (R2). Interestingly, in the three LN obtained we found that the Ki-67 positive fraction R2 is enriched in cells expressing the phosphorylated form $pAKT^{-Thr308}$ whereas non phosphorylated AKT form predominates in the Ki-67 negative fraction (Figure 7C). Supporting our initial hypothesis, we found that the Ki-67 positive fraction with active PI3K/AKT pathway expresses higher Survivin levels as it is depicted in a representative CLL case in figure 7D. Concerning the expression of p27 cell cycle regulator, we finally evaluated the existence of a negative correlation between the PF from LN (R2) and the Ki-67 negative fraction (R1). As it is depicted in figure 7E the Ki-67 negative fraction showed higher expression levels of p27, whereas low or no expression of this marker is found in the PF extracted from this LN.

Altogether, these results confirm the importance of the PI3K/AKT pathway activation in the proliferative subset of CLL and suggest that the same regulatory loop involving miR-22/PTEN/AKT/FOXO1 which was demonstrated to be activated in the PF ongoing CSR, could be relevant in an *in vivo* scenario in these UM CLL patients.

DISCUSSION

CLL can be defined as a low-grade B-cell tumor, where tumoral cells have previously encountered the antigen, escaped programmed cell death and undergone cell cycle arrest in the G0/G1 phase. Despite the fact that, that most leukemic cells are arrested in cell cycle G0/G1 stages (Caligaris-Cappio & Ghia, 2008), Messmer et al demonstrated that CLL is not only a static disease but also a disease in which different proliferative subsets coexist (Messmer et al, 2005); (Palacios et al, 2010). These observations have turned the attention towards the generation of different sub-populations inside the tumoral clone that either reach a homeostatic balance in patients with good clinical course or an imbalance in patients with poor outcome (Caligaris-Cappio, 2011).

The PI3K signaling pathway is involved in a wide variety of normal cellular processes including cell death, migration, protein synthesis, and metabolism. This enzyme is also an important driver of cell proliferation and cell survival, most notably in cells that are responding to growth-factor–receptor engagement in tumor microenvironments (Cully et al, 2006). PI3-K activity recruits and activates phosphatidylinositol-dependent kinase 1 (PDK1). PDK1 phosphorylates and activates the serine-threonine protein kinase AKT/protein kinase B (AKT1), which inhibits the activities of the family of transcription factors FoxO, which in turn are mediators of apoptosis and cell-cycle arrest. In CLL, PI3K has been described as a key intermediary signaling molecule between the microenvironment and the leukemic clone, transmitting signals from membrane receptors such as the BCR, CXCR4 and CD40 (Longo et al, 2008). A variety of novel kinase inhibitors directed to slow down AKT signaling have recently generated considerable excitement in CLL (Amrein et al, 2013; Lannutti et al, 2011), and demonstrated that this pathway could have a broad impact in the treatment of the disease (Hoellenriegel et al, 2011). The main negative regulator of the PI3K/AKT pathway is PTEN. This tumour suppressor is frequently inactivated in human cancer as a result of genetic lesions or absent by postranscripcional and/or postraductional mechanisms (Cully et al, 2006). FoxO proteins are phosphorylated

by AKT,and translocated from the nucleus to the cytoplasm where they are degraded via the ubiquitin–proteasome pathway (Calnan & Brunet, 2008; Greer & Brunet, 2005; Stahl et al, 2002). When PTEN is active and AKT activity is suppressed, FoxO proteins are able to enter the nucleus and upregulate genes such as p27 promoting cell cycle arrest (Tzivion & Hay, 2011);(Bar & Dikstein, 2010).

Recently, an interesting regulatory loop in PTEN/AKT/FOXO1 pathway has been described through the overexpression of miR-22 microRNA (Bar & Dikstein, 2010). MicroRNAs can reduce the levels of their target transcripts as well as the amount of protein encoded by these transcriptsand there is consistent evidences demonstrating the important role of these molecules in the pathogenesis and progression of various human cancers (Cho, 2010). In CLL, microRNA signature is not only important at the prognostic level but also relevant to CLL pathogenesis (Calin et al, 2005). Understanding the crosstalk between the microenvironment signals that trigger specific microRNAs and mRNAs signatures and the malignant subsets, could give us new keys in the cellular and molecular biology of CLL and lead to design novel strategies in the treatment of this disease. Our group succeeded to identify in PB from progressive UM CLL cases, a small leukemic proliferative subset associated to a clinical poor outcome which is characterized by an active CSR process and high expression levels of AID enzyme and Ki-67 progression marker (Palacios et al, 2010). The present work addresses the genomic and the molecular characterization of this subset, provides novel information about the proliferative behaviour of these leukemic cells and suggests that microRNA-22 plays a key role in the proliferation mediated by AKT pathway in CLL and thus may be a useful therapeutic target.

Genomic characterization at the mRNA and microRNAs levels of this PF showed that AKT signaling pathway appeared to be active in this leukemic subset and that miR-22 is the most overexpressed microRNAs, (Figure 3). Since, miR-22 appears to regulate the signaling kinetics of PTEN/AKT/FOXO1 pathway (Bar & Dikstein, 2010), we speculated that the proliferative behaviour of this CLL subset might be linked with miR-22 overexpression which in turn, might impact via PTEN downregulation in AKT signaling activation. To deep insight into the molecular mechanism of this process we characterized the PTEN protein expression as well as the phosphorylated form of AKT ($pAKT^{-Thr308}$) and the localization of FOXO1 transcription factor in order to confirm the activation of the PTEN/AKT/FOXO1 pathway. Our results showed that upregulation of miR-22 is associated with a low PTEN expression not only at the mRNA level but also at the protein level. In line with these results, $pAKT^{-Thr308}$ form is increased in the PF compared with the RF of the same patients (figure 4). Recent work addressed the issue of PTEN/AKT signaling pathway in CLL (Shehata et al, 2010; Zou et al, 2013), but only few evidence concerning FoxO proteins expression in this leukemia is available (Xie et al, 2012). Since, cytoplasmic localization of FoxO proteins is associated to a degradation pathway and is a hallmark of AKT activity (Greer & Brunet, 2005), we proceeded to evaluate the FOXO1 localization in the proliferative and resting fraction of these UM patients. As has been described in other tumor cells (Ho et al, 2012; Stahl et al, 2002; Tzivion & Hay, 2011), our results clearly showed that FOXO1 protein was mostly translocated to the cytoplasmic compartment in the PF. These results are in agreement with previous evidence linking inhibition of PTEN expression, PI3K/AKT pathway activation and cancer proliferation (Cully et al, 2006) and support the work of Bar *et al*. (Bar & Dikstein, 2010) involving the microRNA miR-22 as a novel regulatory molecule in this cascade.

FoxO proteins are also associated with cell cycle arrest by direct downregulation of p27 protein (Ho et al, 2012; Stahl et al, 2002). Since, mRNA arrays analysis displayed low expression of p27 and high expression of Survivin (figure 3), we evaluated whether this differential expression pattern is maintained at the protein levels and if this characteristic is related with an active PTEN/AKT/FOXO1 pathway. Our results showed that in the PF p27 protein is downregulated and that Survivin molecule overexpressed (figure 5). Assuming that microenvironment interactions trigger Survivin expression (Granziero et al, 2001) and that it could be also downregulate p27 molecule (Frenquelli et al, 2010), our results suggest a link between Survivin and p27 proteins. The view that these two key effectors molecules in CLL could be dependent of an activated PTEN/AKT/FOXO1 pathway and might be are inversely implicated in the leukemic proliferation is an interesting proposal to taking into account.

To gain insight into the regulatory role of miRNA-22 over PTEN/PI3K/AKT/FOXO1 pathway and consequently in the downstream expression of p27 and Survivin molecules in CLL, we proceeded to transfect purified CD19 B-CLL cells. Our results confirmed the assumption that miR-22 expression could be at the origin of the PI3K/AKT pathway activation and suggest the this cascade is responsible for Survivin expression and p27 downregulationin CLL. This was supported by significant increased levels of PTEN expression and p27 upregulation after specific inhibition of miRNA-22, whit the antagomir-22 whereas irrelevant miR failed to affect these expressions (figure 6). In addition, transfection with miR-22 upregulate Survivin expression and a clear cytosolic expression of this protein after mir-22 transfection was found.Reconstitution of PTEN activity in CLL in order to counteract the proliferative behavior of AKT signaling has been described by Shehata et al. (Shehata et al, 2010) and Martin et al. (Martins et al, 2010) Concerning p27 molecule recent data proposed that another microRNAs like 221/222 cluster could also regulate p27 and helps to maintain the CLL B-cells in a resting condition (Frenquelli et al, 2010).

Proliferative leukemic cells are placed in LN and BM, where receive through microenvironment interactions survival signals aiming to avoid apoptosis and acquire favourable tumoralgrowing conditions (Caligaris-Cappio et al, 2013). Our results concerning the origins of this proliferative behaviour in CLL suggest that at least in part, the T-dependent CD40L microenvironment signal could be responsible for the overexpression of the miR-22 in the leukemic clone. This stimulus appears to be also responsible for the threonine AKT1 phosphorylation ($pAKT^{-Thr308}$) confirming the activation of this pathway and also of the Survivin increased levels. Supporting these observations some reports suggest that CD40L is responsible for the AKT activation signaling (Benson et al, 2006) whereas others have demonstrated that PI3K regulates Survivin through AKT activation (Asanuma et al, 2005; Zhao et al, 2010). Altogether, these data confirm previous results (Granziero et al, 2001) showing that the proliferative behavior of some CLL B-cells triggered by CD40 engagement are associated to Survivin expression, but in addition, propose that this proliferative potential is dependent of a PI3K/AKT activated pathway which in turn was switched on by miR-22 overexpression. In this line, our results also showed that in three of four CLL cases, either with CD40 engagement or miR-22 transfection, the expression levels of Ki-67 were increased. More important, CD40L and mir-22 appear to act in a synergic form increasing the expression levels of Ki-67 marker as shown in figure 7. Although these results are the *in vitro* experiments and downregulation of Ki-67 by the Antagomir is not complete, altogether these data underline the importance of the miR-22 in CLL proliferation and suggest that additional studies evaluating the putative role of this microRNA as a therapeutic target become an interesting issue.

The notion that optimal B-cell proliferation requires activation of PI3K/AKT pathway has been previously suggested (reviewed in (Rodon et al, 2013)). However, the activation of this cascade in a proliferative CLL subpopulation which was previously triggered by CD40 engagement, overexpression of miR-22 as well as the involvement of different molecules such as PTEN, FOXO1, p27 and Survivin, all of them linked in an activated PI3K/AKT pathway, constitutes a novel and original contribution of this work. The model proposed to illustrate the putative mechanism responsible for the proliferative behavior in the proliferative pool found in progressive UM CLL patients is summarized in the figure 8.

In addition, the relevance of these data are supported by the *in vivo* results obtained from the LN of three different UM and progressive CLL cases. Despite the fact that we could not study miR-22 expression in these samples, since we could not obtain enough RNA, we could demonstrate that the proliferative subset from different LN as assessed by Ki-67 expression is associated with downregulation of PTEN, activation of PI3K/AKT pathway, Survivin expression and p27 downregulation.

A recent study of Herishanu et al. identified LN as a key site for CLL B-cell proliferation (Herishanu et al, 2011). In this site, the leukemic clone showed up-regulation of gene signatures indicating BCR and NF-kβ activation as well as expression of other genes as E2F, c-MYC and Ki-67. The proliferative subset (IgG+, Ki-67+, AID+) described by our group displays very similar markers to those found by this work, which could suggest that this tumoral subset could have recently aggressed from a PCs in LN. Preliminary results from our laboratory, confirm that the loss of the nourishing microenvironment protection compromises the survival of this particular subset (data not shown). Shehata *et al*. works (Shehata et al, 2010) sugges that microenvironment signals also modulate the PTEN/PI3K/AKT cascade to maintain the proliferative subset in CLL support this view.

Taken together, the data here reported sustain the model of B-CLL growth/proliferation and provide conclusive evidence on the role of microenvironment interactions in the induction of the proliferative pool in CLL. They also underline the physiological importance of maintaining a stringent regulation of the different cascade signals such as PI3K/AKT, and suggest that Survivin overexpression and p27 downregulation are two faces of the same coin that could have a key role in the CLL proliferation. Finally, we propose a novel microRNA (miR-22) as an important modulator of the PI3K/AKT cellular signalling and we advise that a better understanding of the biology of this regulation on an important pathway as PI3K/AKT should help in the design of new therapeutic strategies in CLL.

LEGEND TO FIGURES

Figure 1. Proliferative and quiescent CLL fractions in progressive Unmutated CLL patients. (A-B) Representative flow cytometry profile from CLL patient number 03. Intracytoplasmatic expression of IgM, IgM/IgG and IgG as well as the surface expression of IgG/CD5 are depicted. **(C)** RT-PCR from the IgM and IgG cell sorter isolated subsets. Clonal isotype switch transcripts with tumor-related VH and Cμ or Cγ primers and GAPDH as internal control are shown.**(D)** Quiescent and proliferative CLL fractions. Plot of cell fractionation by cell sorter showing the quiescent fraction characterized by surface IgM expression and low Ki-67 (R3) and the proliferative fraction characterized by surface IgG expression and high Ki-67 marker (R4). **(E)** AID RNAm expression in the PBMC, in the quiescent and in the proliferative fractions of patient 03. GAPDH was used as internal control.

Figure 2. (A) Q- PCR of miR-22, miR15b and miR-107 expression in proliferative and quiescent fraction of CLL B-cells isolated of CLL patients (01-04 in table 1). **(B)**Up regulated and down regulated genes (showing $P<0.01$) in proliferative fraction relative to quiescent fraction were used in an ontology analysis. Ontology classes with an over-representation in our dataset are shown. **(C)** Gene-set enrichment analysis (GSEA) for AKT pathway. Microarray data was analyzed using GSEA software to verify if AKT gene set was significantly enriched in one of the phenotypes. AKT gene set was enriched in proliferative fraction with FDR<0.01. The X-axis of the curve for enrichment scores includes 17840 genes, with those correlating best with proliferative fraction on the left and those correlating best with quiescent fraction on the right. Each vertical blue line represents one of the AKT pathway genes. The left-to-right position of each line indicates the relative position of the gene expression value within the rank ordering of all genes. The cumulative enrichment score as a function of position in the gene list is shown in green reaching a maximum enrichment at a score of 0.55. **(D)** Heatmap representing relative proliferative/quiescent gene expression levels for genes in the AKT pathway. Relative expression levels where obtained from microarray data analysis. Red represents high gene expression in proliferative fraction relative to gene expression in quiescent cells while green represents low gene expression in proliferative fraction relative to gene expression in quiescent cells. Color scale is provided at the bottom of the figure. In bold are the genes whose expression level was confirmed by Q-PCR.

Figure 3. Characterization of expression levels of miR22, PTEN, AKT and FOXO1 molecules in quiescent (Q) and proliferative (P) cells from progressive UM CLL cases.(A) Fold change expression of miR22 and PTEN mRNA in proliferative cells compared with quiescent fraction evaluated by Q-PCR in 6 CLL cases **(B)** PTEN protein expression in Q (open circle) and P (black triangle) cells from 6 CLL cases evaluated by flow cytometry. MFI: mean fluorescence intensities. **(C)** Representative image of confocal microscopy showing PTEN expression in Q and P cells from CLL 06. Scale bar: 5μm, green: PTEN and red: DNA. **(D-E)** Phosphorylated AKT form in Threonine 308(pAKT$^{-Thr308}$) and total AKT protein expression evaluated byflow cytometry in Q (open circle) and P (black triangle) from 7 CLL cases. MFI: mean fluorescence intensities. **(F)** Representative pAKT$^{-Thr308}$ histogram from CLL 02 showing mean fluorescence intensities (MFI) in the

proliferative fraction (black peak), quiescent fraction (white peak) and isotype control (dashed peak). **(G)** Graph depicting the ratio of cytosolic and nuclear pattern of FOXO1 in Q and P cells from 6 CLL cases evaluated by confocal microscopy. **(H)** Subcelular localization of FOXO1 in Q and P cells. Representative confocal microscopy from CLL 06, scale bar: 5µm, green: FOXO1 and red: DNA.

Figure 4.Characterization of expression levels of p27 and Survivin molecules in quiescent (Q) and proliferative (P) cells from progressive UM CLL cases.(A, C). Representative confocal microscopy of p27 and Survivin expression in quiescent (Q) and proliferative (P) fractions. Scale bar: 5µm. **(A)** Green: p27 and red: DNA. **(B)** Red: Survivin and cyan: DNA. **(B, D)** p27 and Survivin protein expression evaluated byflow cytometry in Q (open circle) and P (black triangle) from 6 CLL cases. MFI: mean fluorescence intensities.

Figure 5. MiR-22 and antagomir-22 transfection regulates Survivin and p27 protein expression through the PTEN/AKT pathway. (A) Phosphorylated $AKT^{-Thr308}$ levels in 12 progressive and 10 indolent CLL cases evaluated by flow cytometry MFI: mean fluorescence intensities. **(B)** PTEN protein expression evaluated by flow cytometry in progressive (n=10) and indolent (n=10) CLL cases. PTEN protein expression after transfection with antagomiR-22, irrelevant miR and untrasfected (UT) cells in 5 progressive CLL cases with low PTEN expression. **(C)** Representative histogram of PTEN expression in CLL B-cells transfected with antagomir-22, irrelevant miR and untransfected cells. **(D)** p27 protein expression evaluated by confocal microscopy in progressive (n=6) and indolent (n=5) CLL cases. p27 protein expression after transfection with antagomiR-22, irrelevant miR and untrasfected (UT) cells in 5 progressive CLL cases. **(E)** Survivin protein expression evaluated by confocal microscopy in progressive (n=6) and indolent (n = 5) CLL cases. Survivin protein expression after transfection with miR-22, irrelevant miR and untrasfected (UT) cells of 5 progressive CLL cases. **(F)** Representative confocal microscopy of Survivin (red) and p27 (green) are depicted in a representative sample obtained from the CLL patient number 04 after transfection with antagomir-22, miR-22, irrelevant miR and untrasfected cells. (**) = Statistically significant data, $p \leq 0.05$ and (*) = statistically non significant data.

Figure 6. CD40 engagement induce miR-22 expression, activation of AKT pathway, Survivin expression and CLL proliferation. (A) miR-1202, miR-15b and miR-22 fold change expression of CD40L/IL-4 relative to unstimulated cells. **(B-C)** $pAKT^{-Thr308}$ and Survivin protein expression evaluated byflow cytometry and confocal microscopy respectively in unstimulated (US) and CD40L/IL-4 stimulated cells from four CLL cases. MFI: mean fluorescence intensities. **(D)** Ki-67 expression evaluated by flow cytometry in CLL-B cells from four indolent cases after CD40L/IL-4 stimulation, miR-22 transfection and stimulation with CD40L/IL-4 followed by miR-22 or antagomir-22 transfection. MFI: mean fluorescence intensities. Ctr: unstimulated and untransfected cells.

Figure 7. Isolation of Ki-67 positive and negative fractions from B-CLL lymph nodes highlight activation of PTEN/AKT cascade, Survivin expression and downregulation of p27 protein. (A) Representative dot plot of CD19 and Ki-67 protein expression in lymph node (LN) of an UM CLL patient. R1 and R2 show the gates settled for the sorting of negative and positive Ki-67 cells. **(B)** PTEN protein expression evaluated by confocal microscopy in Ki-67 negative and positive cells from a representative LN. Green: PTEN, magenta: DNA. Scale bar: 5µm. **(C)** Flow cytometry histograms depicting expression of $pAKT^{-}{}^{Thr308}$ expression in Ki-67 negative (R1, grey) and Ki-67 positive (R2, black) CLL B-cells. **(D-E)** Characterization of the expression levelsofSurvivin (left panels) and p27 (right panels) by confocal microscopy in the Ki-67 negative and in the Ki-67 positive CLL B-cells from a representative LN. Scale bar: 5µm.

Figure 8. Hypothetical model of the cell biology of B cell of CLL patients. (1)(2) MiR22 expresssion is induced by the interaction of B-CLL cells with the tumoral microenvironment (CD40L/IL4) in secondary lymphoid organs. **(3)**MiR22 downregulate PTEN and the PI3-K phosphorylates and converts the PIP_2 into PIP_3, which recruits and activates PDK1. **(4)**PDK1 phosphorylates and activates the serine-threonine protein kinase AKT, which inhibits the activities of the family of transcription factors FoxO, which in turn are mediators of apoptosis and cell-cycle arrest. Phosphorilated AKT activates survivin, wich contribiuts to cell progression and proliferation. AKT could also activate the cascade NFkB wich might stimulate the expression of AID.

Table 1: Clinical and molecular characterization of CLL patients. (a) ≤ 2% difference from germline gene defined UM patients, **≥** 2% difference define MUT patients. **(b)** Obtained results in cytometry assays using CD38 cut-off = 30%; **(c)** Time from initial diagnosis to first treatment (TFT); **Neg** = **≤** to 1% of $IgG^{(+)}$ CLL B-cells by cytometry assays.

Table 2. Signature microRNAs expression comparison between proliferative fraction and quiescent fraction of UM CLL patients. MicroRNAs with*P*-value ≤ 0.05 were selected**.** Function and the molecular mechanism described for each microRNAs are shown.

Table 3. Set of mRNAs differentially expressed among proliferative and quiescent fractions linked with tumor progression and/or cell proliferation. Fold change evaluated by Q-PCR comparing proliferative vs quiescent cells of UM CLL patients.

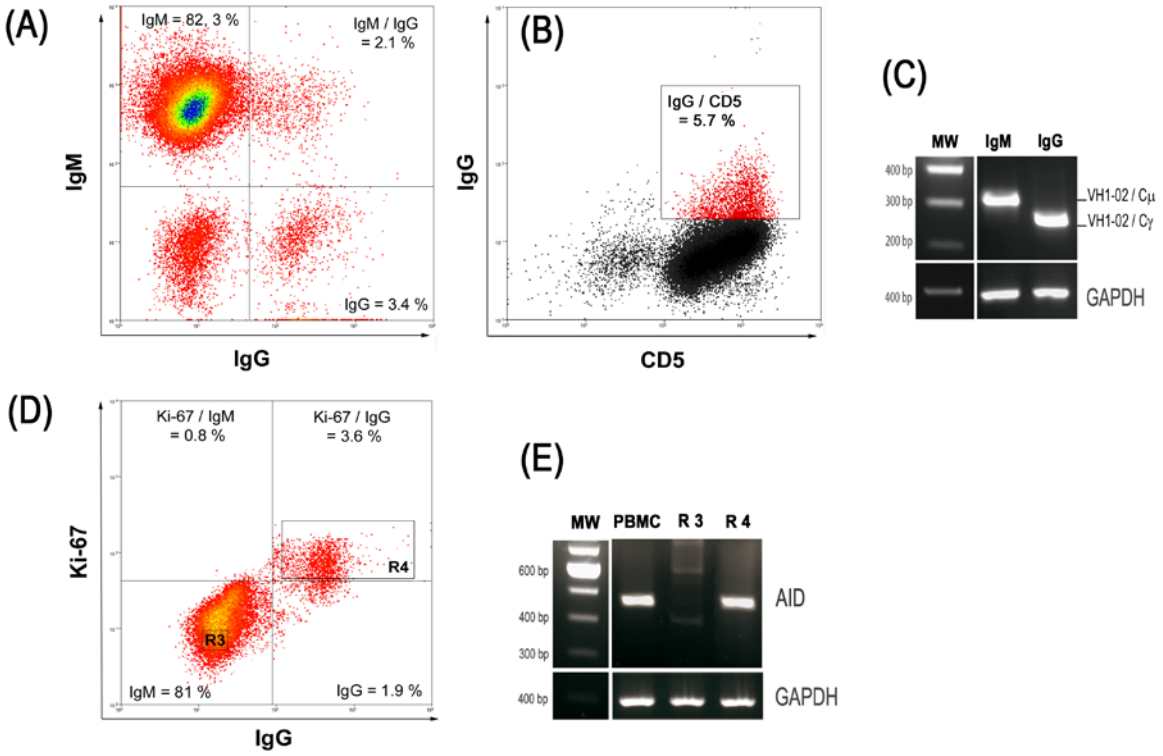
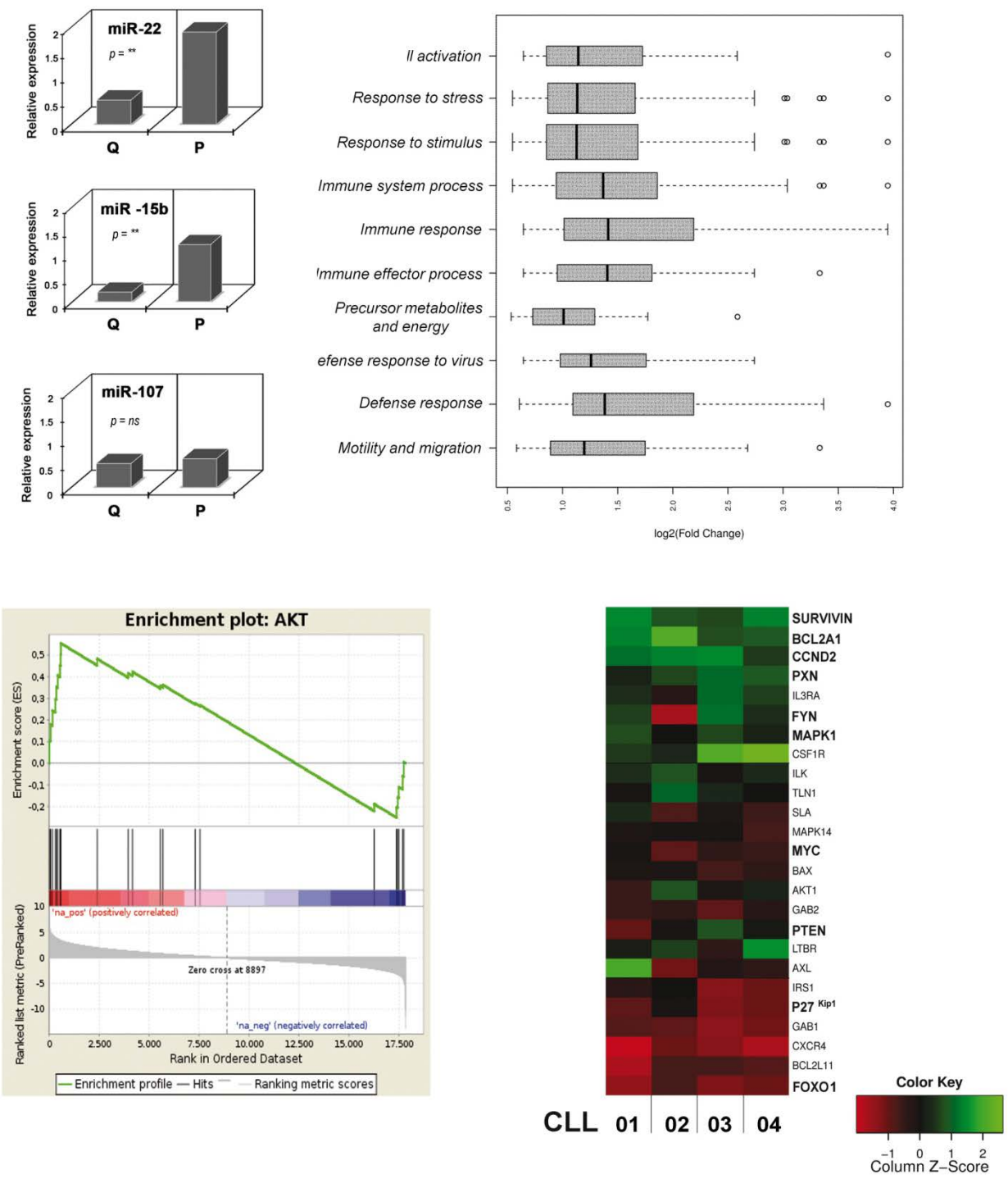

(A)
IgM = 82, 3 %
IgM / IgG
= 2.1 %
IgG = 3.4 %
IgM
IgG
(B)
IgG / CD5
= 5.7 %
IgG
CD5
(C)
MW
IgM
IgG
400 bp
300 bp
200 bp
VH1-02 / Cμ
VH1-02 / Cγ
400 bp
GAPDH
(D)
Ki-67 / IgM
= 0.8 %
Ki-67 / IgG
= 3.6 %
R4
R3
IgM = 81 %
IgG = 1.9 %
Ki-67
IgG
(E)
MW
PBMC
R 3
R 4
600 bp
400 bp
300 bp
AID
400 bp
GAPDH

Figure 1


(A)
(B)
miR-22
p = **
Relative expression
Q
P
miR -15b
p = **
miR-107
p = ns
Il activation
Response to stress
Response to stimulus
Immune system process
Immune response
Immune effector process
Precursor metabolites and energy
efense response to virus
Defense response
Motility and migration
log2(Fold Change)
Enrichment plot: AKT
Enrichment score (ES)
'na_pos' (positively correlated)
Zero cross at 8897
'na_neg' (negatively correlated)
Ranked list metric (PreRanked)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores
SURVIVIN
BCL2A1
CCND2
PXN
IL3RA
FYN
MAPK1
CSF1R
ILK
TLN1
SLA
MAPK14
MYC
BAX
AKT1
GAB2
PTEN
LTBR
AXL
IRS1
P27 Kip1
GAB1
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BCL2L11
FOXO1
CLL 01 02 03 04
Color Key
Column Z-Score
(C)
(D)

Figure 2

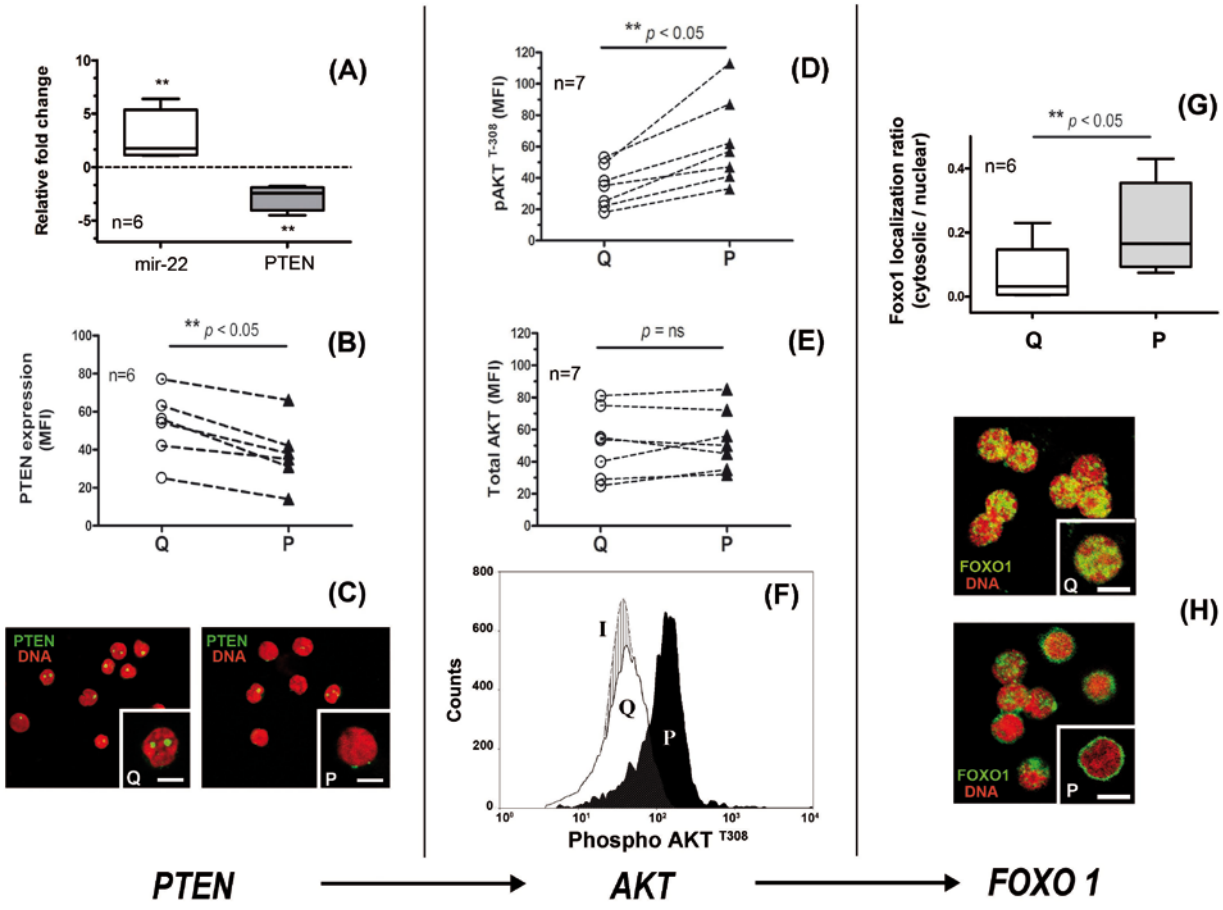
(A)
Relative fold change
n=6
mir-22
PTEN
(B)
** p < 0.05
PTEN expression (MFI)
n=6
Q
P
(C)
PTEN
DNA
Q
P
(D)
** p < 0.05
pAKT T-308 (MFI)
n=7
Q
P
(E)
p = ns
Total AKT (MFI)
n=7
Q
P
(F)
Counts
I
Q
P
Phospho AKT T308
(G)
** p < 0.05
Foxo1 localization ratio (cytosolic / nuclear)
n=6
Q
P
(H)
FOXO1
DNA
Q
FOXO1
DNA
P
PTEN
AKT
FOXO 1

Figure 3

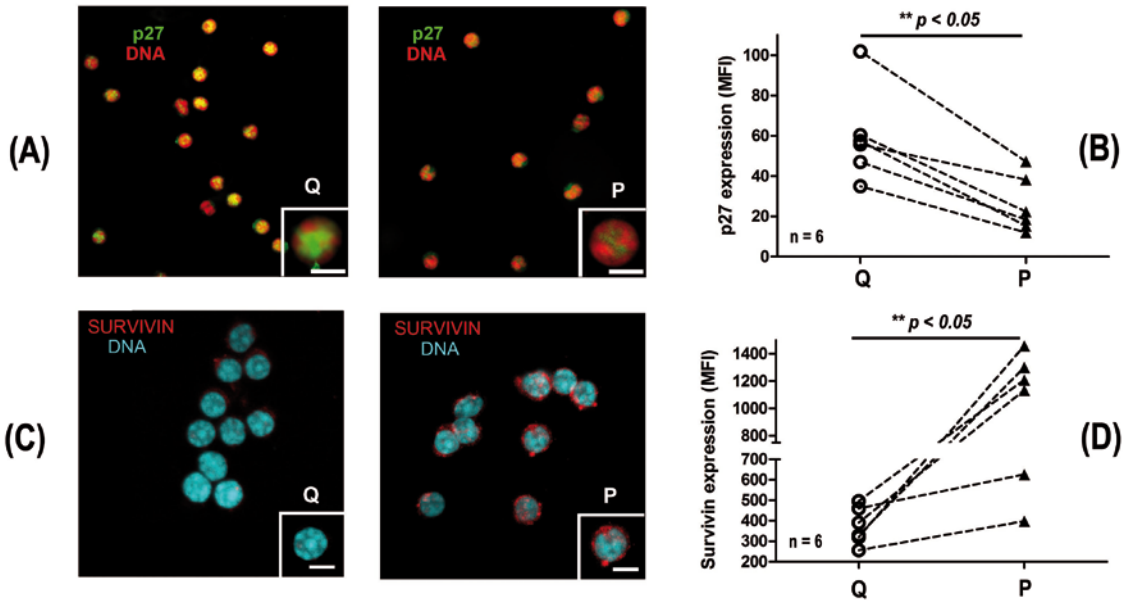
(A)
p27
DNA
Q
p27
DNA
P
(B)
** p < 0.05
p27 expression (MFI)
100
80
60
40
20
0
n = 6
Q
P
(C)
SURVIVIN
DNA
Q
SURVIVIN
DNA
P
(D)
** p < 0.05
Survivin expression (MFI)
1400
1200
1000
800
700
600
500
400
300
200
n = 6
Q
P

Figure 4

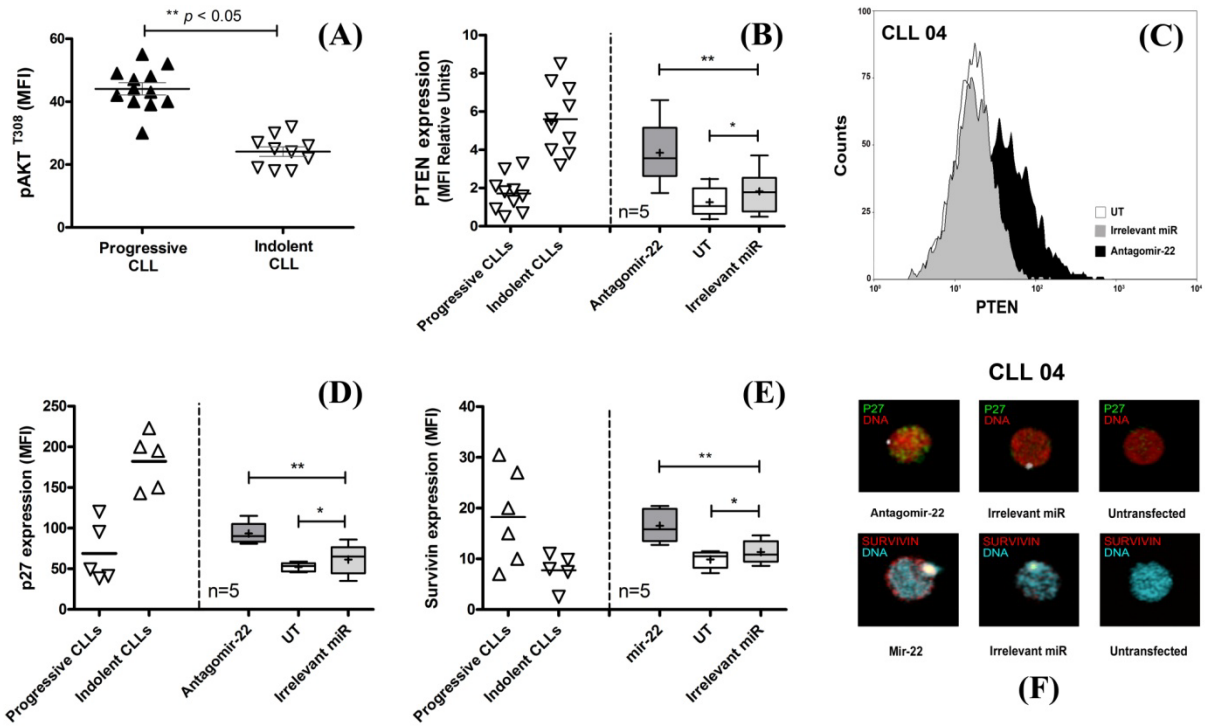
** p < 0.05
(A)
pAKT T308 (MFI)
Progressive CLL
Indolent CLL
(B)
PTEN expression (MFI Relative Units)
n=5
Progressive CLLs
Indolent CLLs
Antagomir-22
UT
Irrelevant miR
(C)
CLL 04
Counts
PTEN
UT
Irrelevant miR
Antagomir-22
(D)
p27 expression (MFI)
n=5
Progressive CLLs
Indolent CLLs
Antagomir-22
UT
Irrelevant miR
(E)
Survivin expression (MFI)
n=5
Progressive CLLs
Indolent CLLs
mir-22
UT
Irrelevant miR
CLL 04
P27
DNA
Antagomir-22
Irrelevant miR
Untransfected
SURVIVIN
DNA
Mir-22
Irrelevant miR
Untransfected
(F)

Figure 5

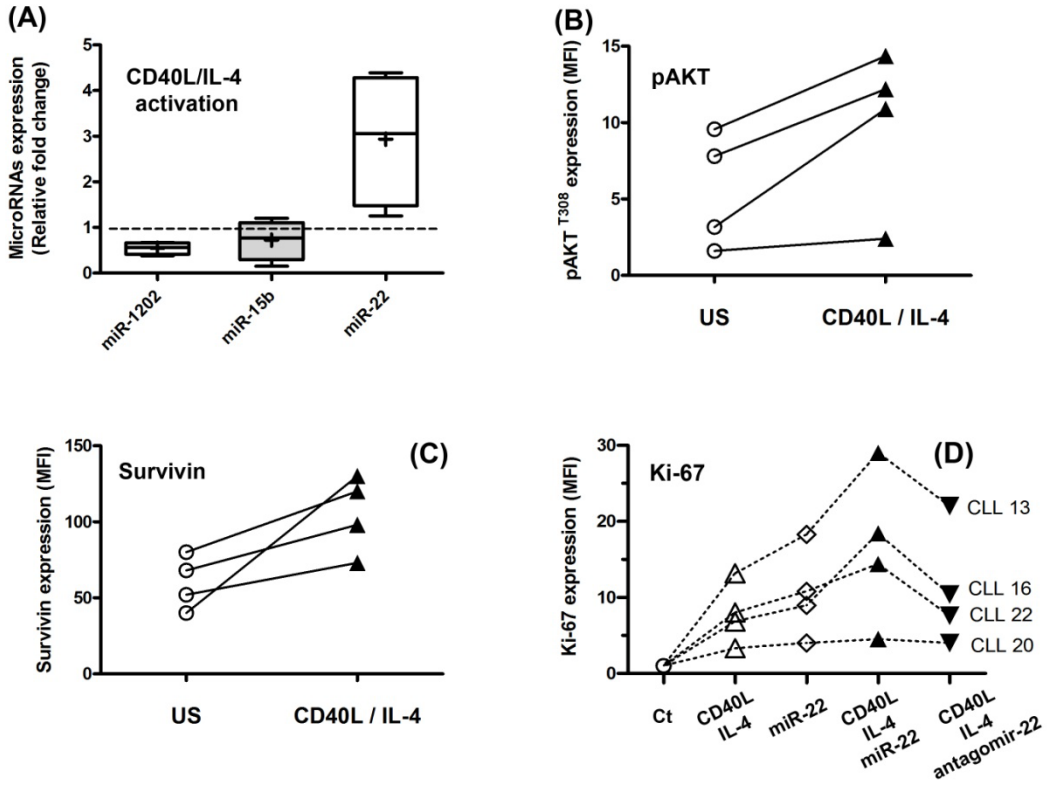
(A)
MicroRNAs expression (Relative fold change)
CD40L/IL-4 activation
miR-1202
miR-15b
miR-22
(B)
pAKT T308 expression (MFI)
pAKT
US
CD40L / IL-4
(C)
Survivin expression (MFI)
Survivin
US
CD40L / IL-4
(D)
Ki-67 expression (MFI)
Ki-67
CLL 13
CLL 16
CLL 22
CLL 20
Ct
CD40L IL-4
miR-22
CD40L IL-4 miR-22
CD40L IL-4 antagomir-22

Figure 6

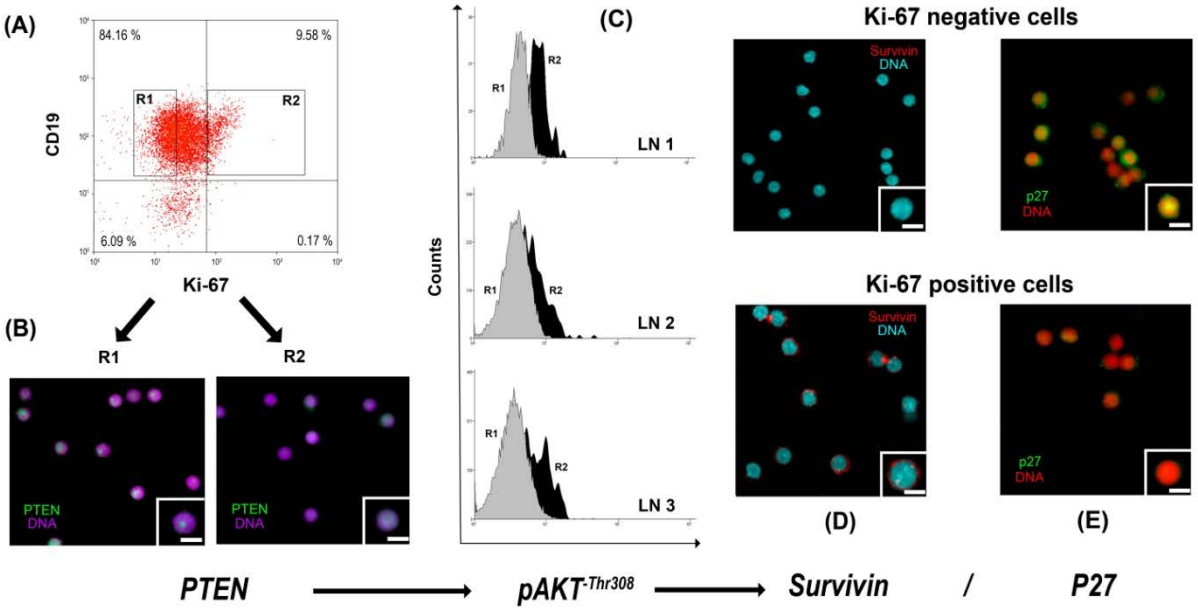
(A)
84.16 %
9.58 %
R1
R2
6.09 %
0.17 %
CD19
Ki-67
(B)
R1
R2
PTEN
DNA
PTEN
DNA
(C)
R1
R2
LN 1
R1
R2
LN 2
R1
R2
LN 3
Counts
Ki-67 negative cells
Survivin
DNA
p27
DNA
Ki-67 positive cells
Survivin
DNA
p27
DNA
(D)
(E)
PTEN
pAKT-Thr308
Survivin
/
P27

Figure 7

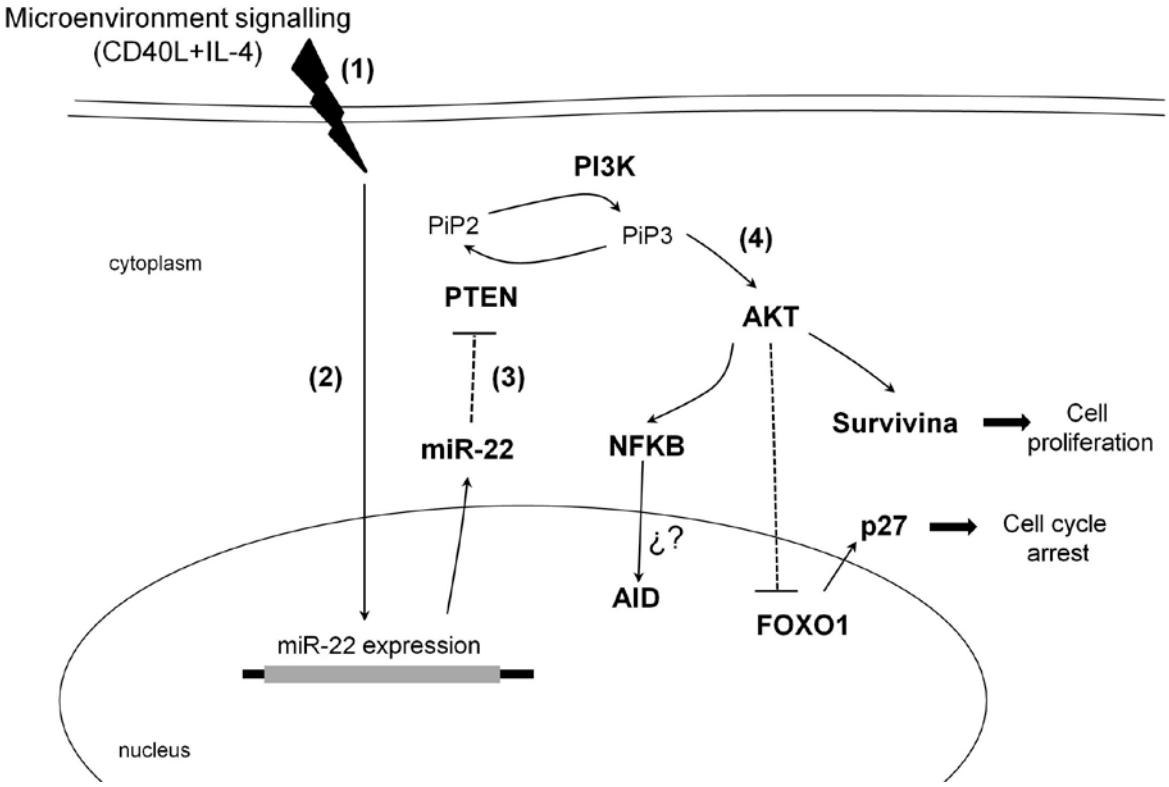
Microenvironment signalling
(CD40L+IL-4)
(1)
PI3K
PiP2
PiP3
(4)
cytoplasm
PTEN
AKT
(2)
(3)
Survivina
Cell
proliferation
miR-22
NFKB
¿?
p27
Cell cycle
arrest
AID
FOXO1
miR-22 expression
nucleus

Figure 8

Table 1

CLL	Diagnostic date / Binet stage	Sample date / Binet stage	Lymphocyte count, x 10^3/μl	VDJ rearrangement (a)	FISH	CD38 % (b)	LPL (Q-PCR)	AID (Q-PCR)	$IgG^{(+)}$ subset(%)	$IgG^{(+)}$/Ki-$67^{(+)}$ subset (%)	Treatment/ TFT, months (c)	Disease related death
01	2002 / A	2003 / C	128	**Um -**VH1-02	del 11q	38	(+)	(+)	6.2	2.2	Yes / 9	Yes
02	2000 / B	2010 / B	220	**Um -**VH1-69*01	del 17p	16	(+/-)	(+)	7.5	2.8	Yes / 8	Yes
03	2009 / A	2009 / A	54	**Um -**VH2-5*10	No	46	(+)	(+)	5.5	3.6	Yes / 24	Yes
04	2005 / A	2010 / B	72	**Um -**VH1-69*01	No	19.5	(+)	(+)	5.1	3.0	Yes / 18	Alive
05	2009 / A	2011 / B	140	**Um -**VH2-5*10	del 17p	36	(+/-)	(+)	3.8	1.5	Yes / 24	No
06	2008 / B	2010 / B	170	**Um -**VH3-48*03	del 11q	22.5	(+)	(+)	5.0	1.2	Yes / 18	Yes
07	2010 / B	2010 / B	130	**Um -**VH3-30*04	Tris 12	45.5	(+)	(+)	3.7	3.0	Yes /13	Yes
08	01-2011 / B	04-2011 / C	48	**Um -**VH3-11*01	No	1.9	(+/-)	(+)	2.5	1.7	Yes / 16	Yes
09	2011 / A	2011 / A	105	**Um -**VH1-2*02	No	2.8	(+)	(+)	2.4	0.5	No	Alive
10	2012 / A	2012 / A	130	**Um -**VH1-69*01	N/A	46.5	(+)	(+/-)	3.5	0.3	No	Alive
11	2012 / B	2012 / B	160	**Um -**VH1-69*01	No	26.2	(+)	(+/-)	2.2	0.7	No	Alive
12	2005 / A	2012 / C	98	**Um -**VH4-34	No	3.9	(+)	(+)	3.2	2.7	Yes / 72	Yes
13	2011 / A	2011 / A	14	**Mut -**VH3-48*02	N/D	N/D	(-)	(-)	Neg	Neg	No	Yes
14	2011 / A	2011 / A	8.9	**Mut -**VH1-03	del 13q	4.5	(-)	(-)	Neg	Neg	No	Yes
15	2011 /A	2012 / A	12	**Mut -**VH4-59*01	No	N/D	(-)	(-)	Neg	Neg	No	Yes
16	2010 / A	2011 / A	54.3	**Mut -**VH3-9*01	N/D	14	(-)	(-)	Neg	Neg	No	Yes
17	2008 / A	2010 / A	24.2	**Mut -**VH1-18*1	No	7.8	(-)	(-)	Neg	Neg	No	Yes
18	2012 / A	2012 / A	10.0	**Mut -**VH4-59*1	No	3.5	(-)	(-)	Neg	Neg	No	Yes
19	2012 / A	2012 / A	26.7	**Mut -**VH1-2*04	del 13q	N/D	(-)	(-)	Neg	Neg	No	Yes
20	2012 / B	2012 / B	21.9	**Mut -**VH3-23	No	32	(-)	(-)	Neg	Neg	No	Yes
21	2012 / A	2012 / A	17.9	**Mut -**VH3-48*02	No	8.9	(-)	(-)	Neg	Neg	No	Yes
22	2010 / A	2012 / A	6.5	**Mut -**VH6-1*01	No	N/D	(-)	(-)	Neg	Neg	No	Yes

Table 2

Systematic Name	Fold Change	P Value	Deregulation in cáncer	Molecular mechanism	References
Up-regulated microRNAS					
hsa-miR-22	3,216	0,0002	Upregulated in breast cancer	- Represses estrogen receptor alpha expression by targeting the estrogen receptor alpha mRNA 3`region	(Cho, 2010)
hsa-miR-107	1,010	0,0385	Overexpressed in Colorectal Cancer	- Promotes tumor progression by targeting the let-7 microRNA. - Upregulate tumour invasion and metastasis by targeting DICER 1 protein	(Chen et al, 2012) (Li et al, 2011)
hsa-miR-15b	0,984	0,0045	Overexpressed in Colorectal and Cervical cancers	Data not available	(Wilting et al, 2013) (Xi et al, 2006)
Down-regulated microRNAS					
hsa-miR-26a	-1,534	0,0362	Downregulated in B-cell Lymphomas and hepatocellular carcinoma	- Inhibits cancer cell proliferation and induce tumor-specific apoptosis. - Mir-26a dowregulation resulted in MYC-induced lymphomagenesis	(Zhang et al, 2012) (Sander et al, 2008)
hsa-miR-29a	-0,801	0,0444	Downregulated in B-cell Lymphomas and progressive CLLs	- miR-29 downregulation resulted in induction of CDK6 and IGF-1R and mediated MYC-driven lymphomagenesis	(Zhang et al, 2012)
hsa-miR-150	-1,5171	0,0014	Downregulated in Acute Myeloid Leukemia	- miR-150 Inhibits Cell Proliferation and Leukemogenesis targeting FLT3 and Myb genes	(Jiang et al, 2012)

Table 3

Symbol	Q-PCR Fold Change[IgM/ IgG] vs Zero	Status	Entrez Gene Name	Molecular Function	References (** related to CLL)
SURVIVIN	8.0	up	Survivin	Apoptosis inhibitor	(**) (Granziero et al, 2001),
BCL2A1	6.3	up	BCL2-related protein	Cell cycle progression	(Choi et al, 1995) (**) (Kipps, 1997)
CCND2	5.0	up	Cyclin D2	Cell-matrix adhesion	(**) (Igawa et al, 2011)
PXN	4.5	up	Paxillin	Apoptosis inhibitor	(Metalli et al, 2010)
FYN	4.2	up	Proto-oncogene tyrosine-protein kinase	Immune response regulation	(Picard et al, 2004)
MAPK1	2.9	up	mitogen-activated protein kinase 1	Intracellular protein kinase cascade	(**) (de Totero et al, 2008),
c-myc	1.1	up	v-myc myelocytomatosis viral oncogene homolog	Cellular proliferation and differentiation	(**) (Larsson et al, 1991),
FOXO1	- 6.5	down	Forkhead box O1	Regulation of cell proliferation	(Greer & Brunet, 2005)
p27	- 3.2	down	Cyclin-dependent kinase inhibitor 1B	Controls cell cycle progression	(**) (Decker et al, 2002),
PTEN	- 3.0	down	Phosphatase and tensin homolog	Regulation of B cell apoptotic process	(**) (Shehata et al, 2010)

DISCUSIÓN Y CONCLUSIONES

La LLC es la forma más frecuente de leucemia del adulto, en las poblaciones de origen caucásiso. Tanto el modo de presentación como la evolución no son uniformes. Alrededor de un tercio de los pacientes nunca requieren tratamiento y mueren de causas ajenas a la enfermedad. Un segundo tercio, comienza con una fase indolente con una evolución benigna, pero luego se agrava y necesita tratamiento. En general estos pacientes mueren por causas vinculadas a la enfermedad. El último tercio se presenta como una enfermedad grave desde el inicio, necesita de un tratamiento inmediato y muere de causas vinculadas a la enfermedad (Vasconcelos et al, 2003).

Esta leucemia puede ser definida como una hemopatía del linfocito B de bajo grado de malignidad cuyas células tumorales encontraron previamente el Ag, lograron escapar al proceso de apoptosis y quedaron congeladas en fase G0/G1, como lo muestra los niveles elevados del regulador negativo de ciclinas p27 (Vrhovac et al, 1998). Dado el rol central de esta proteína en la progresión del ciclo celular, su aumento de expresión podría ser responsable de la acumulación de células tumorales en etapas precoces del ciclo celular. En favor de esta hipótesis está la sobre-expresión de moléculas anti-apoptóticas como BCL-2, BCL-XL, BAG-1 y MCL-1 (Dighiero & Hamblin, 2008). Pese al hecho de que la mayoría de las células leucémicas están bloqueadas en la fase inicial del ciclo celular, Messmer y col demostraron que la LLC no es exclusivamente una enfermedad acumulativa sino que junto a este pool de células quiescentes co-existe un segundo pool de células que proliferan activamente (Messmer et al, 2005).

En los últimos años han surgido evidencias que resaltan la importancia del microambiente inmunogénico en la progresión de la enfermedad. En particular el dialogo entre linfocitos B de LLC con células accesorias en microambientes especializados dentro de la médula ósea o de los órganos linfoides secundarios, favorecen la progresión de la enfermedad promoviendo el crecimiento del clon maligno. Conocer y entender cuáles son las moléculas implicadas en ese diálogo nos puede proporcionar pistas para poder diseñar nuevas estrategias terapéuticas para esta enfermedad.

La observación de que las células de LLC se acumulan progresivamente *in vivo* pero mueren cuando se las cultiva *in vitro* sin ningún tipo de estimulación, resalta la importancia del microambiente tumoral en la habilidad de entregar señales que colaboran con la sobrevida y crecimiento del clon maligno. En este sentido es que se ha descrito que debe existir un equilibrio entre las células quiescentes en SP y las células en división, proliferantes que recientemente recibieron señales del microambiente de los CPs. El balance entre los dos compartimentos podría contribuir al curso clínico variable de los pacientes de LLC (Caligaris-Cappio, 2003).

Es importante destacar que este trabajo nos ha permitido sumar evidencias de la importancia que existe entre las señales del microambiente y el curso clínico de la LLC. En particular, las señales del microambiente en los CPs son capaces de gatillar la inducción de la expresión de enzimas necesarias para generar una respuesta inmune efectiva. En este sentido es que la expresión de AID, que juega un rol fundamental en la generación de la diversidad de los anticuerpos (Okazaki et al, 2002; Yoshikawa et al, 2002), puede ser inducida por señales de linfocitos T (CD40L/IL4), mediante estímulo de de los receptores de tipo Toll o también mediante interacción con células estromales (TACI, BAFFR). Dado que Oppezzo y col. describieron que las células tumorales de pacientes de mal pronóstico, presentaban un proceso activo de CC y expresaban AID en muestras de SP (Oppezzo et al, 2005a; Oppezzo et al, 2003) y al hecho de que el grupo de Chiorazzi describió que AID se encuentra restringida a una fracción del clon tumoral (Albesiano et al, 2003), nos llevó a preguntarnos si esta sub-población es la responsable de la expresión anómala de AID, la cual a su vez sería la consecuencia de un contacto con señales del microambiente tumoral en estos pacientes NM de mal pronóstico.

Nuestros resultados muestran que la expresión de AID se encuentra mayormente restringida en la sub-población tumoral con CC activo en pacientes NM. Cuando se compara ésta sub-población leucémica minoritaria (2-5%) con la sub-población mayoritaria que expresa exclusivamente IgM, se puede observar una alta expresión de moléculas relacionadas con la progresión celular como Ki-67, c-myc, y Bcl2 así como también genes relacionados con el tráfico o migración linfocitaria como la integrina CD49d y las quimioquinas CCL3/4. Asimismo, ésta sub-población se caracteriza por la expresión reducida de p27. Finalmente, la presencia de esta sub-población en SP de pacientes con LLC, se observa en el caso de pacientes progresores y de mal pronóstico.

Debido a que los resultados nos sugirieron que la población AID^{pos} podría ser parte del compartimento proliferante de la LLC, se procedió a aislar la población quiescente de la proliferante de un mismo paciente y estudiar el perfil de expresión génica a nivel de ARNm y de miRNAs en cuatro pacientes diferentes. En una primera parte, nuestros resultados confirman el perfil proliferante de la población AID^{pos} a través de la sobre-expresión de un conjunto de genes relacionados al ciclo celular y la activación de una célula B que está siendo estimulada en un CG. Así como también, la regulación negativa de otros tantos genes cuya baja expresión es característica de una célula no proliferante. Además de ello, los resultados aportan datos nuevos sugiriendo que la sobre-expresión del miR-22 podría estar implicada en la generación de un circulo activador de la proliferación celular a través de la vía PI3K/AKT. Dado que miR-22 regula la expresión de PTEN y por consiguiente activa la vía de señalización PTEN/PI3K/AKT, la cual se haya directamente relacionada con sobrevida y proliferación, se procedió a estudiar en más detalle la expresión de PTEN y de AKT en las fracciones leucémicas proliferantes y quiescentes. Los resultados mostraron que en las células proliferantes existe una asociación entre la alta expresión del miR-22, baja expresión de PTEN tanto a nivel de ARNm como de proteína y una alta expresión de AKT fosforilada o activada. Con el propósito de confirmar la activación de esta vía de señalización se procedió también a estudiar algunas de las moléculas implicadas río abajo de la vía PI3K/AKT, como FOXO, p27 y survivina. Los resultados muestran que la sub-población AID^{pos} presenta la vía de señalización PI3K/AKT activada. Con el propósito de estudiar el rol del miR-22 en la activación de la vía realizamos ensayos de transfección con el miR-22 y antagomiR-22 (molécula inhibitoria del miR-22) los cuales mostraron que efectivamente el miR-22 regula la expresión de PTEN en células B de LLC. A su vez, dado de que este subset de células presenta características de haber estado en contacto reciente con los centros proliferativos, nos preguntamos si la expresión del miR-22 podía ser gatillada por señales del microambiente tumoral. Los resultados nos indican que un estímulo T dependiente del tipo CD40L e IL-4 puede inducir la expresión del miR-22 y por lo tanto favorecer la activación de AKT en estas células proliferantes. Por último determinamos que le comportamiento proliferativo de las células B de LLC de nódulos linfáticos se encuentra asociado con ausencia de la expresión de PTEN y activación de PI3K/AKT resaltando la importancia de esta vía en la activación de la sub-población proliferante en LLC.

En resumen este trabajo resalta la importancia del microambiente tumoral en brindar estímulos capaces de favorecer la sobrevida de una sub-población de linfocitos B de pacientes NM de LLC. Posiblemente estas señales del microambiente colaboran con la inducción de la expresión de AID, enzima mutagénica, que podría también estar relacionada con la progresión de la enfermedad, así como también con la inducción de la expresión del miR-22 capaz de activar la vía de señalización PI3K/AKT asociada con sobrevida y proliferación.

Debido a los resultados planeados anteriormente la discusión de este trabajo va a estar centrada en los siguientes temas: 1- Rol de AID en la población proliferante de pacientes NM progresores; 2-Importancia del miR-22 en la activación de la vía PTEN/AKT en la población proliferante AID^{pos}.

1-Rol de AID en la población proliferante de pacientes NM progresores

La buena comprensión de los mecanismos que gobiernan la inter-relación entre el microambiente y las células tumorales se ha convertido, dado el rol que el microambiente parece jugar en la evolución de la enfermedad, en uno de los grandes desafíos para comprender la fisiopatología de la LLC y poder diseñar nuevas terapéuticas. Así, el aislamiento y el análisis de la sub-población tumoral proliferante inducida por el contacto con el microambiente se ha constituido en un centro de gran interés. Distintos grupos han examinado esta cuestión a través del estudio de diferentes sub-poblaciones expresando distintos marcadores fenotípicos como CD38 (Damle et al, 2007; Pepper et al, 2007), o la intensidad de expresión de las moléculas CD5/CXCL4 (Calissano et al, 2011).

Oppezzo y col. demostraron que los linfocitos tumorales de los pacientes con LLC NM expresaban en forma preferencial la enzima AID y que un porcentaje bajo de estas células tumorales habían sufrido un proceso activo de CC (Oppezzo et al, 2003). Esto nos llevó a efectuar la hipótesis que las células tumorales presentes en SP y que habían llevado a cabo este último proceso, como consecuencia de la acción de la AID, podrían ser representativas y de gran interés en la identificación de genes que, relacionados con la proliferación celular, podrían ser blancos terapéuticos para una posible terapia centrada en las poblaciones proliferantes de la LLC.

A partir de células B de SP de pacientes de LLC NM progresores aislamos la población IgM^{pos} de la $IgM^{pos}IgG^{pos}$ y la IgG^{pos} y estudiamos a nivel de ARNm la expresión de AID. Nuestros resultados muestran que la expresión de AID se encuentra principalmente restringida a la población de linfocitos B $IgM^{pos}IgG^{pos}$ y a la IgG^{pos}. Dado que normalmente la expresión de AID se encuentra en linfocitos B de los CPs de los órganos linfoides secundarios, la presencia de AID en linfocitos B de pacientes de LLC en SP nos llevan a hacernos ciertas preguntas relacionadas con la regulación de la expresión de esta enzima, pero también sobre la función de AID en esta sub-población.

En relación a la regulación de la expresión de AID nos preguntamos lo siguiente:

1- ¿AID se encuentra expresada de forma constitutiva en esta sub-población de linfocitos B de LLC NM progresores? o ¿es una señal de que estas células recientemente estuvieron en contacto con el centro proliferante recibiendo estímulos que gatillaron su expresión?
2- ¿Existen defectos en moléculas implicadas en la regulación de la expresión de AID que favorecen su expresión anómala en linfocitos B de LLC NM?
3- ¿La expresión de AID es causada por un evento único o es mantenida en curso del tiempo en estos pacientes?

Para contestar alguna de estas preguntas a partir de pacientes NM progresores que expresan AID, aislamos las células B, las cultivamos *in vitro* con y sin estímulo (análogos de ADN bacteriano y estimulación con CD40L+IL-4) y estudiamos la expresión de AID en el correr de los días. Los resultados nos mostraron que la expresión de AID en estas células de pacientes era mantenida en cultivo con estímulo. Sin embargo, en las células cultivadas sin estímulo se observó que la expresión de AID decrece con el tiempo (datos no mostrados). Estos resultados sugieren que la expresión de AID en estas células no es constitutiva sino que señales del microambiente son esenciales para el mantenimiento de su expresión. De la misma manera estos resultados apoyan la idea de que estas células en un proceso activo de CC fueron recientemente estimuladas por señales del microambiente inmunológico.

En relación a la regulación de la expresión de AID a nivel molecular, hoy en día se sabe que la expresión de AID se encuentra regulada a nivel de factores de transcripción y miRNAs con funciones activadores o inhibitorias. En este sentido, NFkB y STAT6 (Dedeoglu et al, 2004), HoxC4 (Park et al, 2009), Pax5 (Gonda et al, 2003; Oppezzo et al, 2005a), Bcl6 y IRF8 (Basso et al, 2012)

son los factores de transcripción activadores, mientras que IRF4, Blimp1 y ID2 son factores inhibitorios (Lee-Theilen & Chaudhuri, 2010). En LLC, es importante destacar que la expresión de AID en estos pacientes NM con un proceso activo de CC se encuentra asociada con la expresión del gen Pax5a completo en pacientes de LLC (Oppezzo et al, 2005a) . En este trabajo se mostró que la ausencia de AID y CC se asocia con la reducción de transcriptos de Pax5a y la presencia de una segunda forma de "splicing" que presenta una completa deleción del exón 8 (Pax5/ΔEx8). Lo que resulta más interesante aún es que ambas isoformas (Pax5 y Pax5/ΔEx8) presentan el sitio de unión al promotor de AID y por lo tanto estos datos sugieren que Pax5/ΔEx8 podría jugar un rol en su propia regulación y de forma indirecta regularía AID y el proceso de CC (Oppezzo et al, 2005a).

En relación a la regulación de la expresión de AID y con respecto a la pregunta de si la expresión de AID es mantenida en el curso del tiempo, nuestros resultados muestran que en esta sub-población de linfocitos B de pacientes NM la expresión de AID es mantenida *in vivo* en el tiempo. Esto podría ser la consecuencia de un contacto reciente con el microambiente tumoral en los ganglios linfáticos o la médula ósea, donde las células tumorales están en estrecho contacto con linfocitos T que expresan CD40L, que es capaz a través del contacto con CD40 expresado por el linfocito B, de inducir la expresión de AID. Alternativamente, la expresión de AID podría resultar de un contacto con un posible autoantígeno (revisado en (Oppezzo & Dighiero, 2013) capaz de estimular al linfocito B de LLC mediante el BCR o TLRs y que de alguna manera favorece la sobrevida del clon tumoral. Comprender cuales son las señales capaces de inducir la expresión de AID en estos pacientes progresores puede ser de gran ayuda a la hora de diseñar una estrategia terapéutica que impida el diálogo entre el linfocito B y el microambiente tumoral. Sin embargo, a pesar de que muchos esfuerzos se han puesto en la búsqueda del posible auntoantígeno, aún su identificación no ha sido posible.

La determinación de la sub-población de linfocitos B de LLC que expresa la enzima AID genera muchas interrogantes acerca de cuál es el rol de AID en esta sub-población. Lo cierto es que AID es una enzima implicada en los procesos de generación de diversidad de anticuerpos, participa en el CC y la HS en diferentes lugares anatómicos y diferentes tiempos dentro de los órganos linfoides secundarios. Curiosamente estos pacientes que presentan un proceso activo de CC no presentan HS, ya que las Igs no presentan mutaciones somáticas y se parecen a la línea germinal.

Estos resultados podrían sugerir la existencia de posibles co-factores necesarios para realizar uno u otro de los procesos (Oppezzo et al, 2003). En este sentido poder encontrar e identificar posibles co-factores podría aportar datos sobre la biología del linfocito B más allá de la LLC.

A su vez, es importante destacar que la funcionalidad de AID en pacientes de LLC fue demostrada por la existencia de mutaciones en la región pre-switch (pre-Sμ) del ADN (Oppezzo et al, 2003). Más recientemente Patten y col. determinaron la funcionalidad de AID en pacientes NM y MUT debido a que observaron en células B de LLC estimuladas con CD40L/IL4 generan roturas de doble hebra en el ADN, un aumento del proceso de CC así como también mutaciones en la región variable de las Igs indicando HS de novo (Patten et al, 2012). Pese a que el perfil mutacional de la Ig tumoral no sufre prácticamente ninguna alteración durante un período largo de evolución de la enfermedad, un trabajo del mismo grupo, mostró que la estimulación de células B de LLC de pacientes MUT o NM con células T, tanto *in vitro* como *in vivo*, resultó de nuevas mutaciones en los genes de VH de las Igs evidenciando la funcionalidad de AID. De esta manera ellos proponen que este podría ser un modelo para estudiar la funcionalidad de AID fuera del locus de las Igs y de esta manera asociar su expresión con la LLC más agresiva (Chu et al, 2013).

Aunque al día de hoy no existen evidencias directas demostrando que AID es la responsable de las aberraciones cromosómicas observadas en los pacientes de LLC, numerosos trabajos asocian la expresión de AID con un aumento de mutaciones cromosómicas y la presencia de una enfermedad más agresiva (Leuenberger et al, 2010). Por esta razón, es que se cree que AID podría ser en parte responsable de la evolución clonal en pacientes con a un curso agresivo de la enfermedad (Patten et al, 2008; Patten et al, 2012).

Por otro lado, también en relación a la funcionalidad de AID, se ha descrito que la misma juega un rol importante en la regulación de la expresión de genes, en particular, es capaz de desmetilar islas CpG del ADN e inducir su expresión (Bhutani et al, 2009; Munoz et al, 2013). En este sentido, es que parte de nuestro grupo de trabajo intenta determinar si AID en esta población de linfocitos B juega un rol en la regulación de la expresión de genes que podrían contribuir a la proliferación y expansión del tumor.

La población AID^{pos} que presenta un proceso activo de CC presenta un perfil diferencial de moléculas relacionadas con ciclo celular y apoptosis. En este trabajo determinamos que existe un aumento de Ki-67, c-myc, Bcl2 así como una baja expresión del regulador del ciclo celular p27 en la población de linfocitos B AID^{pos} con un proceso de CC activo en comparación con la población AID^{neg}. En particular, Ki-67 es una proteína nuclear que se encuentra expresada en las fases G1, S, G2 y M del ciclo celular, mientras que se encuentra ausente en reposo o G0. El proto-oncogen c-myc codifica para el factor de transcripción MYC, quien regula la expresión de una gran cantidad de genes involucrados en división celular, crecimiento celular (ARN ribosómico y proteína) y apoptosis. Una desregulación de la expresión de c-myc ha sido asociado al cáncer (Dang et al, 1999). A su vez, se ha descrito que pacientes de LLC que presentan una evolución clínica desfavorable presentan una alta expresión de la proteína anti-apoptótica Bcl2, así como también del inhibidor de quinasas dependientes de ciclinas p27 (Vrhovac et al, 1998). En nuestro trabajo la evidencia de una alta expresión de Ki-67 y Bcl2 y del ARNm de c-myc, así como una disminución del p27 estarían sugiriendo que las células AID^{pos} con un activo proceso de CC presentan características de una célula proliferante de un CG, posiblemente jugando un rol en la sobrevida del clon tumoral en estos paciente NM de mal pronóstico.

Por otro lado, es importante destacar que la sub-población AID^{pos} en pacientes de LLC NM mostró una expresión de moléculas relacionadas con el microambiente inmunogénico activado. En particular se determinó que ésta sub-población presenta una sobre-expresión de CD49d y que la mitad de esta subpoblación (50-60 %) es CD38 positiva. Además de esto estos linfocitos B muestran alta expresión de las quimioquinas CCL3/4 en comparación con su contrapartida en reposo IgM^{pos}. CD49d forma parte de la superfamilia de las integrinas capaz de unirse a la molécula de adhesión celular vascular 1 (VCAM-1) regulando la unión de las células a la matriz extracelular. Su expresión ha sido asociada a la expresión con CD38 quien por su parte se une a CD31 estimulando la sobrevida y crecimiento celular. La sobreexpresión de CD38 y CD49d, están asociadas a un pronóstico negativo para la LLC (Damle et al, 1999; Gattei et al, 2008) y están implicados en el dialogo entre las células B y el microambiente. A su vez, las quimioquinas CCL3 y CCL4 son pequeñas proteínas (8-10 kDa) producidas por células hematopoyéticas que actúan como potentes quimioatrayentes para monocitos, macrófagos, células dendríticas, T y "natural killer". Es interesante destacar que un trabajo de Zuccheto y col. determinaron que células B $CD38^{pos}/CD49d^{pos}$ de pacientes con LLC sobre-expresan las quimioquinas CCL3/4, a diferencia de

la población $CD38^{neg}$/$CD49d^{neg}$ (Zucchetto et al, 2009). En este trabajo los autores proponen que los linfocitos B reciben señales del microambiente que activan cascadas se señalización que inducen la expresión de las quimioquinas CCL3/4 capaces de reclutar células del linaje mieloide (Zucchetto et al, 2010). Nuestros resultados en conjunto con los de Zucchetto resaltan la importancia de estas moléculas como jugadores importantes de la activación de células B generadas por las interacciones con el microambiente tumoral.

Estos resultados en conjunto sugieren que la sub-población AID^{pos} que presenta un proceso activo de CC y que expresa moléculas relacionadas con la proliferación y un microambiente tumoral activado en pacientes NM progresores forma parte del compartimento proliferante de la LLC.

En este sentido es importante recordar que grandes esfuerzos se han hecho con el propósito de identificar y caracterizar la población proliferante. En particular, Damle y col. han propuesto a la población $CD38^{pos}$ como parte de la población proliferante (Damle et al, 2007). Dado que la expresión de CD38 varía con la evolución de los pacientes, que se expresa en un gran porcentaje en células en pacientes NM y el hecho que estos autores no pudieron establecer una relación clara cuando compararon ambas poblaciones con el pronóstico hace que al día de hoy las células $CD38^{pos}$ se las considera como una población heterogénea, donde una fracción de las células $CD38^{pos}$ presentan característica de proliferantes y mientras que la otra fracción representa las células en reposo, quiescentes. En particular, nuestros resultados muestran que una fracción de la sub-población AID^{pos} expresa CD38 y recordando los resultados de Messmer donde sólo un pequeño porcentaje de entre 0,08-1,7% de linfocitos B proliferan *in vivo*(Messmer et al, 2005), estos datos en conjunto sugieren que una fracción de la población con el proceso activo de CC y expresando AID puede formar parte del pool proliferante en pacientes con LLC.

Más recientemente Calissano y col. proponen un modelo sobre la biología celular de la LLC. En este modelo, la fracción proliferante se encuentra formada por células recientemente divididas que han emigrado del ganglio linfático ($CXCR4^{low}CD5^{high}$). La fracción quiescente ($CXCR4^{high}$ $CD5^{low}$) se encuentra enriquecida por células menos vitales que necesitan migrar hacia los ganglios linfáticos para recibir señales de sobrevida o directamente morir en la periferia (Calissano et al, 2011). Nuestros resultados en el tema apoyan de alguna manera el modelo de

Calissano debido a que la sub-población de linfocitos B AID^{pos} son también $CD5^{pos}$ y expresan moléculas relacionadas con un microambiente tumoral activado (CD38, CD49d, CCL3/4). En conjunto estos resultados resaltan la importancia del microambiente tumoral en la sobrevida de las diferentes poblaciones proliferantes.

Lo cierto es que si la población AID^{pos} es una población proliferante, sería de esperar que ésta sub-población se volviera con el tiempo la población tumoral dominante, sin embargo esto no es lo que sucede. Por lo tanto, proponemos que la sub-población AID^{pos} que se observa en SP en el caso de formas muy agresivas de la enfermedad, es una población que emigró recientemente del microambiente tumoral y que al perder la estimulación positiva de éste, está condenada a la apoptosis. En apoyo a esta idea se ha determinado que AID puede introducir mutaciones que inducen la apoptosis de las células (Zaheen et al, 2009). Recientemente se ha descrito que AID es capaz de generar una recombinación, llamada recombinación del locus suicida, que elimina por completo las regiones constantes de las Igs (Peron et al, 2012). Esto genera que el linfocito B no produzca más Igs lo que lo lleva a la muerte. En este sentido podríamos pensar que al menos una fracción de la sub-población AID^{pos} podría estar recombinando el locus suicida lo que conduciría a la muerte de las células.

Lo cierto es que a pesar de los grandes avances en la identificación y caracterización de las sub-poblaciones proliferantes de la LLC, entender el rol biológico de las mismas sigue siendo una de las interrogantes sin responder en el área. En este trabajo nosotros logramos establecer una correlación entre los pacientes que presentan la sub-población con el proceso activo de CC y expresan AID y la sobrevida. La presencia de esta sub-población en SP se observa en pacientes con una progresión rápida de la enfermedad.

En suma este trabajo agrega nuevas evidencias que apoyan la hipótesis de que la LLC no es solamente una enfermedad acumulativa de linfocitos B quiescentes (Burger et al, 2009a; Chiorazzi, 2007) sino que existen linfocitos tumorales con una alta capacidad proliferante. El equilibrio entre ambos compartimientos celulares, podría ser el responsable de la heterogeneidad clínica observada así como del proceso evolutivo de esta leucemia. La caracterización de la población proliferante permitiría comprender cuales son las vías de señalización activadas que juegan un rol importante en la sobrevida y proliferación celular,

permitiendo identificar posibles moléculas blancos especificas con el objetivo de diseñar una terapia finalmente efectiva para esta leucemia (Burger et al, 2009a).

Con el propósito de caracterizar la población proliferante AID^{pos} que presentan un CC activo en estos pacientes NM progresores aislamos la población proliferante (Ki-67^{pos}, IgG^{pos}, AID^{pos}) y quiescente (Ki-67^{neg}, IgM^{pos}, AID^{neg}) de un mismo paciente y estudiamos el perfil de expresión a nivel ARNm y microRNA por microarreglos.Los resultados de esta parte del trabajo constituye el segundo tema que pretendo describir en la discusión.

2- Importancia del miR-22 en la activación de la vía PTEN/PI3K/AKT en la población AID^{pos} proliferante

El análisis bioinformático del perfil de expresión génica mostró que los genes (miRNAs y ARNm) diferencialmente expresados se encuentran relacionados con ciclo y proliferación celular así como también a la progresión tumoral. Estos resultados fueron posteriormente confirmados por PCR cuantitativa y señalaron al miR-22 como el microRNA más diferencialmente expresado entre ambas poblaciones.

Curiosamente la función del miR-22 es un tanto controversial. Algunos trabajos lo describen como supresor de tumor mientras que otros lo proponen como un oncogen. En particular, el miR-22 inhibe la invasión y migración del tumor en cáncer gástrico, hepático o de pulmón (Guo et al, 2013; Ling et al, 2012; Zhang et al, 2010). Sin embargo, por otro lado también se ha descrito que miR-22 puede jugar un rol oncogénco inhibiendo al supresor de tumor PTEN y activando la vía PI3K/AKT, relacionada con sobrevida y proliferación celular (Bar & Dikstein, 2010). A su vez, se ha descrito que el miR-22 promueve la sobrevida celular vía la represión de PTEN en respuesta a radiación UV (Tan et al, 2011). El miR-22 también pude ser inducido por el carcinógeno "benzo[a]pyrene-7,8-diol-9,10-epoxide", producto de la combustión y característico del humo del cigarro. Se observó un incremento de la expresión del miR-22 en células transformadas del epitelio bronquial tratadas con el carcinógeno. En estas células epiteliales el miR-22 también es capaz de regular negativamente la expresión de PTEN (Liu et al, 2010). Más recientemente Song ha identificado al miR-22 como un potente proto-oncogen para enfermedades hematopoyéticas

(Song et al, 2013a). Asimismo, propone que el miR-22 promueve y potencia la invasividad y metástasis en cáncer de mama (Song et al, 2013b).

Es importante destacar que a pesar de que varios estudios se han centrado en la búsqueda de miRNAs relacionados con la progresión tumoral, no existen hasta el momento publicaciones relacionando miR-22 con la proliferación en LLC. Tanto el trabajo de Calin (Calin et al, 2005) donde determinaron miRNAs diferencialmente expresados en pacientes de mal y de buen pronóstico (NM ZAP70^{pos} y MUT ZAP70^{neg} respectivamente) y más recientemente el estudio Mraz y col. donde describieron miRNAs asociados a pacientes con la deleción 17p (Mraz et al, 2009), ninguno de estos trabajos mostró al miR-22 como molécula relacionada a la proliferación tumoral. De todas maneras la diferencia importante entre los trabajos de Calin y Mraz con el nuestro, es que nosotros estamos describiendo una sobre-expresión del miR-22 en una sub-población del clon tumoral que presenta características proliferantes en pacientes NM de mal pronóstico sobre-expresando la enzima AID. En este caso nuestro estudio está centrado en el perfil de expresión de miRNAs diferenciales de distintas sub-poblaciones dentro del clon tumoral y no en la totalidad del clon leucémico. Dado que la población de interés en donde se describe la sobre-expresion del miR-22 es una parte muy minoritaria del clon, dicha sobreexpresión podría ser pasada por alto al realizar estudios más generales que involucran a todo el clon leucémico.

Por otro lado, es importante destacar que el estudio del perfil de expresión a nivel de ARNm mostró la presencia de 25 genes asociados a la vía de señalización PI3K/AKT, que se encontraron diferencialmente expresados entre la población quiescente y proliferante. La misma se encuentra relacionada a diferentes procesos incluyendo, migración celular, síntesis y metabolismo de proteínas, muerte y sobrevida celular. Hecho interesante, en distintas leucemias la vía PI3K/AKT se encuentra constitutivamente activada, presumiblemente debido a señales del microambiente a través del BCR, CD40 o CXCR4 (Longo et al, 2007). En este sentido es que la PI3K de esta manera permite una activación basal de la quinasa AKT relacionada con la sobrevida y proliferación celular (Barragan et al, 2006). Una vez que la célula recibe señales de activación, la PI3K cataliza la fosforilación del segundo mensajero lipidico PIP_2 en PIP_3, el cual recluta y activa la quinasa PDK1. Esta enzima es capaz de fosforilar y activar la serina-treonina quinasa AKT relacionada con la sobrevida y proliferación celular.

La vía PI3K/AKT se encuentra regulada por el gen supresor de tumor PTEN. Esta fosfatasa es un antagonista de PI3K, que cataliza la formación de PiP_2 a partir de PiP_3. Debido al importante rol que cumple PTEN en el control de la quinasa AKT su desregulación ha sido asociada a diferentes tipos de cánceres humanos (Cully et al, 2006). Incluso, la inactivación de PTEN generado por mutaciones en el ADN o modificaciones pos-traduccionales han sido asociados a cáncer (Bar & Dikstein, 2010; Cully et al, 2006). Un trabajo reciente en LLC mostró que un aumento de las funciones de PTEN podrían inhibir la actividad proliferante de la quinasa AKT (Shehata et al, 2010), resaltando la importancia de PTEN en la regulación de la proliferación celular de linfocitos B de pacientes de LLC.

Debido a que el miR-22 regula negativamente PTEN y activa AKT, nos preguntamos si existe una relación entre el miR-22 y la vía PTEN/PI3K/AKT en células B de pacientes de LLC y en particular en la sub-población proliferante AID^{pos} de pacientes NM. En este sentido, nuestros resultados mostraron que el miR-22 se encuentra sobre-expresado y PTEN disminuido tanto a nivel de ARNm como proteína en la sub-población proliferante en comparación con linfocitos B quiescentes. A su vez, determinamos que la expresión de AKT total no varía ente ambas poblaciones, sin embargo la fracción proliferante presenta más AKT en su estado activado, a través de la fosforilación de la treonina 308 ($pAKT^{Thr308}$). Esta asociación, entre el miR-22 y PTEN, fue confirmada mediante ensayos de transfecciones con miR-22 y antagomiR-22 (molécula complementaria al miRNA), donde determinamos que el miR-22 efectivamente regula la expresión de PTEN en células B de LLC.

Con el propósito de determinar si $pAKT^{Thr308}$ determina una mayor proliferación celular en la sub-población AID^{pos}, estudiamos la expresión de proteínas río abajo de la vía de traducción de señales como ser el factor de transcripción FOXO1, el regulador negativo del ciclo celular p27 y el inhibidor de la apoptosis survivina.

FOXO1 es un factor de transcripción que regula genes involucrados en el estrés oxidativo, reparación de ADN, ciclo celular y apoptosis. Se ha determinado que $pAKT^{Thr308}$ es capaz de fosforilar a FOXO, lo que determina su translocación del núcleo hacia el citoplasma donde es degradado por la vía de ubiquitina-proteasoma (revisado en (Tzivion & Hay, 2011)). Cuando PTEN es funcional, la actividad de AKT es suprimida y FOXO es capaz de entrar al núcleo y regular la

expresión de genes que promueven el arresto del ciclo celular (Bar & Dikstein, 2010; Greer & Brunet, 2005; Tzivion & Hay, 2011). En este sentido es que la localización subcelular de FOXO1 es una evidencia indirecta de la activación de AKT. Por esta razón, estudiamos la localización sub-celular de FOXO y observamos que en la población proliferante este factor de transcripción se encuentra mayoritariamente en el citoplasma indicando que AKT se encuentra activada.

Dado que FOXO regula la expresión de p27 (Ho et al, 2012; Stahl et al, 2002), molécula sobreexpresada en células B de pacientes de LLC (Vrhovac et al, 1998), estudiamos la expresión de p27 en ambas poblaciones, la proliferante y la quiescente. Los resultados mostraron que este regulador negativo de la entrada al ciclo celular se encuentra disminuido en la población proliferante. A su vez, dado que AKT puede regular al inhibidor de la apoptosis survivina a través del NFkB (Hideshima et al, 2007) y al hecho de que los resultados del perfil de expresión génica mostraron una sobre-expresión de survivina en la población proliferante, es que estudiamos la expresión de survivina a nivel de proteína en ambas poblaciones. Los resultados mostraron que hay una mayor expresión de survivina en la sub-población proliferante AID^{pos}. Es importante destacar que a pesar de que esta descrito que survivina presenta diferentes formas de splaicing, las cuales no todas ellas son formas activas (Li, 2005; Nakagawa et al, 2004), en este trabajo no nos centramos en el estudio de las distintas formas. Sin embargo, es importante señalar un trabajo de Ganziero y col. mostraron que la mayoría de los pacientes de LLC no expresan survivina y que curiosamente solamente un grupo de pacientes de LLC clasificados como respondedores, eran capaces de expresar survivina luego de una estimulación CD40L/IL4 (Granziero et al, 2001). Por esta razón, es que en conjunto estos resultados resaltan la importancia de las interacciones del microambiente inmunogénico en la sobrevida del clon tumoral. En este sentido, la sub-población con un proceso activo de CC, expresa AID, CD49d, CCL3/4 también survivina indicando que esta sub-población recientemente recibió señales de sobrevida en un centro proliferante de un órgano linfoide secundario.

En resumen, hasta el momento determinamos que la sub-población AID^{pos} presenta una alta expresión del miR-22, una disminución de la expresión de PTEN, mayor cantidad de $pAK^{Tthr308}$, una disminución de FOXO y de p27, así como un aumento de survivina. Dado que confirmamos que el miR-22 regula negativamente la expresión de PTEN mediante ensayos *in vitro*, estos resultados sugieren que el miR-22 es una molécula clave en la regulación de la vía de traducción

de señales PTEN/PI3K/AKT en las células proliferantes AID^{pos} de los pacientes NM de LLC de mal pronóstico.

Finalmente, dado que la población proliferante AID^{pos} presenta características de células activadas recientemente en CG es que nos preguntamos si los estímulos del microambiente tumoral pueden gatillar la expresión del miR-22 y por consiguiente activar la vía AKT. Los resultados mostraron que estímulos T dependientes (CD40L/IL4) pueden inducir la expresión del miR-22, la activación de $pAKT^{Thr308}$ y de survivina en células B de pacientes de LLC. A su vez, mediante ensayos de activación (CD40L/IL4) y transfecciones con el miR-22 y su inhibidor (el antagomiR), observamos que hay una mayor proliferación, aumento de Ki-67, en células B de pacientes estimulados con CD40L/IL4 y transfectados con el miR-22, a diferencia de células B estimuladas con CD40L/IL4 junto con el inhibidor del miR-22 (antagomiR-22). Estos resultados indican que existe una relación entre el miR-22 y la proliferación tumoral en células B de pacientes de LLC.

En resumen y de acuerdo a los resultados planteados proponemos que pacientes NM progresores que presentan una sub-población con un proceso activo de CC recientemente estuvieron en contacto en un CG recibiendo señales de proliferación y sobrevida. Los estímulos recibidos en el microambiente activado permitieron probablemente la inducción de la expresión de AID así como del miR-22. Debido a que AID es una enzima mutagénica que en células B de pacientes de LLC es completamente funcional (Chu et al, 2013 ; Oppezzo et al, 2003; Patten et al, 2012) una sobre-expresión de AID por tiempos prolongados podría favorecer la inducción de las aberraciones genéticas observadas en pacientes con un perfil agresivo y de esta manera contribuir a la evolución clonal en la LLC (Albesiano et al, 2003; Oppezzo et al, 2003; Patten et al, 2012). Por otro lado, el miR-22 parece jugar un rol importante en la regulación de la via PTEN/PI3K/AKT relacionada con la sobrevida y proliferación. A pesar de que existe una activación basal de AKT en células B de pacientes de LLC, en este trabajo observamos que la sub-población AID^{pos} presenta una mayor activación de AKT comparado a la población quiescente dado que observamos una disminución del factor de transcripción FOXO1 y el regulador negativo del ciclo celular p27 así como una activación del inhibidor de la apoptosis survivina en la población proliferante AID.Finalmente este trabajo propone al miR-22 como una molécula clave en la regulación de la vía PTEN/PI3K/AKT en células B de pacientes de LLC.

Dada la asociación de la expresión de AID y del miR-22 en la proliferación de las células B de LLC sería interesante estudiar si existe alguna conexión entre ambas vías de traducción de señales. En este sentido es importante recordar que AID puede ser activad a través del factor NFkB (Dedeoglu et al, 2004) y que dependiendo de la señal externa recibida este factor de transcripción puede ser inducido por la vía AKT. Nuestros resultados sugieren que el miR-22 podría estimular, al menos en parte y de manera indirecta, la expresión de AID, sin embargo más estudios serían necesarios para poder confirmar esta sugerencia.

A partir de nuestros resultados y en conjunto con los observados por Shehata (Shehata et al, 2010) se desprende la idea de que una restauración de las funciones de PTEN podría ser clave para inducir la inhibición de la vía AKT y por consiguiente la muerte del clon tumoral. En este sentido una inhibición del miR-22 en las células proliferantes podría funcionar como una posible terapia para pacientes NM progresores. Sin embargo, creemos que aunque es una estrategia prometedora queda mucho aún por aprender sobre las funciones del miR-22 en células de LLC.

Un progreso mayor en el tratamiento de la LLC está dado por el advenimiento de nuevas terapéuticas inhibidoras de la quinasa BTK (PCI-32765, Ibrutinib) y de la quinasa PI3K-δ (CAL-101, idelalisib), cuyos resultados son muy promisorios (Hallek, 2013). Estas drogas se caracterizan por un patrón de respuesta dado por una reducción muy importante de la masa tumoral y por la irrupción en SP de una hiperlinfocitosis de linfocitos tumorales. Este último fenómeno podría ser la consecuencia de una recirculación de los linfocitos tumorales, que serían obligados de salir de sus nichos en los órganos linfoides, que los protegen de la apoptosis y los ayudan a proliferar. Al ser privadas de esta protección, estas células tumorales estarían condenadas a la apoptosis espontánea. La idea de bloquear el efecto del microambiente sobre las células tumorales con inhibidores de quinasas, BTK y de PI3K-δ ha constituido un avance significativo en las últimas pruebas de tratamiento en esta leucemia.

En esta línea de pensamiento nuestros resultados aportan nuevos argumentos a favor del uso de inhibidores de la vía PI3K y subrayan la importancia de seguir estudiando posibles efectos inhibitorios sobre el micro RNA miR-22, el cual parecería ser responsable al menos en parte del perfil proliferante de la población leucémica descrita en este trabajo de Tesis.

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LISTA DE ABREVIATURAS

Ags	antígenos
AKT	serin-treonin quinasa AKT
ATM	ataxia telangiectasia
Bcl2	"B-cell lymphoma 2"
BCR	recptor de célula B
BIRC3	baculovirus IAP conteniendo el repetido3
Btk	Bruton tirosin-quinasa
CD19	**"C**luster of **D**ifferentiation **19"**
CD20	**"C**luster of **D**ifferentiation **20"**
CD22	**"C**luster of **D**ifferentiation **22"**
CD23	**"C**luster of **D**ifferentiation **23"**
CD5	**"C**luster of **D**ifferentiation **5"**
CGs	centros germinales
CLL	"Chronic Lymphocytic Leukaemia"
CUL5	cullina 5
FOXO	**"Forkhead box protein O1"**
Hb	Hemoglobina
IAP	"inhibitor of apoptosis"
Igs	Inmunoglóbulinas
IL2	interleuquina 2
IL4	interleuquina 4
INFγ	interferon γ
KLH6	"Kelch-like 6"
LDH	lactato deshidrogenasa
LLC	Leucemis Linfoide Crónica
miRNAs	microRNAs
MYD88	diferenciación mieloide gen 88 de respuesta primaria,

NFkB	factor nuclear kappa B
NLC	¨nurse like cells¨
NPAT	proteína nuclear ataxia telangeiectasia
p27	"cyclin-dependent kinase inhibitor 1B", p27, Kip1
PI3K	"Phosphatidylinositide 3-kinases"
PIP_2	fosfatidil-insitol (4, 5) bifosfato
PIP_3	fosfatidil-inositol (3, 4, 5) trifosfato
PLEKHG5	"pleckstrin homology domain containing, family G"
PPP2R1B	fosfatasa 2, subunidad regulatoria A beta,
PTEN	fosfatasa y tensin homóloga deletado en el cromosoma 10
SF3B1	factor 3 de splicing, unidad B1, SF3B1
SLL	Small Lymphocytic Lymphoma
SP	sangre periférica
Survivina	**"**baculoviral inhibitor of apoptosis repeat-containing 5",BIRC5
TGM	transglutaminasa7
TLRs	"Toll like receptors"
TRAF2	TNF asociado al factor 2
TRAF3	TNF asociado al factor 3

ANEXOS

1-"Microenvironment Interactions in Chronic Lymphocytic Leukemia: A Delicate EquilibriumLinking the Quiescent and the Proliferative Pool" (2011)

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2-"Lipoprotein lipase expression in unmutated CLL patients isthe consequence of a demethylation process induced bythe microenvironment" (2013)

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3-"Origins and Consequences of AID Expression in Lymphoid Neoplasms"(2013)

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2

Microenvironment Interactions in Chronic Lymphocytic Leukemia: A Delicate Equilibrium Linking the Quiescent and the Proliferative Pool

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1. Introduction

Chronic lymphocytic leukemia (CLL) is the commonest form of leukemia in Europe and North America, and mainly, though not exclusively, affects older individuals. It has a very variable course, with survival ranging from months to decades [1]. It is a neoplastic disorder, characterized by progressive accumulation of monoclonal B lymphocytes, expressing CD5 and CD23 molecules and low amounts of surface membrane Ig and CD79b molecules [2]. About one-third of patients never requires treatment, has a long survival and dies of causes unrelated to CLL; in another third an initial indolent phase is followed by progression of the disease; the remaining third of patients has aggressive disease at the onset and requires early treatment [3]

Accumulation of mature B-cells that have escaped programmed cell death and undergone cell-cycle arrest in the G0/G1 phase is the hallmark of CLL [4]. In this leukemia elevated levels of the cyclin negative regulator $p27^{Kip1}$ protein are found in a majority of patients [5]. Given the key role of this protein in cell cycle progression, the over-expression of $p27^{Kip1}$ could account for the accumulation of CLL B-cells in early phases of the cell cycle. Furthermore, it has been postulated that the survival advantage of CLL lymphocytes is also due to aberrant over-expression of antiapoptotic Bcl-2 family proteins in general [6] and Bcl-2 and Mcl-1 proteins in particular [7]. Other members of the Bcl-2 family, such as anti-apoptotic proteins BCL-XL and BAG1 are overexpressed in CLL B-cells whereas proapoptotic proteins, such as BAX and BCL-XS, are underexpressed [4]. These antiapoptotic proteins sequester pro-apoptotic counterparts and a balance between both determines the fate of a cell. Additionally, the most consistent cytogenetic lesion in CLL is chromosomal deletions of 13q14, resulting in loss of microRNAs, miR-15a and miR-16-1. Expression of these microRNAs has been founded inversely correlated to Bcl-2 expression and thus, suggested that translocation 13q14 is associated to survival of CLL B-cells [8]. These observations establish anti-apoptotic Bcl-2 family proteins as key survival factors for CLL [9].

High expression of cyclin cell cycle negative regulator $p27^{Kip1}$, antiapoptotic molecules such as Bcl-2 or Mcl-1, and a characteristic non activated phenotype of CLL B- lymphocytes (low surface immunoglobulin (Ig) expression and absence of activated lymphocyte molecules) led to the assumption that CLL disease is a leukemia resulting from accumulation rather than from proliferation. However this traditional view that CLL is a disease deriving from an inherent defect in apoptosis has being called into question [10]. Recent studies suggest that CLL is a dynamic process, comprising leukemic cells that multiply and die at measurable rates. Furthermore, since CLL cells do not appear to be inherently immortal, patient´s compromise does not occur from passive accumulation, but from active generation of subclones that over time develop dangerous genetic abnormalities which further change the birth/death ratios [10,11].

These observations have turned the attention towards the occurrence of different sub-populations inside the tumoral clone. It is clear that most, if not all, proliferative events occur in tissues where leukemic cells are able to exploit microenvironment interactions in order to avoid apoptosis and acquire tumoral growing conditions [12]. This concept is supported by reports showing that, despite their monoclonal origin, there are different subpopulations within clonal CLL B-cells [13,14 and 15].

These works which underline the presence of a proliferative B-cell subset within the tumoral clone, furnish new strength to the hypothesis that the microenvironment plays a central role in the maintenance and progression of this disease. Thus, upregulation of antiapoptotic proteins such as Survivin [16], Mcl-1, Bcl-2, as well as specific chemokines and cytokines in CLL (reviewed in [17]), like CCL2 [18], CCL3/CCL4 [19,20] CXCR4–CXCL12 [21], and IL-4 [22] among others, support a process of activation and reinforcement of the malignant cells by the microenvironment. These key interactions provide survival signals to the leukemic cells leading to the progression and treatment resistance of the tumoral clone. Therefore, the development and design of therapeutic agents with the goal of disrupting the crosstalk between malignant B cells and their microenvironment is an attractive novel strategy in the treatment of CLL, a heterogeneous disease that as yet remains incurable.

In this chapter we will compile the available evidence related to the main B-cell/microenvironment interactions responsible to maintain a CLL proliferative subset. We will discuss the present knowledge about the proliferative B-cell subsets and how they are preserved within the tumoral CLL clone.

2. Role of the microenvironment in CLL-B cell survival

All the physiological processes during which B-cells encounter their antigen (Ag) occur in specific anatomical sites so-called "specialized microenvironments". Germinal centers are the typical immunological picture of these activation places. In this environment B-cell stimulation is totally dependent on complex supportive interactions with both Ag-specific and Ag-non-specific accessory populations. T cells and a variety of different types of adherent cells, generally defined as ‘stromal cells’, are the main elements of this microenvironment.

In CLL disease, the proliferating compartment is represented by focal aggregates of proliferating prolymphocytes and para-immunoblasts that give rise to the called pseudo-follicles or proliferation centres [23]. Pseudo-follicles are the histological CLL hallmark in lymph nodes (LN), splenic white pulp and bone marrow (BM) where they appear as

vaguely nodular areas never surrounded by a mantle zone. These areas are usually infiltrated with an important number of CLL B-cells that after interaction with T-cells and/or stromal/follicular dendritic cells, are able to express the proliferation marker Ki-67 and the progression disease molecules such as CD38 [24] and CD49d [25].

The general observation that CLL B-cells rapidly dye by apoptosis after culture in the absence of accessory cells strongly indicate that CLL B-cells maintain their capacity to respond to selected external stimuli that confer to leukemic cells a growth advantage and an extended survival. Furthermore, numerous *in-vitro* evidence indicate a predominant role of the microenvironment in CLL cell survival [26].

T lymphocytes, the bone marrow stromal cells, and the follicular dendritic cells are involved in the natural history of the disease and appear to be major players in delivering key signals for the proliferation of tumoral clone and disease progression [27]. The exposure of malignant cell subclones to microenvironmental stimuli results in increased proliferation, a prerequisite for the occurrence of new genetic abnormalities that lead to the development of a more aggressive disease.

The pattern of tissue infiltration by CLL cells may be variable. More frequently, malignant cells are seen only or predominantly in the peripheral blood (PB) and the BM. In some instances a vast LN involvement is observed together with a modest PB involvement. These clinical observations point to the existence of mechanisms that selectively control the trafficking and homing of malignant lymphocytes to distinct microenvironments. One such mechanism might be accounted by chemokines and chemokine receptors. Recent data indicate that CLL cells may express specific sets of chemokine receptors and/or respond to specific chemokines produced by microenvironmental elements that selectively attract individual cells to explicit anatomical sites [17].

Chemokines constitute a growing family of chemotactic cytokines that are generally involved in leukocyte migration. According to a current classification based on their function, they are subdivided into three different groups: (1) the homeostatic chemokines regulating lymphocyte migration and homing processes under physiological conditions, (2) the inducible chemokines expressed during inflammation and (3) an overlapping group involved in both processes. Their expression can be induced by various stimuli, including growth factors and inflammatory cytokines. Besides these general aspects, chemokines are also associated to a variety of pathological processes. During tumourigenesis, they are known to play a crucial role informing and modifying the tumour stroma by inducing the infiltration of various hematopoietic cells (e.g.macrophages, natural killer (NK) cells, eosinophils, B and T lymphocytes) as well as fibroblasts and endothelial cells. They also contribute to the neovascularisation, the growth and the spreading of tumours [17].

2.1 Role of T-cells in the CLL microenvironment

The peripheral T-cell repertoire in CLL is significantly altered with a marked increase in oligoclonality in both CD4 and CD8 positive cells [28]. A multitude of *in-vitro* findings indicate that T lymphocytes are attractive candidates to play a role in the inhibition of the malignant B-cell apoptosis and to favour disease progression [29,30]. The weight of evidence points to a dialogue between malignant CLL B-cells and $CD4^{pos}$ T-cells, based upon

bidirectional interactions that are regulated by adhesion molecules and chemokines and translate into the production of several cytokines by both cell types (reviewed by [26]). T-cell cytokines, including IL-4, IFN-γ, and IL-2 inhibit CLL B-cell apoptosis by upregulating Bcl-2 protein, reinforcing the concept that the ability of CLL cell to avoid apoptosis may be strongly influenced by external stimuli provided by the microenvironment [26].

Within pseudofollicular proliferation centers, proliferating leukemic lymphocytes are in contact with numerous $CD3^{pos}$ T-cells, most of which are $CD4^{pos}$, and express CD40L, which can support the growth of CLL B-cells through CD40 ligation. CD40 is a member of the tumour necrosis factor (TNF) receptor superfamily that is expressed by B-cells, dendritic cells and monocytes [31]. The stimulation of CD40 and interleukin 4 (IL-4) rescues CLL B-cells from apoptosis and induces their proliferation [32]. Moreover, CD40 crosslinking on CLL B-cells induces up-regulation of CD80 and CD54 and turns nonimmunogenic CLL cells into effective T-cell stimulators [33]. Later studies of Granziero *et al*. have shown that this proliferative CLL B-cells activated through CD40 also express survivin, a member of the family protein of inhibitor of apoptosis, (IAPs) [16]. This protein is the only IAP whose expression is induced in CLL B-cells by CD40L. The survivin positive cells have an extended survival, an increased proliferative rate and retain Bcl-2 positivity.

It is unclear why and how $CD4^{pos}$ T-cells that gather in CLL pseudo-follicles are activated. Under normal circumstances, $CD4^{pos}$ T-cells that cooperate with B lymphocytes in primary follicles recognize the antigenic peptide in the context of MHC-II class molecules (peptide MHCII binds to T cell receptor, TCR). This interaction results in the transient up-regulation of CD40L. However, T-cells in CLL patients exhibit defective immunological synapse formation which may account for the defects in T-cell helper function seen in earlier studies [30]. Whatever causes their activation, $CD40L^{pos}$ T-cells are in close physical contact with $CD40^{pos}$ CLL within proliferation centers [24]; hence the physiological stimulus provided by CD40L is available to malignant B-cells. Subsequent research has shown that these activated $CD4^{pos}$ T-cells tend to assemble in pseudo-follicles attracted by the chemokines, CCL17 and CCL22 [22] and CCL3 and CCL4 [19,20] produced by proliferating CLL B-cells themselves, (Figure 1A).

In regard to CCL17 and CCL22, it is interesting that leukemic cells purified from LN and BM, but not from PB, constitutively express mRNA for both of them. The CD40-crosslinking of PB CLL cells induces the expression of these chemokines at RNA level [22]. Of them, CCL22 is released and is capable of attracting activated $CD4^{pos}$ /$CD40L^{pos}$ T-cells, while CCL17 is released only when IL-4 is added to the *in-vitro* system [16] (Figure 1A).

3. Role of stromal cells in the CLL microenvironment

T cells are not the only active responsible partners for leukemic B-cells. A number of adherent accessory cells present in different microenvironments are gaining increasing attention in the last years in the CLL progression. It has been convincingly demonstrated that a direct physical contact between BM stromal cells and leukaemic cells extends the survival of CLL B-cell [34]. Stromal cells are key regulators of normal B lymphopoiesis. However, even if they are known to provide binding sites and growth factors to developing B-cells, the precise nature of ligand–receptor interactions are not fully known. The interest has been initially focused upon

adhesion molecules. *In-vitro*, it has been shown that malignant CLL B-cells interact with BM stromal cells via β1 and β2 integrins [34]. This binding rescues CLL cells from apoptosis and extends their lifespan, suggesting a potential mechanism for the preferential *in-vivo* accumulation and survival of CLL cells within the BM.

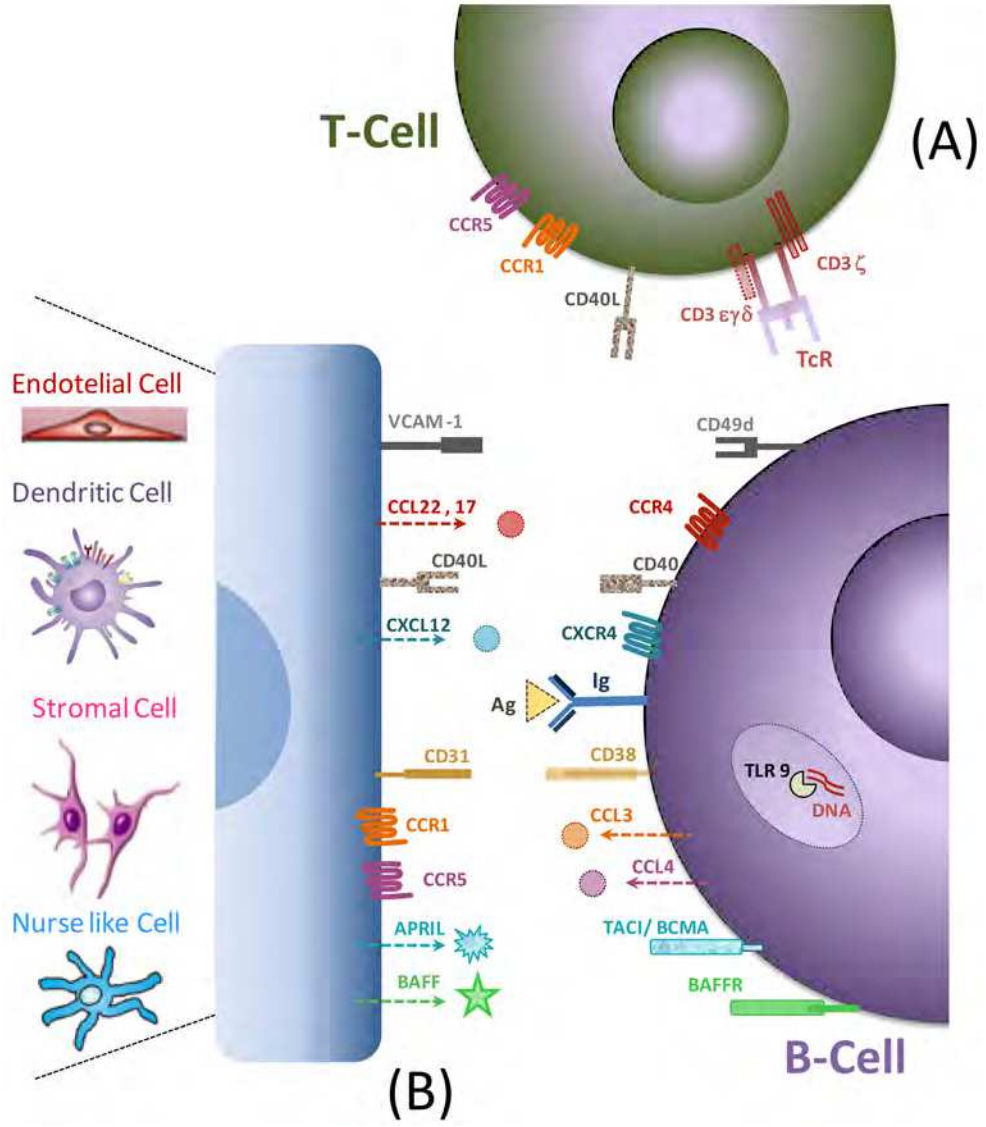

Fig. 1. The microenvironment stimuli on CLL B-cells . Main signaling interactions regulating the survival and the proliferation of leukemic clone. A) T-cells signals to CLL B-cells. B) Endothelial, dendritic and nurse like cells signals to CLL B-cells.

The PB of CLL patients has been shown to contain cells that *in-vitro* can differentiate into adherent nurse-like cells, endowed with the capacity of protecting the attached leukaemic B-cells from spontaneous apoptosis [21]. Blood-derived nurse cells protect CLL B-cells from apoptosis by utilizing a mechanism dependent on SDF-1 (CXCL12), a CXC chemokine that is constitutively secreted by BM stromal cells and regulates B lymphopoiesis upon binding its receptor CXCR4 (CD184). CXCR4 is consistently over expressed by CLL B-cells [21]. In this sense, a recent work of Vaisitti et al., clearly shows that CD38 synergizes with the CXCR4 pathway supporting the working hypothesis that migration is a central step in disease progression and that expression of CD38 is correlated to this expression [35].

Using gene expression profiles comparing $CD38^{pos}/CD49d^{pos}$ versus $CD38^{neg}/CD49d^{neg}$ CLL B-cells, Zucchetto *et al.* [19] showed an over expression of the CCL3 and CCL4 chemokines in leukemic cells from the $CD38^{pos}/CD49d^{pos}$ subset. CCL3 and CCL4 are up-regulated by CD38 engagement in $CD38^{pos}/CD49d^{pos}$ CLL B-cells and also CCL3 was found to be expressed by CLL B-cells from bone marrow biopsies (BMB) of $CD38^{pos}/CD49d^{pos}$ but not $CD38^{neg}/CD49d^{neg}$ cases. High levels of CCR1 and, to a lesser extent, CCR5, the receptors for CCL3 and CCL4, were found in CLL-derived monocyte-macrophages. Consistently, CCL3 induced monocyte migration and CD68+ macrophage infiltration was particularly high in BMB from $CD38^{pos}/CD49d^{pos}$ CLL B-cells. Conditioned media from CCL3-stimulated macrophages induced endothelial cells to express vascular cell adhesion molecule-1 (VCAM1), the CD49d ligand, likely through TNF-α over production. These effects were apparent in BMB from $CD38^{pos}/CD49d^{pos}$ CLL, where lymphoid infiltrates were characterized by a prominent meshwork of VCAM-1+ stromal/endothelial cells. It appears that the CD31/CD38/ZAP-70 axis may represent a point of convergence of proliferative and migratory signals. CD38/CD31 interactions are followed by a marked upregulation of the semaphorin family member CD100, which in turn interacts with the plexin B1 ligand expressed by stromal cells and contributes to further sustain proliferation and survival of CLL B-cells [36].

Underlying the role of stromal cells in the CLL survival signals, a recent work of Zuchetto et al., show that T-cells do not emerge as relevant players in CCL3/CCL4-driven dynamics in CLL BM microenvironment. Rather, this work proposes that CCL3/CCL4 chemokines preferentially target monocytes/macrophages, which are recruited by this/these chemokine/s, in the context of microenvironmental sites of CCL3/CLL4-producing CLL [19]. CCL3 and CCL4 are small (8–10 kDa), structurally related chemokines that, under normal conditions, are secreted by mature hematopoietic cells. Biologically, CCL3 and CCL4 have overlapping effects and act as potent chemoattractants for monocyte, macrophages, dendritic, T, and natural killer cells [37]. Highlighting the importance of the expression of these chemokines in CLL progression, a recent work of Sivina *et al.*, proposes CCL3 chemokine as a novel prognostic marker in CLL, suggesting that its evaluation might become useful for risk-assessment in patients with CLL [38] (Figure 1B).

Leukemic CLL B-cells are not only exposed to signals delivered by accessory, non-malignant cells in the lymphoid tissues, but they are also capable of sensing pathogen associated molecular patterns through a variety of membrane or cytosolic receptors. Toll-like receptors (TLR) are probably the best characterized. TLR7 and TLR9, which recognize single stranded RNA and bacterial DNA respectively, are virtually always expressed (Figure 1). Other evidence which reinforces the importance of the microenvironment on the survival of B-

cells came from Decker *et al.* These authors have been shown that stimulation of CLL B-cells with an analog of bacterial DNA (CpG –ODN) induces the expression of cyclin D2 and cyclin D3 and reduces the expression of p27-kip1 associated with cell cycling. Both cyclins were associated with cdk4, which is the catalytic partner of D-type cyclins in normal B cells. Moreover, immune complexes consisting of cyclin D2-cdk4 or cyclin D3-cdk4 were both functional and phosphorylated the RB protein *in-vitro* [39].

Finally, not only signals delivered by stromal cells appear to be essential in the microenvironment crosstalk with the leukemic B lymphocyte. Cytokine array and enzyme-linked immunosorbent assay studies revealed increased expression of soluble CD14 by monocytes in the presence of CLL B-cells. This work shows that monocytes help in the survival of CLL B-cells by secreting soluble CD14, which induces nuclear factor κ β activation in these cells [40].

Overall, these data provide a link between microenvironmental factors and the proliferation/apoptosis dilemma of CLL B-cells. CLL is now revealing itself to be an environment-dependent hematological malignancy. This idea could be in agreement with a model of selective survival of certain clonal submembers, which would receive survival signals in these particular lymphoid sites.

3.1 Other microenvironment soluble factors involved in CLL progression

Several works in the last years, display the importance of soluble factor regulating the balance between stability and progression of this disease. It is known that CLL B-cells themselves can secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin (Ang) which are involved in the formation of new blood vessels. These newly formed vessels are characterized by increased permeability, and thus contribute to disease dissemination [12]. CLL B-cells can also express receptors for some of these pro-angiogenetic factors, including VEGF receptors VEGFR1 and 2 as well as the Ang-receptor. Signaling through these receptors significantly prolongs cell survival [41]. Additionally, it has been described that thioredoxin (Trx) is expressed in LN of CLL patients and that this expression can increase the CLL survival clone. In this work, the authors found that adding Trx at CLL B-cells increased in a dose-dependent fashion the release of TNF-α, which has been suggested to be an autocrine growth factor for these cells. Secretion of TNF-α maintained Bcl-2, and diminish the apoptosis in the CLL B-cells. [42].

4. Proliferative pool in CLL

It is well established that CLL is a heterogeneous disease: some patients experience a slowly progressive clinical course, but most will eventually enter an advanced phase requiring repeated treatment. Different groups have suggested that cytoskeletal organization, cellular adhesion and the migratory potential of the leukemic clone regulate tissue distribution of CLL cells, possibly influencing a patient's outcome [43,44]. This highlights the significance of topographical issues in disease progression and provides convincing evidence that CLL B-cells with enhanced motility are associated with aggressive disease. Independent confirmation of these results comes from data generated in patients, showing that a

significant proportion of the leukemic clone proliferates and that proliferation occurs predominantly in lymphoid organs.

Messmer and col. clearly demonstrate that a proliferative compartment exists in CLL, [11] although major part probably resides in the solid tissues [14]. Further, it is self-evident that the accumulated CLL B-cells in the PB are constantly nourished by an upstream proliferation cell compartment. It is reasonable to assume that the balance between the two compartments may be at the bases of the highly variable clinical course of CLL, which may behave as a stable and indolent monoclonal lymphocytosis, or as an aggressive disease.

At present, two proliferative subsets related to disease progression has been described in CLL. Chiorazzi´s group proposed that the subset CD38 positive/Ki67 positive CLL B-cells could be a proliferative pool in this disease [14]. Additionally a recent work of Palacios *et al.*, also describe a proliferative subset in UM CLL patients characterized by the presence of active class switch recombination process and anomalous expression of the Activation-Induced cytidine Deaminase (AID) enzyme [15].

4.1 Proliferative CD38 positive CLL B-cells

Despite the large number of surface markers described in the CLL, the expression of CD38 and its association with the disease has been intensively studied. CD38 is accepted as a dependable marker of unfavorable prognosis and as an indicator of activation and possibly proliferation of CLL cells at the time of analysis. Leukemic clones with higher numbers of CD38 positive cells are more responsive to BCR signaling and are characterized by enhanced migration. *In-vitro* activation through CD38 drives CLL proliferation and chemotaxis, via activation of a signaling pathway that includes ZAP-70 and ERK1/2. *In-vivo* interaction of CD38 with CD31, its cognate receptor, have an important role in cell-cell interactions activating survival pathways in normal and leukemic lymphocytes [45].

An important work of Chiorazzi´s group highlights the cell-cycling status of CLL cells, focusing on those leukemic cells expressing CD38 [14]. In order to going deeper in this area Pepper *et al.* extended these observations by comparing gene profile of $CD38^{pos}$ and $CD38^{neg}$ CLL B-cells of a single patients. The results showed that $CD38^{pos}$ CLL cells possess a distinct gene expression profile compared with their $CD38^{neg}$ sub-clones. $CD38^{pos}$ CLL B-cells relatively overexpress vascular endothelial growth factor (VEGF), which is associated with increased expression of the anti-apoptotic protein Mcl-1 [13]. Detailed characterization of the proliferating CLL B-cell convincingly demonstrated a close association between CD38 expression and increased percentages of Ki-67 and ZAP-70 positive cells, suggesting that $CD38^{pos}$ clonal members are more highly activated and prone to enter the cell cycle than their negative counterpart [13].

However, further studies of the same laboratory failed to establish a strong correlation between the percentage of $CD38^{pos}$ proliferating cells in CLL clones and survival and disease progression [46]. The fact that CD38 is expressed in a high percentage of tumoral cells in UM patients indicate that $CD38^{pos}$ leukemic cells constitute a heterogeneous population including a small fraction of cells with an increased proliferative potential. Results from Messmer *et al.* show that leukemic CLL proliferating rates range from 0.08% to 1.7% [11] suggesting that not all CD38 positive cells, are proliferating.

The scenario outlined by these data indicates that the $CD38^{pos}$ cells subpopulation involve a discrete and small subset of cells, also CD38 positive, that have recently exited a solid tissue, and have received freshly proliferation signals.

4.2 Proliferative AID positive CLL B-cells

Recent evidences from our group outline the importance of another cellular subset, characterized by an anomalous expression of the mutagenic molecule AID in a proliferative leukemic clone [15]. This protein is a B cell–restricted enzyme, induced principally through the contact of T and B-cells via CD40-CD40L interactions, despite that recent works also show that the innate immune response via TLR receptor is able to trigger their expression [47]. The physiological expression of this enzyme is responsible for somatic hypermutation (HMS) and class switch recombination (CSR) process in B lymphocytes [48]. However, the mutational activity of AID identifies this enzyme as the first genome mutator in humans with oncogenic potential [49]. Supporting this view, different works report that constitutive AID expression is associated with a loss in the target specificity and with lymphoproliferative disorders [49,50].

In the CLL disease we have reported that AID is anomalous expressed in the PB of some patients with UM VH genes, active CSR and clinical poor outcome [51]. Despite expression of a functional AID as assessed by an active CSR and mutations induced in the preswitch region, CLL B-cells in these patients did not succeed to achieve the SHM process [52]. Although clonal CSR has been described in CLL B-cells long ago [53,54] and different works have shown that this process occurs principally in patients with UM disease [52,55], the origin and the biologic implications of this subpopulation in the physiopathology of CLL remain elusive.

Because AID expression in CLL is associated with ongoing CSR in patients with UM disease, we investigated the relation of AID expression, CSR process, and microenvironment activation in the PB of CLL patients with different clinical profiles. Our results show that high expression of AID is almost exclusively restricted to the subpopulation of tumoral B-cells having an active CSR process (IgG^{pos} CLL B-cells). This subset expresses high levels of proliferation and antiapoptotic molecules such as Ki-67, c-*Myc*, and Bcl-2. In addition, this particular subset of leukemic cells display high levels of CD49d and CCL3/CCL4 chemokines, as well as a decreased expression of cell cycle inhibitor $p27_{kip1}$ compared with their quiescent counterpart IgM B-cells. Finally, the presence of this subpopulation in patients with UM CLL is closely related to an aggressive course of the disease [15]. Additionally to this, over-expression of anti-apoptotic and proliferative molecules as well as expression of molecules implicated in the microenvironment interactions has also been established. Thus, this tumoral CLL subset appears to be a hallmark of a recent contact with an activated microenvironment exclusively found in UM CLL patients with a poor clinical outcome [15].

It is difficult to determine the precise role of these highly proliferating activated tumoral B-cells. Since the presence of this subset is clearly associated to poor prognosis, it might have an adjuvant role in the maintenance of the CLL proliferative pool. However, given their increased proliferative potential they should normally outnumber the IgM^{pos} cells and this is not the case. Thus, we could assume that these cells should undergo apoptosis

once leaving the pseudo-follicles. A recent work suggesting a link between AID expression and B-cell apoptosis in GC favour this view [56]. In these conditions, the IgG^{pos} subset could reflect the existence of an active microenvironment leading to permanent stimulation of the IgM^{pos} pool, which would be turn on the CSR machinery maintaining this IgG^{pos} subset in the PB. Alternatively, an adjuvant role in the maintenance of the CLL IgM proliferative pool by this subset could be considered. Recently, evidence indicates that outside the GC, there is a fraction of AID^{pos} B-cells subset of interfollicular large B-lymphocyte and in the thymic medullae of tonsils [57]. Interestingly, these AID positive B-cells ongoing CSR form prominent cytoplasmic extensions, lending them to a "dendritic cell-like" appearance [57]. In this respect, unpublished results from our laboratory indicate that *in-vitro* stimulation with CD40L/IL-4 not only induces B-cells to proliferate, but also activates lymphocytes to adopt a morphological aspect of "pseudo-dendritic" cells expressing B-cell markers. If the stimulation through CD40L or other stimulation molecules are able to induce these "pseudo-dendritic" cells to become efficient antigen presenting cells remains elusive yet. Whatever the case, the hypothesis that in the UM subgroup stimulation of BCR takes place by an unknown auto-antigen [27,58] and that this is responsible for consecutives stimulations sustaining survival/expansion signals in the tumoral clone, results an interesting issue highlighted by these results.

In this context, we hypothesize that the survival signals of this AID^{pos} CLL B-cells subset could be constitutively triggered by the recognition of an autoantigen present in LN and/or BM (figure 2). In order to explain, why an active AID^{pos} tumor clone is unable to carry out the SHM process, we propose that an unidentified cofactor of AID is absent in the AID^{pos}, UM CLL subset. The correct expression of both, AID and its cofactor, enables the leukemic clone to achieve the SHM process. Once mutated, the clone loses its ability to recognize the autoantigen and, consequently it loses the possibility to receive pro-survival and proliferative signals (figure 2 panel A). In contrast, the expression of AID in the absence of its cofactor prevents BCR mutations, allowing a persistent interaction of the leukemic cells with the autoantigen (figure 2 panel B). The positive signaling through the BCR together with pro-survival and proliferative factors from the microenvironment leads to the accumulation of CLL B-cells and the progression of the disease. The high proliferation rate, the over-expression of AID and other factors, could favor DNA translocations and oncogenic mutations finally associated with progressive and refractory disease (figure 2 panel B).

Inhibition of apoptosis may occur *in-vivo* in pseudo-follicles observed in the lymph nodes, and in the cell clusters described in the bone marrow. These pseudo-follicles include proliferating B-cells in close contact with increased numbers of CD4 T-cells expressing CD40L, which is necessary for AID expression. These activated CD4 T-cells could be recruited by tumor B-cells through the expression of T cell–attracting chemokines such as CCL17 and CCL22 [22] and/or CCL3 and CCL4 [20]. Besides this, the CD38 and CD49d proteins appear to be important additional players interacting with nurse-like cells, stromal, and endothelial cells to complete the activation pathway within the proliferative centers [19]. Overall, these observations favor the view that certain cellular subsets in CLL could receive survival signals in the specific microenvironments, increasing their proliferative potential and consequently associated with a more aggressive disease.

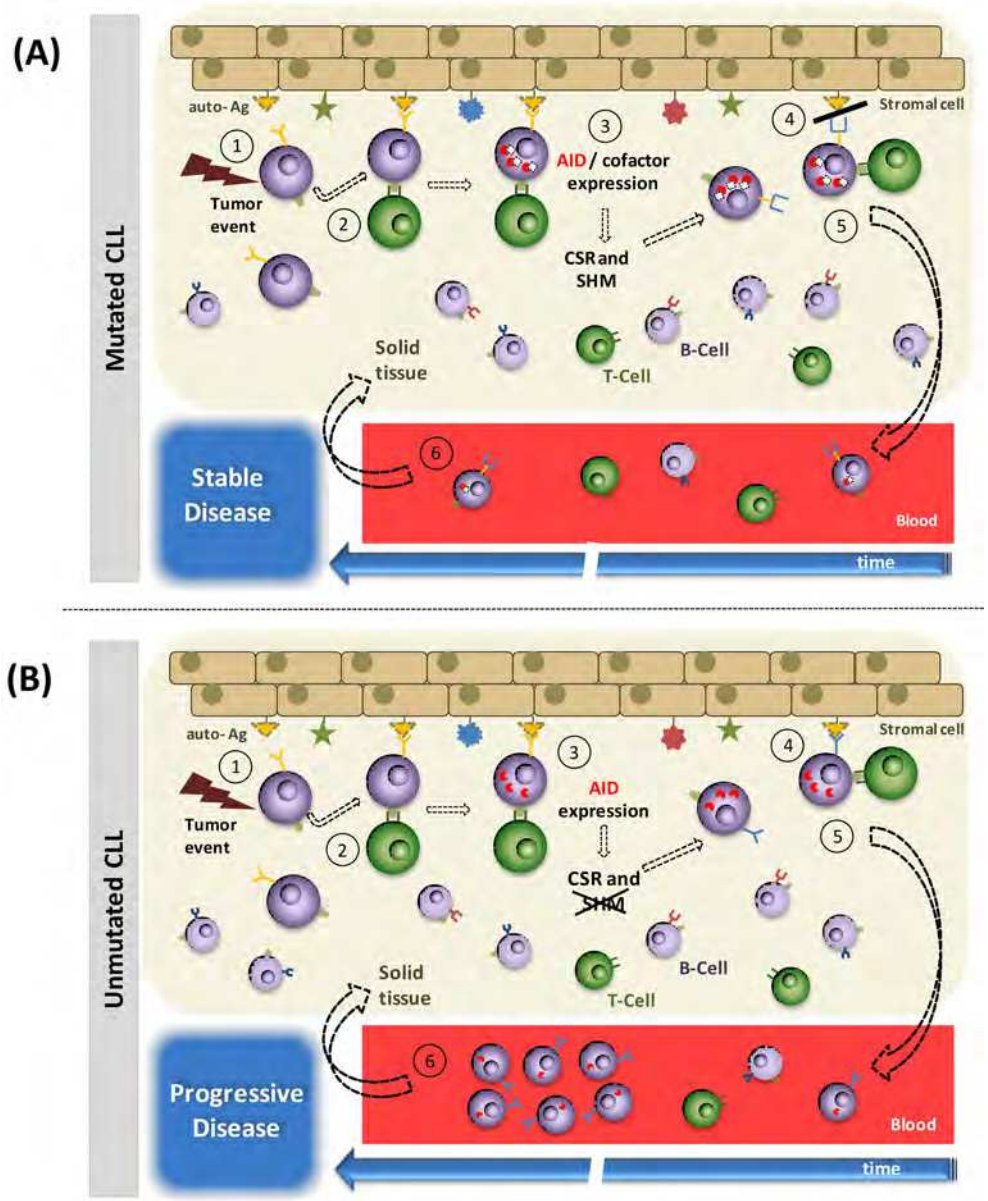

Fig. 2. Anomalous AID expression in UM CLL patients: potential role of autoantigen in the progression diseases.

The survival signals of the proliferative AID^{pos} CLL B-cells subset could be constitutively triggered by the recognition of an autoantigen (auto-Ag) present in LN and/or BM.

In the mutated cases we propose that after an unknown tumor event (1), the tumoral B-cell could be recognize an auto-Ag through BCR and receive collaboration from other cells such as T follicular helper cells or antigen-presenting cell (APC) (2). At this level proliferation centers could be initiated and after this activation the tumor clone might trigger AID expression and its unknown partners in order to achieve SHM and CSR (3) Once mutated the VDJ regions of BCR, the leukemic clone loses its ability to recognize the auto-Ag (4) and, consequently also loses the possibility to receive survival and proliferative signals.

In UM patients, panel B, tumor event occurs in a B-cell (1), BCR of this leukemic cell recognizes the auto-Ag and is induced to proliferate with the help of another T-cells or APC. (2) The leukemic clone expresses AID and their partners, but not the specific cofactor necessary to achieve a correct SHM process (3). Constitutive AID expression in this scenario only is able to trigger CSR, but it cannot mutate the VDJ region of BCR (4). This persistent activation of the leukemic clone leads to the existence of this proliferative subset IgG^{pos}/AID^{pos}**.** The increasing number of these leukemic, switched cells in the proliferative centers leds to the leukemic cells extravasation to peripheral blood (5). These circulating cells might home to solid tissues eventually and thus, they would receive proliferation/survival signals again (6). Cycles of these last two events overtime, produce an increase in the number of proliferating AID^{pos} CLL B-cells (detectable in peripheral blood), which is considered as a hallmark of a proliferative and progressive leukemia.

5. Inflammation role in an activated CLL microenvironment

The relationship between antigen stimulation/inflammation and the natural history of CLL is not surprising considering that inflammation is involved in the initiation and progression of several chronic lymphoid malignancies of B-cell type [59].

Chronic inflammation and CLL are inter-related in many aspects. The malfunctioning of the immune system helps the first few cancer cells to establish into a full-fledged CLL. In comparison to normal B-cells, leukemic cells are rescued from apoptosis by bone-marrow stromal cells, signifying the selectivity of microenvironment for malignant cells. Compelling evidences show us that CLL progression is originated in an inflammatory microenvironment in which many cells (T-cells, stromal cells, monocytes, macrophage and dendritic cells) are all able to delivered survival signals supporting the tumoral clone. These microenvironmental responses are often brought about by the interplay of different chemokines, cytokines, transcriptional factors or post-translational modifications [9].

The inflammatory chemokines are expressed in inflamed tissues and signal for recruitment of neutrophils. On the other hand, homeostatic chemokines produced constitutively in distinct tissue microenvironments to sustain traffic of mature lymphocytes in lymphoid and nonlymphoid tissues [17]. Despite the protective function it has on the CLL B-cells through apoptosis inhibition this factor also allows the spontaneous migration of malignant cells towards BM stromal cells, suggesting that CLL B-cells may utilize this mechanism to infiltrate the BM [21]. SDF-1 and other chemokines such as CCL3 and CCL4 secreted proteins, appear to form a pro-survival circuitry by regulating leukocyte trafficking, extravagating into sites of tissue inflammation and maintaining extended lymphocyte survival [19].

Cytokines are signaling key mediators of inflammation or an immune response, involved in accelerating inflammation and also are present in high levels in CLL patients. They are classified as pro-inflammatory (IL1, IL6, IL15, IL17, IL23 and TNF-α [61,62]), or anti-inflammatory (IL4, IL10, IL13, transforming growth factor(TGFβ) and TNF-α depending on their function in tumorigenesis [60]). Another work, recently performed by Schulz *et al.* [61] touch upon the issue of inflammatory cytokines and signaling pathways associated with CLL survival. Consistent with this possibility inflamatory cytokines genes are upregulated in this work. Among these genes chemokine (C-C motif) ligand 2 (CCL2) was shown to be induced in monocytes by the presence of CLL cells *in- vitro*.

In addition to chemokines and cytokines, the key mediators of inflammation-induced cancer include activation of transcription factors. There are a wide range of transcriptional factors that bind to the promoter region of target genes and activate transcription of these oncogenes. Aberrant expression of the transcription factors like MYC, STAT and NF-kB are associated to inflammatory immune response but also in carcinogenesis and poor prognosis in CLL [62].

The fact that inflammatory receptors such as Toll-like receptors (TLR) can be engaged concomitantly with the BCR, it becomes reasonable to presume that TLR may also play a role in BCR co-stimulation of CLL cells. Indeed, bacterial lipopeptides protect CLL cells from spontaneous apoptosis mediated by TLR signaling [63]. On the other hand, post-translational modifications may affect the activity and longevity of the proteins anti- and pro-apoptotic proteins in an inflammatory microenvironment. Bcl-2 protein undergoes phosphorylation at sites Thr56, Thr69, Ser70, Thr74 and Ser87 in response to different stimuli [64]. Taken together, the extracellular signals from cytokines and chemokines, the contribution of transcriptional factors and post-translational modifications on anti-apoptotic proteins ultimately form a complex network to deliver microenvironmental support to the malignant cells [9].

6. Conclusion

Important progress resulting in high levels of clinical and even molecular remissions has been recently achieved in CLL treatment. However, CLL remains an incurable disease. Compelling evidence suggests that crosstalk with accessory cells in specialized tissue microenvironments, such as the BM and secondary lymphoid organs, favours disease progression by promoting malignant B-cell growth and drug resistance. We are starting to understand which genes, molecules and accessory cells are involved in CLL B-cell/microenvironment interactions and what roles they play. Nevertheless, we need a more proper knowledge about the signals received and/or transmitted by CLL B-lymphocyte, interacting with T lymphocytes, and/or with stromal, endothelial, dendritic and nurse-like cells in the particular CLL microenvironment. Therefore, understanding the crosstalk between malignant B-cells and their milieu could give us new keys in the cellular and molecular biology of CLL that can finally lead to novel strategies in the treatment of this disease.

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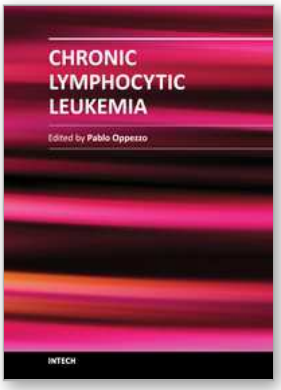

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B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancitopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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candidate *stemness* genes using either inhibitor or RNAi-based approaches.

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Lipoprotein lipase expression in unmutated CLL patients is the consequence of a demethylation process induced by the microenvironment

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Chronic lymphocytic leukaemia (CLL) can be defined as a low-grade B-cell tumor with antigen-experienced monoclonal CD5$^+$ B cells that, having escaped programmed cell death and undergone cell cycle arrest in the G0/G1 phase, relentlessly accumulate in lymphoid organs and circulate into the peripheral blood.[1] This leukemic B-cell accumulation results from a complex balance between activation of cell proliferation and inhibition of apoptotic death.[2] During the past few years, several new prognostic markers have emerged in CLL. Among them, the mutational status of the immunoglobulin heavy-chain variable (*IGHV*) genes is considered one of the strongest.[3] Results from gene expression profile in CLL led us to propose that expression of the lipoprotein lipase (*LPL*) gene could constitute a suitable surrogate marker of the mutational status of IGHV.[4] Despite the usefulness of LPL for CLL prognosis,[5–8] its functional role and the molecular mechanism regulating its expression remain elusive as yet.

LPL has a central role in lipid metabolism by catalyzing the hydrolysis of chylomicrons and very-low-density lipoproteins. In addition to its catalytic function, LPL acts as a bridging protein between cell surface proteins and lipoproteins, by increasing the contact between monocytes and endothelial cell surface through its interaction with heparan sulfate proteoglycans.[9] In CLL B cells, LPL expression has been related to functional pathways involved in fatty acid degradation and signaling, which may influence CLL biology and clinical outcome.[10]

There is increasing evidence that regulation of gene expression during normal lymphocyte development is mediated through changes in chromatin structure and/or through the methylated patterns of CpG islands. Tissue-specific patterns of methylated cytosine residues can be altered by environmental factors, and are often abnormal in tumor disorders.[11,12]

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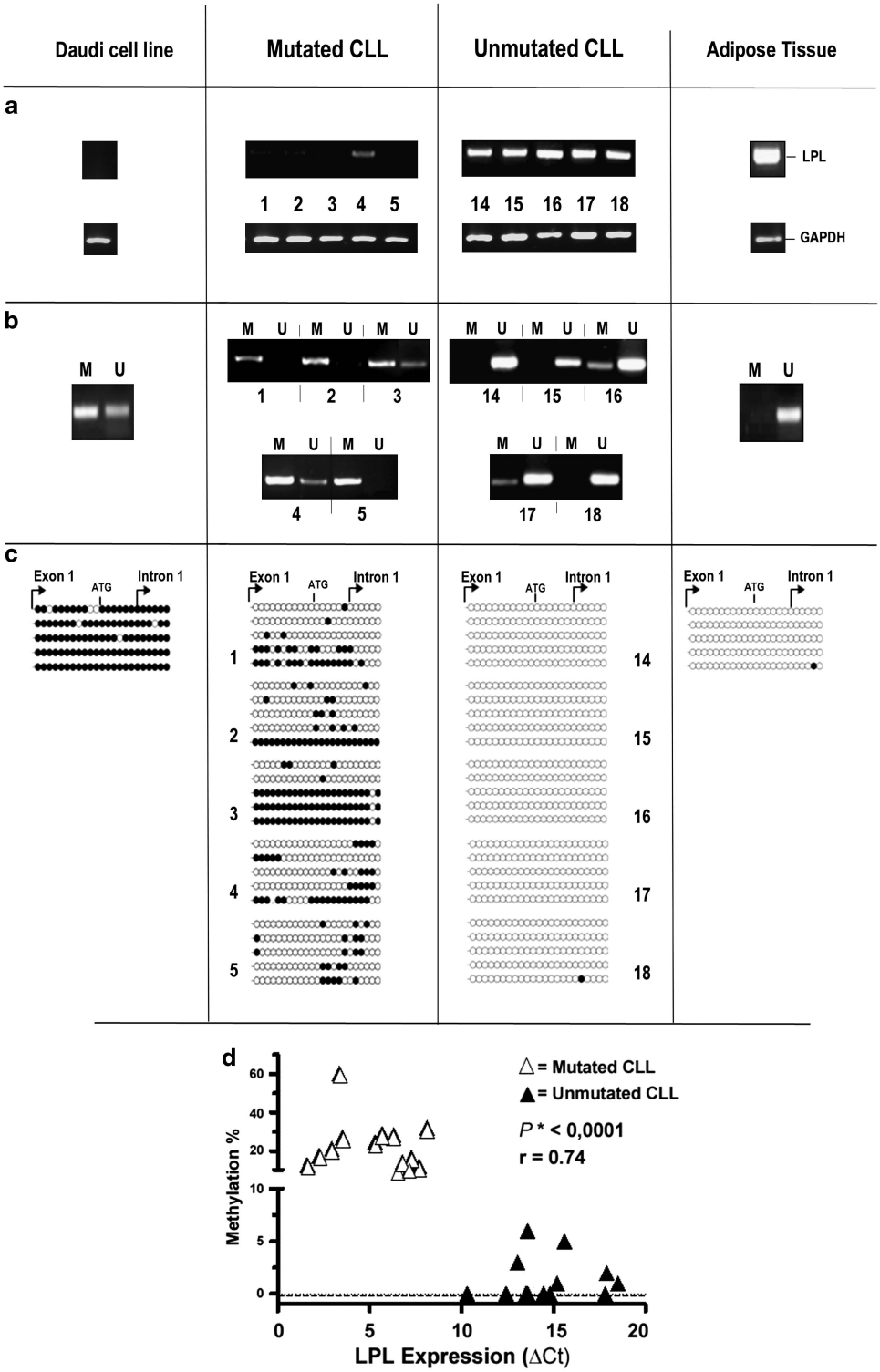

Figure 1. Differential expression of *LPL* gene and methylation status in Mut and Um CLL patients. (**a**–**c**) Results from five Mut (1–5) and five Um (14–18) representative CLL patients, Daudi cell line and adipose tissue samples as negative and positive controls, respectively, are depicted. (**a**) LPL mRNA expression evaluated by RT-PCR is shown in agarose gel stained with ethidium bromide. GAPDH was amplified in all cases as internal control. (**b**) Methylation-specific PCR analysis for R1-LPL region. U, unmethylated and M, methylated. (**c**) Results of bisulphite sequencing of R1-LPL region. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. (**d**) Correlation between LPL mRNA expression of 14 Mut and 12 Um CLL patients evaluated by quantitative reverse transcription PCR and methylation percentage in R1-LPL region is shown. Statistical analysis indicating a significant correlation (*) by Spearman's rank test where *P*-values are $\leqslant 0.001$ is shown. In this case for LPL, expression correlated to methylation status $P < 0.00015$; Spearman's rank coefficient $P = 0.72$.

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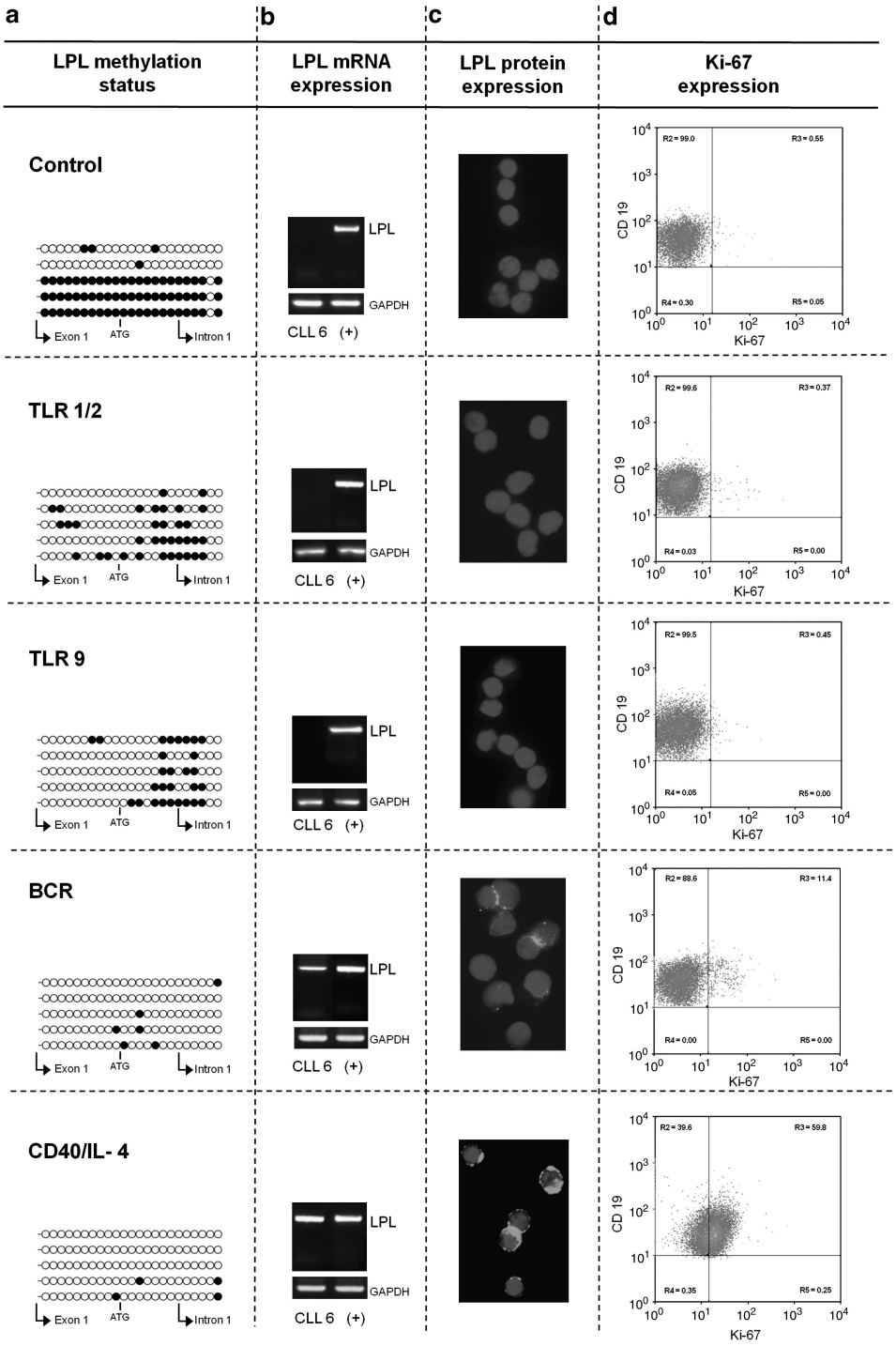

Figure 2. Representative CLL patient after stimulation with different microenvironment signals. (**a**) DNA methylation profile of R1-LPL region of CLL number 6 before and after different activation signals. Each row represents one bacterial clone in which black and white circles represent methylated and unmethylated CpG dinucleotides, respectively. (**b**) LPL mRNA expression by RT-PCR. LPL expression is depicted in agarose gel stained with ethidium bromide. Um/LPL$^{(pos)}$ CLL was used as positive control and GAPDH was used as endogenous control. (**c**) LPL protein expression in CLL patient. Protein expression was visualized by epifluorescence microscopy in Mut/LPL$^{(neg)}$ CLL B cells and in the same CLL case after different stimulations. Green: antibody anti-LPL, blue dye: DAPI. (**d**) Evaluation of Ki-67 expression. Cytometry assays displaying Ki-67 and CD19 expression in CLL patient (Mut/LPL$^{(neg)}$ number 6). Cell populations were discriminated by forward scattering and later B lymphocytes were discriminated by gating CD19 subset. The color reproduction of this figure is available at the *Leukemia* journal online.

To gain insight into the molecular mechanisms responsible for the high LPL expression in Unmutated (Um) CLL B cells, we investigated: (a) the methylation status of the CpG island from this gene in 26 CLL cases and (b) the possibility that LPL expression could be related to specific signals delivered from an activated CLL microenvironment.

In a first step, we analyzed the CpG sites in the *LPL* gene. This analysis revealed CpG-rich sequences encompassing a CpG island of 1163 bp with 112 CpG dinucleotides. This area includes a region within the first exon and the first intron of the *LPL* gene. To better characterize this CpG island, we focused on methylation

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status of CpG dinucleotides in two different regions (R1 = 248 bp, from +87 bp to +335 bp and R2 = 261 bp, from +446 bp to +707 bp). Our results comparing methylation changes between R1 and R2 region in preliminary six CLL samples showed that the main differences appeared to be restricted to exon 1 (CpG dinucleotides number 1–18) and to the first region of intron 1 (CpG dinucleotides number 19–23) (Supplementary Figures 1A–C). Importance of the DNA methylation in the first exon has been recently linked to transcripcional gene expression.[11] To confirm these results, we performed methylation analysis on the R1 region of LPL-CpG island in 26 CLL patients, 14 Um/LPL-positive CLLs and 12 mutated (Mut)/LPL-negative patients (clinical and molecular CLL characterization is shown in Supplementary Table 1). All samples were analyzed following bisulphite DNA conversion, methylation-specific primer-PCR and confirmed by PCR amplification, cloning and sequencing of bisulphite DNA corresponding to R1-LPL region (Material and Methods available as Supplementary Material online). Results have shown that Mut CLL samples and Daudi Human Burkitt's lymphoma cell line (negative control) did not express, or expressed minimal levels of LPL mRNA. In contrast, Um CLL cases expressed high levels of LPL mRNA, though lower than adipocyte cells (AT) (Figure 1a). Interestingly, a different methylation pattern between Um and Mut CLL samples has been found (Figures 1b and c), suggesting that differential methylation status is responsible for *LPL* gene expression in Mut and Um CLL patients. To confirm these results, we studied LPL mRNA expression of these 26 CLL patients by quantitative reverse transcription PCR and correlated LPL expression to the analysis of methylation status by bisulphite sequencing. Results showed a significant correlation ($P < 0.0001$) between LPL expression and demethylated status in Um CLL patients and absence of LPL expression and methylated status in Mut CLL patients (Figure 1d). To further characterize this observation, *in vitro* treatment with DNA methyltransferase inhibitor 5-Aza-dC on Daudi cell line was performed. Results showed that exposure to this drug triggered LPL mRNA expression at significant levels compared with untreated cells and that 5-Aza-dC was capable to induce a clear demethylation of R1-LPL region (Supplementary Figure 2). Overall, these data confirm that demethylation in Exon 1/Intron 1 of *LPL* gene correlates with LPL expression in leukemic CLL B cells.

Previous work suggests that lipid metabolism activation is associated with high LPL expression in Um and progressive CLL patients.[13] Therefore, we investigated whether this anomalous expression could be related with proliferative microenvironment signals delivered to the leukemic clone. For this, we stimulated PBMC from six LPL-negative patients with CD40 ligand plus IL-4, anti-IgM, CpG-ODN or Pam3CSK4, (see Material and Methods in Supplementary Data). Activation through CD40/IL-4 was able to induce high expression of *LPL* gene at mRNA and protein levels (Figures 2b and c). Accordingly, this expression was associated with both, DNA demethylation of R1-LPL region and with proliferation of CLL B cells as evidenced by Ki-67 protein expression (Figures 2a and d). Stimulation through the BCR also increased LPL expression and demethylation of R1-LPL region in four out of six CLL samples, as well as Ki-67 protein expression in three of them. In contrast, stimulation through TLR receptors did not result in DNA demethylation and Ki-67 protein expression, nor induced LPL expression in any of the six samples evaluated (Supplementary Figures 3A–C). Results from one representative LPL-negative CLL patient before and following these different stimulations are shown in Figure 2. To better characterize these results, we evaluated whether LPL methylation status and LPL expression in CLL B cells could be also affected by their interaction with autologous-activated T cells. To this aim, PBMC from two negative LPL CLL samples and one weak positive CLL sample were stimulated with immobilized anti-CD3 for 4 days. We found that both negative cases became positive for LPL mRNA after autologous T-cell activation, whereas the weakly positive CLL sample slightly enhanced LPL expression. Moreover, the methylation status of R1-LPL region turned into a mostly unmethylated pattern of CpG dinucleotides (methylation % in Supplementary Figure 3D), confirming previous results obtained with recombinant CD40L and IL-4. Graphics and statistical analysis of the six stimulated CLL samples with the different signals and of the three CLL activated with autologous T cells are provided in the Supplementary Figure 3. Overall, these results link tumoral cell proliferation to a demethylation process in the CpG island of LPL DNA and suggest that expression of this gene in CLL could be related to specific proliferative microenvironment signals.

Evidences indicate that CLL evolution results from the balance between proliferating cells in specialized tissue microenvironment and circulating cells resisting apoptosis.[2] This equilibrium is finely tuned by a set of surface molecules expressed by CLL B cells and modulated in response to environment signals.[14] High expression of *LPL* gene in Um CLL B cells constitutes an unexpected observation. This specific and anomalous expression constitutes not only a suitable prognostic marker in CLL, but could also help to understand the heterogeneous behavior of this disease. LPL has a bridging function in the formation of a trimolecular complex (lipoprotein particle, LPL and heparan sulfate proteoglycans).[9] This role is a very interesting characteristic, because in addition to its catalytical function, LPL expression in Um CLL patients might be associated with the migratory capacity of a tumoral proliferative cell subset. If true, LPL might also act as a crosstalk factor facilitating specific interactions with accessory cells in the tissue microenvironments. The expression of this protein in concert with integrins, such as CD49d, antiapoptotic molecules (BCL2) as well as chemokines (CCL3, CCL4, CXCL12), implicated in the activation of CLL proliferative pool,[14,15] could be responsible for a circular activation loop in which the leukemic clone is continuously nourished.

The role that abnormal LPL expression could have in disease evolution, has been also addressed by previous work from Pallash *et al.*,[10] demonstrating that lipase-associated genes and triglyceride-specific lipase activity were increased when comparing CLL B cells to normal $CD5^+$ B cells. The same authors suggest that lipid metabolism and lipase activity may be functionally relevant in aggressive CLL.[10] Our results showing proliferation of the tumoral clone associated with demethylation and subsequent LPL expression support these results and highlight the idea that *LPL* gene could constitute a potential therapeutic target in Um CLL cases.

In conclusion, by comparing methylation changes in the LPL-CpG island between Um and Mut CLL patients, we demonstrate a clear association between LPL expression and a demethylation process in the CpG island of the *LPL* gene. This process can be induced in the leukemic clone by specific microenvironment signals, delivered by CD40L/IL-4 and anti-IgM, but not by T-independent related signals delivered through Toll-like receptors. Overall, these results suggest that an epigenetic mechanism, triggered by the microenvironment, regulates LPL expression in CLL B cells.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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A phase 1 study of concomitant high-dose lenalidomide and 5-azacitidine induction in the treatment of AML

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Lenalidomide at a dose of 10 mg daily is approved for treatment of low risk/intermediate risk-1 myelodysplasia (MDS) patients who are red cell transfusion dependent.[1,2] We recently reported on an institutional phase 2 trial treating newly diagnosed elderly acute myeloid leukemia (AML) patients with high-dose single agent lenalidomide as an induction treatment (50 mg/day × 28 days) followed by lower dose maintenance (10 mg daily for 12 months). Thirty percent of patients achieved a complete remission (CR) or complete remission with incomplete blood count recovery (CRi) of 30%.[3,4] We and others have hypothesized that the combination of lenalidomide and azacitidine (AZA) may result in higher rates of sustained CR compared with these drugs individually. Sekeres *et al.*[5] showed feasibility and efficacy of low-dose lenalidomide (10 mg daily) with AZA in high-risk MDS. We read the article 'Safety, efficacy and biological predictors of response to sequential AZA and lenalidomide for elderly patients with acute myeloid leukemia' by Pollyea *et al.*[6] with interest. In that article, the authors report their experience with a phase 1 trial using sequential therapy with AZA and lenalidomide for elderly patients with AML. In the trial, the authors escalated the dose of lenalidomide to 50 mg/day with AZA dose fixed at 75 mg/m^2. Here we report on the findings of our own institutional phase 1 prospective trial of AZA and high-dose lenalidomide (HDL) as an induction regimen followed by maintenance therapy with standard dose AZA and lower dose of lenalidomide. In contrast to Pollyea *et al.*,[6] we (1) used escalating doses of AZA while keeping the lenalidomide dose constant, (2) used concomitant dosing of AZA and HDL and (3) chose to reduce the dose of lenalidomide after the initial two cycles of therapy.

Newly diagnosed elderly AML patients $\geqslant$60 years of age with intermediate- or poor-risk cytogenetics, without isolated 5q abnormalities (elderly AML) and relapsed/refractory AML $\geqslant$18 years of age (relapsed AML) were eligible for this study. Additional inclusion criteria included: Eastern Cooperative Oncology Group performance status of 0–2, and adequate renal (serum creatinine $<1.5\times$ upper limit of normal) and hepatic function (bilirubin <2.0 mg/dl and aspartate aminotransferase/alanine transaminase $<5\times$ upper limit of normal). Patients with acute promyelocytic leukemia, central nervous system leukemia, or prior use of lenalidomide or AZA were excluded. The treatment schedule consisted of two 28-day induction cycles with HDL, 50 mg orally for days 1–28 and AZA given intravenously for days 1–5 at three-dose cohorts (cohort 1, 25 mg/m^2 (cohort 1), 50 mg/m^2; cohort 2 and cohort 3, 75 mg/m^2). Thereafter, patients were given maintenance cycles (every 28 days) with lenalidomide 10 mg

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Origins and Consequences of AID Expression in Lymphoid Neoplasms

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Abstract: The enzyme Activation-induced cytidine deaminase (AID) initiates somatic hypermutation (SHM) and class switch recombination (CSR) of the immunoglobulin (Ig) genes, which are critically important for an effective immune response. In addition, AID seems to contribute to B cell tolerance in mice and humans by, in some still undefined way, eliminating developing autoreactive B cells. As a trade-off for the benefits brought about by its physiological roles, AID can also contribute to cellular transformation and tumor progression through its mutagenic activity.

AID deaminates deoxycytidines at the Ig genes thereby generating deoxyuridine, which as part of the normal mechanism of SHM and CSR is processed by DNA repair enzymes into a larger spectrum of point mutations and also DNA double-strand breaks. Multiple mechanisms regulate AID function to minimize deleterious or pathogenic DNA damage during antibody gene diversification. Despite this, off-target AID activity still makes point mutations and initiates chromosomal translocations that affect tumor suppressor and proto-oncogenes associated with B-cell lymphoid neoplasms. Through this collateral damage, AID is etiological for the development of lymphoma in several mouse models and is expressed in many human malignancies of mature B-cell origin where it may contribute to tumor clonal evolution. Mounting evidences indicate a role for AID also in disease progression and worsening of the prognosis of Chronic Lymphocytic Leukemia (CLL) and Chronic Myelogenous Leukemia (CML). Since these leukemia are not immediately derived from germinal center B cells, normal AID regulation might not be fully functional in those cases. This review discusses recent findings on the role of AID in lymphomagenesis. We describe the multilevel regulation of AID expression and function in normal compared to tumor B cells, specially focusing on the emerging role of AID in CLL and CML.

Keywords: Activation-induced cytidine deaminase, antibody diversity, lymphoid neoplasms, CLL.

ANTIGEN AND ANTIBODY DIVERSITY

How do we defend ourselves against countless pathogens that are constantly evolving and express a potentially infinite variety of antigens? The answer is that we, and all animals, can generate an equally broad variety of antigen-specific receptors in the form of antibodies. A first repertoire of membrane-bound antibodies is generated during B cell development through combinatorial rearrangement of pre-diversified gene fragments by the process of VDJ recombination [1]. The vastness and constant renewal of this primary repertoire ensure that there are always a few B cell clones able to recognize any antigen. This first interaction is of relatively low affinity but there are mechanisms to improve it. Further diversification reactions take place in the secondary lymphoid tissues (lymph nodes, tonsils, spleen, gut associated lymphoid tissue) and modify both the variable (V) and constant (C) domains of the antibody. Thus, after cognate antigen engagement, a second stage of Ig V diversification takes place by the accumulation of point mutations, which combined with selection allows to improve the antibody's affinity. In parallel, isotype switching takes place, which by changing the C domain of the antibody heavy chain permits specific interactions of the antibody with different Fc receptors and with soluble factors to efficiently eliminate each kind of antigen. The molecular mechanisms underpinning affinity maturation and isotype switching, somatic hypermutation (SHM) and class-switch recombination (CSR), respectively, are initiated by the enzyme activation-induced cytidine deaminase (AID) [2, 3]. This mutagenic enzyme that is critical for the immune response, is also an etiological and disease progression factor in lymphomas and leukemia (Fig. **1**).

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ANTIBODY DIVERSIFICATION BY AID: SHM AND CSR

The biochemical activity of AID is to deaminate deoxycytidine (dC) into deoxyuridine (dU) [4-9]. There are two conserved DNA repair pathways that remove uracil from DNA in most species. The most active one is the base excision repair (BER) pathway, initiated by the uracil-DNA glycosylase UNG, which excises the uracil base. Alternatively, the dU:dG mismatch created by dC deamination can be recognized and processed by the mismatch repair (MMR) pathway. AID targeting to the *IgV*

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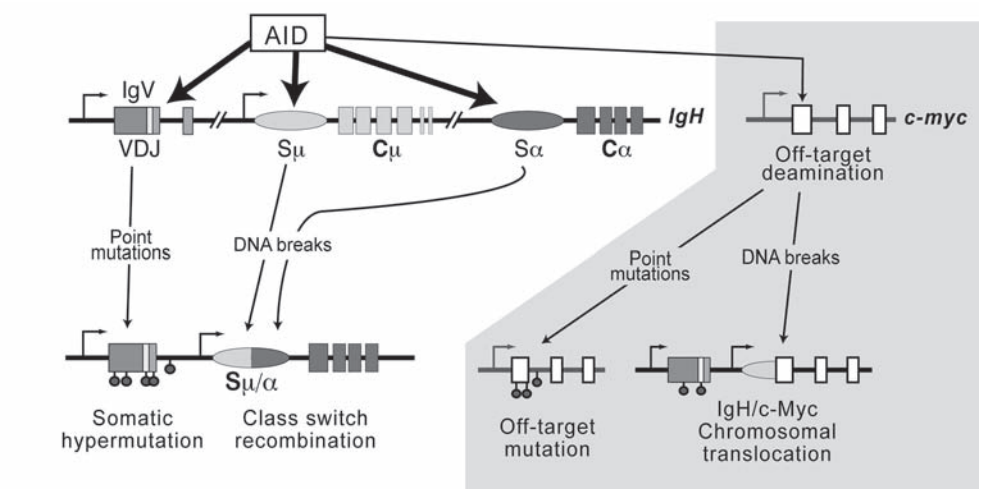

Fig. (1). Antibody diversification by AID. The physiological and pathological outcomes of AID are exemplified using schemes of the *IgH* and *c-Myc*. Targeted deamination leads to the accumulation of mutations at the Ig V and S regions. The architecture and base composition of the S regions favor the accumulation of closely spaced deaminations in both strands leading to DNA breaks. Synapse of two S regions allows the chromosome to be rejoined by non homologous end joining (NHEJ), thus completing the process of isotype switching (IgM to IgA in the example). Less frequent deamination events lead to mutations in other genes and chromosomal translocations as shown for *c-Myc*.

of the antibody heavy and light chain genes creates dU that are also engaged by BER and MMR but, in ways that are incompletely understood, it forces mutagenic instead of the normal faithful repair; thus creating point mutations at high frequency (reviewed in [10, 11]).

Isotype switching is initiated by AID through targeting specific DNA regions known as switch (S) regions. These regions are composed of imperfect repeats and precede the exons encoding for the different classes of C region at the *IgH* (antibody heavy chain) locus. In this case, dU processing in opposite strands by BER and/or MMR components determines the formation of DNA double-strand breaks [12]. The joining of simultaneous chromosomal breaks in two different S regions by non homologous end joining (NHEJ) results in the loss of the intervening sequence and brings exons determining a different isotype next to the IgV exon (reviewed by [12, 13]). By creating antibodies of different isotypes with high affinity for the cognate antigen, CSR and SHM are fundamental for a successful immune response.

AID REGULATION

Gene Induction and Post-Transcriptional Regulation

AID is mostly expressed in germinal center (GC) B cells [14, 15], an anatomical and functional compartment formed by antigen-activated B cells in cooperation with cognate T cells in the secondary lymphoid organs. During the GC reaction B cells cycle between two distinguishable stages: centroblasts, which undergo SHM and show high AID expression, and centrocytes, in which the AID gene (*AICDA*) expression is decreased and that are selected by antigen presenting and T cells based on the affinity of their membrane bound antibody [16]. *AICDA* is normally turned off in plasma cells [15]. Some AID^+ cells can also be detected outside the GC, mostly within the subset of interfollicular large B lymphocytes [17, 18], with unknown significance. As collateral damage during the GC reaction, AID can target non-Ig genes, thus mutating and/or initiating chromosomal translocations, which when affecting tumor suppressors or proto-oncogenes can promote malignancies (see below). As a potently mutagenic but critically important enzyme, the amount and function of AID need to be tightly regulated to balance its physiological and pathological consequences (Fig. **2**).

Aicda$^{-/-}$ mice and AID-deficient patients completely lack switched isotypes and SHM and present lymphoid tissue hyperplasia, recurrent infections and deficient gut mucosal immunity [2, 3, 19]. Partial modification of AID cellular levels also has consequences on its physiological and pathological roles. In engineered mice, AID haploinsufficiency translates into reduced SHM and CSR [20, 21]. However, in less controlled environments, AID haploinsufficiency is not easily detectable and humans with only one functional *Aicda* allele do not show clinical symptoms [22]. Increasing AID expression through transgenic overexpression can lead to increased SHM and CSR but also increased incidence of cancer and B cell lymphomas [13, 23]. Higher AID expression, SHM and CSR has been more reliably achieved by eliminating regulation limiting AID mRNA levels [24, 25] or protein stability [26], as well as by increasing activating phosphorylation by PKA [27]. Not only SHM and CSR are sensitive to downwards and upwards AID dosage changes but also its oncogenic ability, which explains the existence of multiple regulatory mechanisms determining AID cellular levels.

As a first line of control, *AICDA* expression is regulated by multiple transcription factors with activating and inhibiting functions that bind across four regions of the gene. Positive regulators include NFkB and STAT6 [28], HoxC4 [29], Pax5 [30, 31], BCL6 and IRF8 [32]. Negative regulators, such as IRF4, Blimp1 and ID2 appear to restrict AID expression primarily to germinal center B cells (reviewed by [33]). An additional mechanism limiting AID

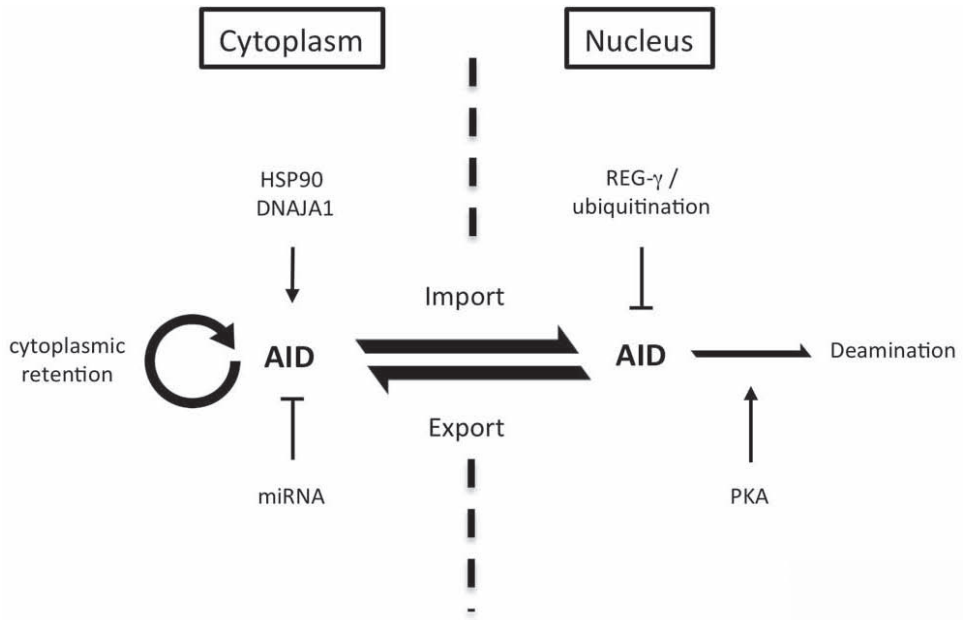

Fig. (2). Major AID posttranslation regulation mechanisms. AID mRNA can be degraded by different miRNAs preventing its accumulation. Cytoplasmic AID stabilized by the Hsp90 chaperoning complex, including DnaJa1, and retained by a poorly characterized, high molecular weight complex, which limits its nuclear import. Nuclear AID can be exported out of the nucleus by CRM1, targeted for degradation by REG-γ or some unknown ubiquitin ligases, all of which limits its activity. Targeting to and deamination of its genomic targets requires multiple interactions and phosphorylation by PKA.

mRNA levels is through miRNAs, most notably miR-155 and miR-181b, which lead to mRNA degradation [24, 25, 34]. In fact, one major role of miR-155 appears to be regulating AID mRNA, as its ablation results in increased CSR and *IgH/c-Myc* translocation [35]. Meanwhile, miR-181b might be implicated in ensuring low AID expression in immature or unstimulated B cells [36, 37], as it is downregulated in activated B cells [32].

Subcellular Localization

A major strategy limiting AID activity is through regulating its presence in the nucleus. This is achieved through several interconnected mechanisms: limiting AID nuclear import through cytoplasmic retention [38] and nuclear export [39, 41] as well as destabilization of AID inside the nucleus [26, 42]. It was early on noticed that ~90% of AID is cytoplasmic in B cells [43]. The identification of a leucine-rich nuclear export signal (NES) in the last ten C-terminal amino acids of AID partly explained this partition. The AID NES is recognized by the soluble shuttle receptor chromosome region maintenance/exportin 1 (CRM1), which exports AID from the nucleus [39, 40]. That treating cells with the CRM1 inhibitor leptomycin B results in nuclear accumulation of AID demonstrated that its cytoplasmic localization is only apparent and that AID is a nucleo-cytoplasmic shuttling protein [39, 41]. Later on it was shown that AID requires active nuclear import despite being smaller than the nuclear pore cut-off [38]. Passive diffusion is in fact prevented by a still incompletely defined cytoplasmic retention mechanism, partly mediated by a C-terminal domain, and which might be the major mechanism of nuclear exclusion for endogenous AID [38]. The translation elongation factor 1α (eEF1A) forms a stoichiometric complex with cytoplasmic AID and may be part of the cytosolic retention complex, although this has not been demonstrated [44]. The nuclear import pathway of AID is not yet fully understood. AID possesses a structural nuclear localization signal that interacts with importin-α [38, 45] but other potential mediators or regulators of AID import have been described such as the NLS-binding protein CTNNBL1 [46] and the GC-associated nuclear protein GANP [47].

Protein Stability and Phosphorylation

AID subcellular localization impacts protein stability. AID has a half-life of 18 h in the cytoplasm compared to only 2.5 h in the nucleus [26]. This is explained by the Hsp90 molecular chaperoning pathway, which actively stabilizes cytoplasmic AID [48]. AID forms a transient complex with Hsp90 that includes or requires DnaJa1 and Hsp70, and which is required to achieve the physiological levels of AID protein in the cytoplasm for successful antibody diversification [48]. In contrast, nuclear AID is actively destabilized by the nuclear protein REG-γ, which targets AID to the proteasome in ubiquitin independent manner [42], and also by unknown ubiquitin ligases [26], although RING finger protein 126 may ubiquitinylate AID [49].

Phosphorylation of Ser and Thr residues in nuclear AID does not seem to affect protein stability but instead controls the formation of the AID complex(es) that perform CSR and SHM. Most notably, Ser38 is phosphorylated by protein kinase A (PKA) at the chromatin, which seems to be required for association with replication protein A (RPA) [50, 51]. In addition, Thr140 phosphorylation, perhaps by PKC, promotes CSR and SHM [52]. In the cytoplasm, AID is phosphorylated at Ser38, which leads to inhibition of AID

activity [53] but the physiological role of this modification, as well as that of Tyr184 phosphorylation [50, 54] remain to be demonstrated.

AID Targeting

AID prefers to, but does not exclusively; deaminate dC within WRC DNA motifs so it can basically mutate any transcribed DNA substrate, at least *in vitro* [4-6]. *In vivo*, unknown mechanisms preferentially target AID to the Ig genes, which nevertheless still allow it to mutate a number of other genes with detectable frequency [51, 55]. A still undefined combination of *cis*-acting motifs at the Ig loci and trans-acting factors probably explain the favored action of AID at the Ig loci (reviewed in [56]), which is linked to transcription [57, 58]. Some of the cis-acting DNA elements have been characterized [59, 60]. Several transcription factors and other AID binding partners have also been implicated in AID targeting to the Ig and non-Ig genes [56, 61]. For example Spt5 and Spt6, factors associated with stalled RNA pol II [62], the RNA pol II elongation PAF complex [63], the RNA exosome [64] and PTBP2 [65] all contribute to transcription-linked AID targeting. However, as they are all general factors, they fail to explain specificity if analyzed in isolation. Mechanistic insight integrating all these findings is largely missing on this central issue.

PATHOLOGICAL EXPRESSION OF AID AND LYMPHOMAGENESIS

Mouse Models

Even in normal B cells, it is well established that AID is not exclusively targeted to the Ig loci. AID chromatin immunoprecipitation (ChIP) shows that it binds to thousands of genes in activated B cells, of which it mutates only a fraction [51, 55]. By inducing DNA double strand breaks, AID can initiate chromosomal translocations involving some 150 genes, many of which are oncogenic [66]. In a normal setting, DSBs are promptly repaired; for instance, homologous recombination prevents widespread DNA breaks by AID [67, 68]. Still, since the main factor influencing the rate of translocations is the formation of DSBs, continual localized DNA damage by AID probably favors recurrent translocations [66]. A prime example is the *IgH/c-Myc* translocation typical of mouse plasmacytoma models and hallmark of Burkitt's lymphoma in humans [13, 69].

The same DNA repair pathways involved in antibody diversification, BER and MMR, can also confer certain protection against the accumulation of mutations at non-Ig genes in mice [55]. That normal DNA repair protects from AID-induced lymphomagenesis is suggested by the modest increase in B cell lymphoma incidence in UNG-deficient [70] as well as by the major increase in lymphoma incidence in UNG-SMUG1-MSH2 triple deficient mice [71], although the oncogenic role of AID has not been formally demonstrated in these systems.

In mouse models, ubiquitous AID transgenic overexpression is sufficient to cause T cell lymphomas, lung adenomas and adenocarcinomas [72], while B cell lymphomas require in addition a p53 deficient background [13]. Thus, p53 greatly moderates the oncogenic potential of AID [13, 73] and p53-deficiency could synergize with AID in human malignancies. Although increased AID expression does lead to increased mutation at the Ig as well as at non-Ig AID targets [20, 54] the endogenous levels of AID are sufficient to predispose B cells for transformation. This has been demonstrated in IL-6 transgenic or pristane-induced plasmacytoma models, in which AID is crucial for the creation of the *IgH/c-Myc* translocation [20, 74]. Similar experiments showed the importance of AID for diffuse large-cell lymphoma (DLBCL)-like malignancies in the Iμ-BCL6 transgenic mouse model [27].

Thus, it is now clear that changes in AID regulation leading to overexpression, but also the combination of normal AID expression with deficiencies in either DNA repair or tumor suppressor mechanisms, can potentiate its oncogenic or disease progression activity.

Human Malignancies and AID

The non-Ig targets of AID characterized in mice most likely explain the aberrant SHM that was described in human B cell lymphomas even before AID was identified as the enzyme producing SHM [75, 76] Aberrant SHM in normal and lymphoma B cells affects many protoconcogenes and tumor suppressors including *MYC, IG alpha, PAX5, BCL6, Rhoh and PIM1* [75-77]. Aberrant SHM was also found in leukemias of B cell origin, most notably B-cell Acute lymphoblastic leukemia (B-ALL) and chronic lymphocytic leukemia (CLL) in which CD95, BCL6, *MYC, PAX5, and Rhoh* among other genes are probably targeted by AID [78, 79]. Despite the impossibility of performing genetics in humans, once AID function and sequence preference was characterized, a great deal of these aberrant SHM could be blamed on AID with reasonable certainty. In fact, many human hematological malignancies express AID; mostly B lineage leukemias and lymphomas, including CLL [80, 81], B-ALL [82], mantle-cell lymphoma [83], follicular lymphoma [84], Diffuse Large B-cells lymphoma (DLBCL) [77] and Burkitt's lymphoma [85]. In addition, AID expression can also be found in a number of non-B cell malignancies including chronic myelogenous leukemia and even epithelial cancers such as *H. pylori*-associated gastric cancer [86], hepatocellular carcinomas [87, 88], and lung carcinomas [89].

AID expression could be especially problematic in chronic diseases, where even a small but continuous level of AID activity can lead to selectable genetic mutations over time, giving rise to more aggressive tumors and treatment resistance. We will focus here in chronic lymphocytic leukemia (CLL) and chronic myelocytic leukemia (CML), two chronic and in most cases incurable malignancies that express AID and are commonly observed in the elderly population [90].

CHRONIC LYMPHOCYTIC LEUKEMIA

CLL Biology

CLL is the commonest leukaemia in Europe and North America. The annual incidence varies with the age and sex structure of the population. A recent analysis of the

Surveillance Epidemiology and End Results (SEER) database puts the annual incidence in the USA at 3.5 per 100,000 (males 5.0: females 2.5) [91]. This leukemia can be defined as a low-grade CD5+ B-cell tumor, where tumoral cells have previously encountered the antigen, escaped programmed cell death and undergone cell cycle arrest in the G0/G1 phase [105]. These cells relentlessly accumulate in lymphoid organs (lymph nodes, spleen and bone marrow) and circulate into the peripheral blood (PB).

The clinical diagnosis of CLL requires an absolute lymphocytosis of at least 5 x 10^9/L mature-appearing lymphocytes and an appropriate immunophenotype. Three main phenotypic features define the CLL B cell: **a**) the predominant population shares B-cell markers (CD19, CD20, and CD23) as well as the expression of CD5 antigen, a molecule normally expressed in T cells and in the subclass of B-cells 1(B1); **b**) the B-cells are monoclonal with regard to expression of either **κ** or **λ** light chains and **c**) low expression of B cell receptor, CD79β, CD20 and low density of CD22 compared with the normal B-cell population(s) levels.

These characteristics are generally adequate for a precise diagnosis of CLL, and they also distinguish CLL from other disorders such as prolymphocytic leukemia, hairy-cell leukemia, mantle-cell lymphoma, and other lymphomas that can mimic CLL [92-94]

Recently, molecular and cellular markers have been identified that may predict the tendency for disease progression. In particular, the mutational profile of Ig genes [95] and some cytogenetic abnormalities [96] display strong prognostic value. However, these biological differences do not separate CLL into two different diseases; it remains a single disease with heterogeneous features [91].

The leukemic B-cells express CD5 and IgM/IgD and thus have a mantle zone-like phenotype of naive cells, which, in normal conditions express unmutated Ig genes (UM) [97]. However, 50%-70% of CLL harbor somatic mutations of IgVH genes [98] as if they had matured in the GC. Interestingly, the presence or absence of SHM is associated with the use of particular IgVH genes. The study of the clonal rearrangement of the B cell receptor (*IGHV* genes) has been one of the major advancements in the identification of molecular prognosis markers that predict the tendency for disease progression in CLL patients [95, 99]. These results allow to speculate that there are two types of CLL: one arising from relatively less differentiated (immunologically naive) B-cells with UM heavy chain genes, which has a poor prognosis; the other evolving from more differentiated B lymphocyte(memory B-cells) with somatically mutated (Mut) heavy chain genes, which has a good prognosis.

Genetic Abnormalities in CLL

Although multiple instances of the disease in some families and the low incidence of the disease among individuals of Japanese origin suggest that genetic influences may be stronger than environmental factors in the pathogenesis of the disease, the nature of this genetic predisposition remains unknown. None of the reported genetic aberrations is constant and it is presently unclear whether they constitute initial events or occur during evolution. In contrast with what is observed in other B cell malignancies, which typically exhibit balanced chromosomal translocations, in CLL the most frequent abnormalities are mutations, deletions or trisomies [100]. Chromosomal aberrations can be detected in interphase cells by fluorescence in situ hybridization (FISH) in 82% of cases [96]. The most frequent alterations are 13q deletions, observed in 55% of patients, followed by trisomy of chromosome 12 (18%) and 11q deletion (16%). The 17p deletion involving the p53 protein is observed less frequently (7%). Interestingly, the presence of a 17p or 11q deletion is associated with poor prognosis and predominates among advanced stages of the disease and among patients displaying UM VH genes, whereas the 13q deletion or a normal karyotype are associated with good prognosis, early disease and mutated VH genes. Deletions of the short arm of chromosome 17 (del 17p13), which contains the *p53* tumor suppressor gene, and of the long arm of chromosome 11(del 11q23), which contains the ataxia telangectasia mutated (*ATM*) gene, result in a loss of function of the *p53* gene. Defects on this pathway constitute the strongest independent predictors for disease that is resistant to standard therapy [101].

The Microenvironment Role in the Leukemic Progression

CLL can be defined as a low-grade CD5+ B-cell tumor, whose tumoral cells have previously encountered cognate antigen, escaped programmed cell death and undergone cell cycle arrest at the G0/G1 phase. Available treatments often induce remissions, though almost all patients relapse and CLL remains an incurable disease [102]. The traditional view has been that CLL derives from a defect in apoptosis, thus allowing slowly proliferating B lymphocytes to accumulate. Increased expression of anti-apoptotic Bcl-2 protein and accumulation of tumoral B-cells in a G0-G1 phase would support this observation [103]. However, recent studies showed that CLL is a dynamic process involving cells that proliferate and die, often at appreciable levels [104]. These observations have turned the attention towards the generation of different sub-populations in the tumoral clone that either reach a homeostatic balance in patients with stable lymphocyte counts and good clinical course or an imbalance in patients with rising lymphocyte counts and poor outcome. It is clear that most, if not all, proliferative events occur in the tissues where leukemic cells are able to exploit microenvironment interactions to avoid apoptosis and acquire tumoral growing conditions [104].

Within the leukemic microenvironment, two cellular components appear to be potential players: stromal cells and T-lymphocytes. *In vitro*, spontaneous apoptosis of B-CLL cells can be rescued by stimulation *via* surface CD40 and IL-4R [105], by the co-culture with stromal cells [106] and/or monocyte-derived nurse like cells (NLCs) [107]. *In vivo*, inhibition of apoptosis may occur in pseudo-follicles observed in the lymph nodes (LN) and in the cell clusters described in the bone marrow (BM). These pseudo-follicles show $CD4^+$ T cells expressing CD40 ligand in close contact with proliferating B-cells. T cells are recruited by the tumor B-cells through constitutive expression of the T cell-attracting chemokines CCL17, CCL22, CCL3 and/or CCL4 [107, 108]. In addition it has been recently demonstrated that another co-stimulatory signals involving TNF-Related

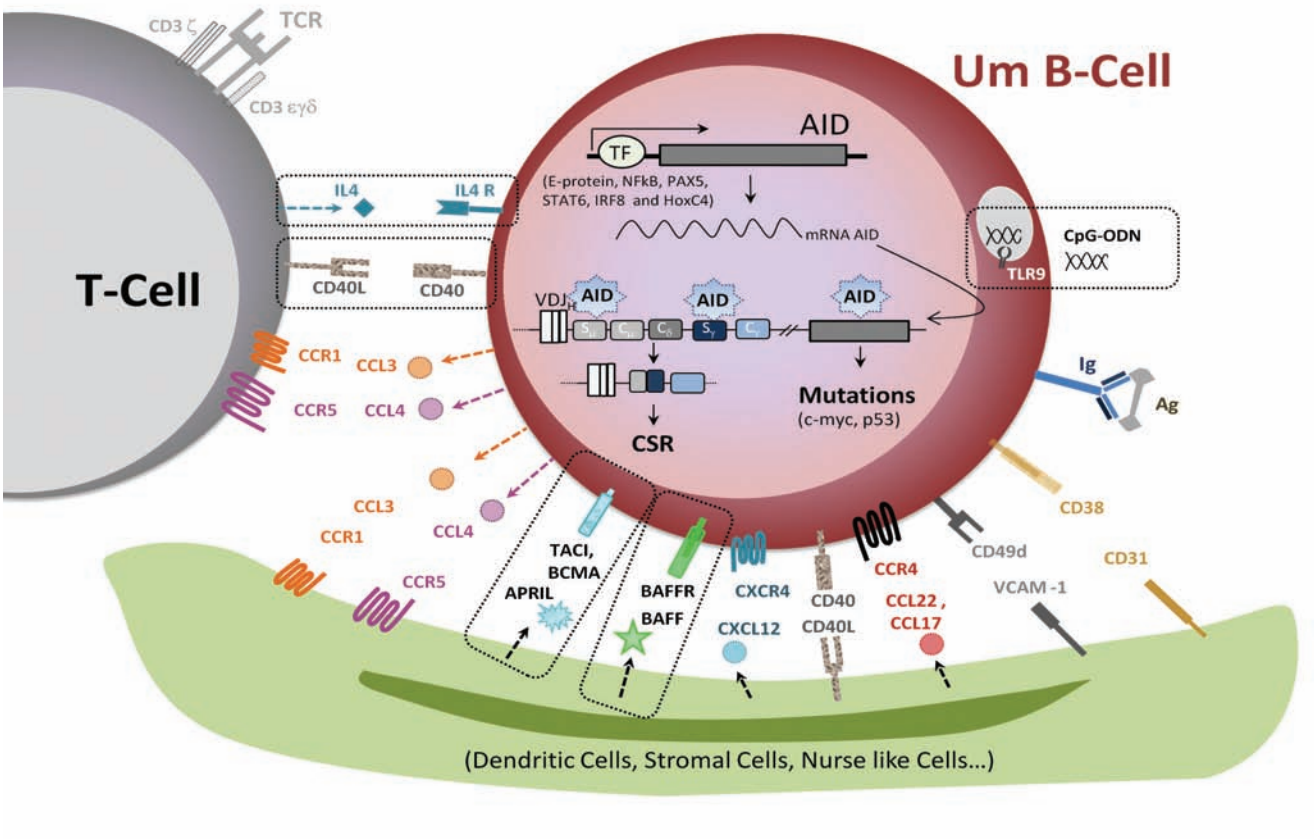

Fig. (3). The microenvironment stimuli on CLL B-cells and AID expression. T-dependent and T-independent signal interactions that regulate the survival and the proliferation of leukemic clone. The signals that have been demonstrated be involved in AID expression are highlighted in dashed line.

Factors such as BAFF (B-cell-activating factor) and APRIL (proliferation-inducing ligand) [109] or TLRs (Toll like receptors) [110] and TLRs and BCR [111] are also able to trigger AID expression and CSR. Thus, ongoing CSR and AID expression in PB of UM progressive CLL cases appears to be a hallmark of a proliferative disease in which B lymphocytes are being constitutively activated in specific tumor microenvironments [112]. This is in agreement with a model of selective persistence of certain clonal sub-members, which would receive survival signals in these particular sites (Fig. **3**).

Implication of AID Expression in CLL

Despite CLL cells do not have proliferative phenotype emulating an activated B-cells profile of the GC reaction, CSR was found in the PB of some UM patients [113, 114]. This led to examine the link between CSR, SHM and AID expression in CLL B-cells. In contrast to normal circulating B-lymphocytes, which mainly express AID transcripts following CD40L stimulation, some of CLL patients show constitutive expression of AID transcripts [80, 81]. Interestingly, almost all these cases corresponded to UM and progressive forms of CLL ongoing CSR, as attested by identical VDJ rearrangements associated to different isotypes [80]. Expression of AID in CLL has been proposed as a marker prognostic factor [115] even though its usefulness for diagnostic remains to be established. Interestingly, AID expression in CLL is confined to a small proportion of the CLL clone [116].

Functional AID expression in UM patients was demonstrated by the existence of mutations in the pre- Sμ region at a rate of 3×10^{-3} [80], which was close to previous reports [117]. Similar frequencies were observed when AID$^-$ normal B-cells and Mut CLL B-cells were stimulated with CD40L and IL-4. The mutation spectrum at the Sμ in UM CLL cases was biased towards transition mutations at C:G pairs in UM CLL [80]. That mutations accumulate at the Sμ of the UM CLL patients indicates dissociation in the targeting or action of AID between the IgVH and the S regions, which explains the existence of CSR without IgVH SHM. Although normally CSR and SHM are temporally and spatially linked, none of them appears to be a prerequisite for the other, since both B-cells in which SHM but not CSR has occurred and *vice versa* have been described in different models [80, 118]. Recent results confirm AID protein expression in PB of CLL cases, which is shown to be functional for CSR and accumulation of mutations in the VDJ regions [119].

There are some indications as to why would AID be expressed in CLL cells. The B cell–specific transcription

factor Pax5 and the E-box transcriptional activator E47 induce AID expression in mouse through elements residing in the first intron of AID [30, 120]. Similarly, the anomalous expression of AID and CSR in CLL patients is associated with high expression of the complete form of *Pax5* gene but not *Id-2* or *prdm-1* in Mut and UM CLL patients [31]. In contrast, absent AID and CSR are consistently associated to a reduction of Pax5a transcripts and the appearance of a second spliced form displaying a deletion in the C-terminal domain (Pax5/ΔEx8). Similar results were found before and after CD40L+IL-4 stimulation in normal and CLL B-cells without expression of AID [31]. These results suggested that Pax5/Δ-Ex8 cloud play an important role in the control of AID enzyme [31, 121]. The relationship between anomalous AID expression in CLL and microenvironment interactions that are normally involved in the disease progression has also been analyzed [122]. The results show that AID expression is almost exclusively restricted to the subpopulation of tumoral B-cells that are undergoing CSR. This subset also expresses high levels of proliferation and antiapoptotic molecules such as Ki-67, c-MYC, and Bcl-2. In addition, this particular subset of leukemic cells display high levels of CD49d and CCL3/CCL4 chemokines, as well as a decreased expression of cell cycle inhibitor $p27^{-kip1}$ compared with their quiescent counterpart IgM B-cells. Overall, these results display a connection between the presence of the subpopulation expressing AID in the PB of UM cases and a clinical poor outcome [122] (Fig. **4**).

A recent study that compared the gene expression profile of tumoral cells from PB, LN and BM in 24 untreated CLL patients identified the LN as a key site for proliferation [123]. Gene signatures of CLL cells in the LN indicated activation of the B cell receptor and NF-kβ pathways, with the expression of these genes being higher in more aggressive CLL. Also tumor proliferation was higher in these cells and was correlated to disease progression, as assessed by E2F, c-MYC and Ki-67 expression [123]. The significant association between AID protein expression and unfavorable clinical outcome in patients with nodal involvement of CLL or small lymphocytic lymphoma [124] also supports the hypothesis that AID expression in CLL is closely related to an aggressive course of the disease. Additionally, this was the first work correlating an unfavorable clinical outcome with the presence of the two more important chromosomic aberrations in CLL such as p53 and ATM deletion, although the number of cases analyzed so far is insufficient for significant correlation [124]. Alternatively, given the relevance of ATM and p53 in controlling AID-initiated DNA damage, their absence could

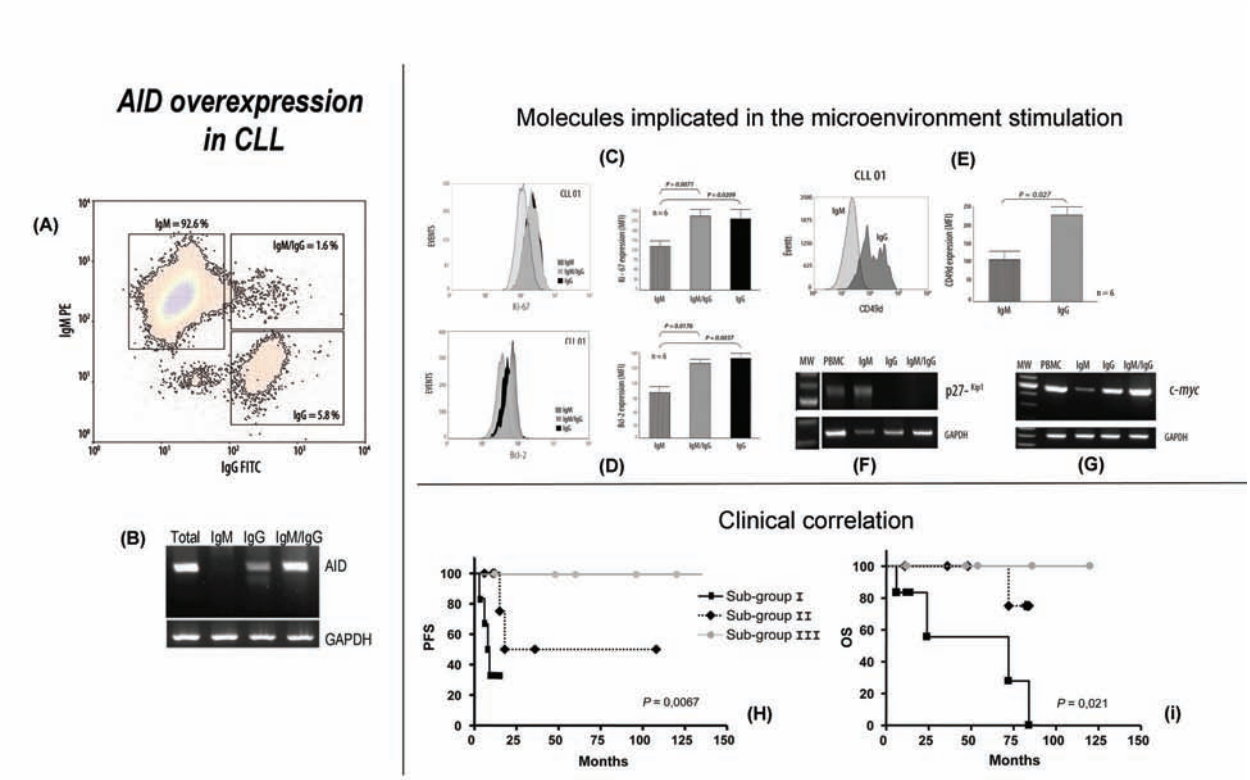

Fig. (4). CLL B-cells subset over-expressing AID enzyme is a hallmark of microenvironment stimulation and is associated to disease progression. (**A**) Representative flow cytometry profile from an UM AID^{++} CLL patient. The three CLL B-cells, IgM^{+}; IgM^{+}/IgG^{+} and IgG^{+} subsets are depicted. (**B**) Semi quantitative RT-PCR from the three cell sorter isolated subpopulations. **Molecules implicated in the microenvironment stimulation:** (**C**-**G**) Flow cytometry analysis and RT-PCR in a representative UM CLL patient, showing the Ki-67, Bcl-2 and CD49d protein expression (**C**, **D**, **E**), as well as p27 and c-myc mRNA expression (F, G). Means values (± SE) of Mean Fluorescence Intensity (MFI) of respectively, Ki-67 and Bcl-2 expression from 6 UM CLL patients with high AID expression levels are shown. **Clinical correlation:** (**H**, **i**) Kaplan-Meier curves based on AID and CSR expression in the 3 subgroups are depicted. Correlations of AID expression and PFS (**H**) and OS (**i**) for the 3 subgroups are plotted. Both analyses indicate a significant negative correlations; (P *) by Spearman rank test for AID expression correlated to PFS and for OS.

actually be synergistic with AID in driving clonal evolution. In fact, it has been also suggested that mutations in *TP53* could be related with AID expression in CLL [125].

At this time and in contrast with another lymphoid neoplasias, more studies are necessary to directly link AID with any etiological role in genomic aberrations in CLL. However, if this assumption turns to be true, the constitutive AID expression in the leukemic clone history could be a key event in the disease progression of this leukemia and a potential target to prevent disease progression.

CHRONIC MYELOGENOUS LEUKEMIA

CML Biology

CML develops from hematopoetic stem cells that can undergo differentiation to multiple lineages; it is a myeloproliferative neoplasm that accounts for about 15% of leukemia cases in adults [126]. CML has an incidence rate of 0.6 to 2.0 cases per 100,000 and is ~1.5-fold more prevalent in males and in the elderly population (reviewed in [127]). Clinical symptoms for CML include splenomegaly (50-60% of cases), hepatomegaly (10-20% of cases), anemia, fatigue, weight loss and upper quadrant fullness or pain. However, most newly diagnosed patients are asymptomatic and the disease is usually diagnosed through routine blood tests. The gold standard for diagnosis is the identification of the Philadephia (Ph) chromosome abnormality, resulting from the translocation t(9;22)(q34;q11), which brings together the *BCR* and *ABL1* genes and creates the constitutively active fusion tyrosine kinase BCR-ABL1 [128]. The Ph chromosome is found in 95% of CML patients and it can be accompanied by other chromosomal changes such as trisomy 8 and isochromosome 17 in 10-15% of patients.

CML can progress through three distinguishable stages: the chronic stage (CML-CP), which is characterized by strong clonal expansion of myeloid cells; an accelerated phase (AP) usually defined by a high percentage of myeloid blasts in the blood or bone marrow (10-19%) [129]; and finally the blast crisis phase (CML-LBC or BP) characterized by rapid disease progression and low survivability. This final stage is usually preceded by a number of chromosomal changes that render the tumor more aggressive including duplication of the Ph chromosome [130] as well as mutations in *CDKN2A* (*p16*) and *TP53* [131, 132]. Once the blast crisis phase is reached, disease progression is extremely rapid, which could be related to the mounting genetic instability observed in the tumor cells.

The fusion gene determined by the Ph chromosome is under constitutive transcriptional control of the *BCR* promoter and produces the BCR-ABL1 fusion protein [133, 134]. BCR-ABL1 is thus formed by the kinase ABL, which is involved in cell cycle regulation [135], cellular response to genotoxic stress [136] and integrin signaling (reviewed in [137]) and BCR, which has serine-threonine kinase activity [138]. Whether or not BCR actually plays a role in CML pathogenesis is still unclear [139]. Through phosphorylation of a wide-range of targets BCR-ABL1 drives the pathogenesis of the tumor by affecting proliferation, survival, DNA repair, *etc.* [140]. Different isoforms of BCR-ABL1 have been identified depending on the site of break in *BCR* and can vary in size from 185 kD to 230 kD [141], with the 190 kD form having the highest tyrosine kinase activity [142] and driving more aggressive tumors in immunosuppressed mice [143]. The 190 kD protein is most highly prevalent in patients with Ph^+ B cell acute lymphoblastic leukemia (B-ALL) whereas most CML patients express the 210 kD protein [141]. This may in part explain the different spectra of Ph^+ leukemias [143].

Since BCR-ABL1 is the driving oncogene in CML, the most effective treatment is the use of tyrosine-kinase inhibitors (TKIs) that trap the catalytic domain of the ABL1 kinase into an inactive form [144]. TKIs such as imatinib, dasatinib and nilotinib are quite effective during the CML-CP but a small number of leukemic cells always persist and patients need to be treated indefinitely [145]. During the blast crisis phase an initial response to TKIs can be observed in most patients but it is usually short-lived and CML-LBC is invariably multidrug resistant towards TKIs [146]. The mechanisms of resistance involve aberrant over-expression of *BCR-ABL1*, forcing the use of pathological levels of TKIs, or the accumulation of point mutations in the *ABL1* tyrosine kinase domain [147, 148]. Due to the chronic nature of the disease, these mutations increase in frequency as the disease progresses and one can anticipate finding more resistance to TKIs in the blast crisis phase than in CML-CP [149, 150]. In fact, mutations affecting the ABL1 kinase domain can be found in 75% of the myeloid blast crisis patients and 83% of lymphoid blast crisis patients compared to 27% amongst chronic phase patients [151]. It should also be noted that point mutations in *BCR-ABL1* could also be found in samples never exposed to TKIs [150, 152] suggesting an endogenous mutator.

Implication of AID in CML

AID is not normally expressed in myeloid lineage cells, at least in mice [37] so it seems unlikely that AID can be etiological in CML. On the other hand, there is good evidence suggesting that it can be an important disease progression factor [153] (Fig. **5**). The clinical evolution of CML from the chronic phase to the blast crisis is marked by the accumulation of mutations that can confer proliferative and survival advantages as well as drug resistance. CML-LBC cells express 4 to 10-fold higher AID mRNA than CML-CP [55, 153], which is presumably related to their partial differentiation towards the lymphoid lineage and induction of the transcription factor PAX5 [153], which defines B cell identity [154]. The correlation between PAX5 and AID expression in CML is reminiscent of what happens in CLL (see above). Accordingly, expression of AID from bone marrow-derived cells *in vitro* required both transduction with *BCR-ABL1*-transduced and the B cell defining cytokine IL-7, but did not happen in the presence of cytokines driving myeloid differentiation [153].

Although only a few publications have analyzed the relationship between AID and CML so far, it has been proposed that AID actually promotes the lymphoid blast crisis in CML. This is based on correlations with the appearance of mutations in tumor suppressor and DNA repair genes, and most importantly in *BCR-ABL1* [153] but more work is necessary to firmly establish at which point AID expression comes into play. On one side, the low but

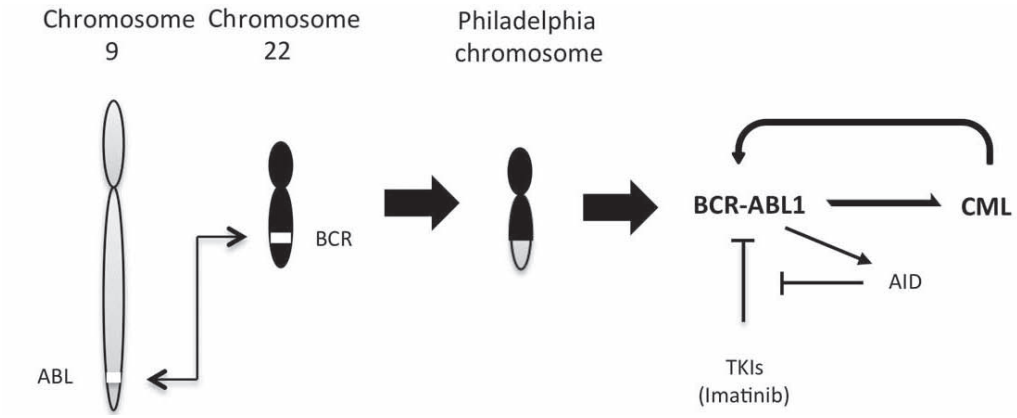

Fig. (5). The Philadelphia chromosome is a hallmark of CML and some B-ALL. The fusion of human chromosomes 9 and 22 leads to the formation of the Philadelphia chromosome. The joining of the breakpoints in each chromosome fuse the *BCR* and *ABL1* genes, creating the new *BCR-ABL1* oncogene that produces an oncogenic kinase. The main therapy for CML uses TKIs blocking BCR-ABL1 activity. Mutate *BCR-ABL1* and produce BCR-ABL1 variants that do not bind TKIs, leading to treatment resistance. There is evidence that BCR-ABL1 induces the expression of AID in leukemic cells.

chronic expression of AID detected during CML-CP could contribute to genomic instability. The combination of a mutator acting over the term of years on a large population of CML cells could in principle generate more aggressive clones, which if additionally rendered TKI-resistant would be selected during therapy. On the other side, it is also possible that the expression of pathologically relevant AID levels happens at a late stage, perhaps already committed to lymphoid lineage. The AID-induced mutations in *BCR-ABL1* would generate TKI resistance leading to selection of the cells expressing AID, which can drive progression of the disease through additional mutations. In any case, once AID is expressed it is clear that it confers advantages to the tumor cell population expressing it [153]. A subset of patients suffering from B-ALL also carries the Ph chromosome with 25% incidence in adults but only 3% in pediatric patients [155]. AID may also a disease progression factor in B-ALL patients despite in this case it may not mutate *BCR-ABL1* [82]. In a murine bone marrow transplant model of B-ALL, AID^+ leukemia showed a more aggressive phenotype when compared to the $Aicda^{-/-}$ leukemia, including more genetic lesions and the presence of aberrant SHM in various genes [82]. There was also a noticeable difference in gene expression profile between $Aicda^{+/+}$ and $Aicda^{-/-}$ leukemic cells [82], which may or may not be related to a proposed activity of AID in regulating transcription through demethylation [156, 157]. Although *BCR-ABL1*+ B-ALL arises from pre-B-cells, which normally do not express AID, AID expression was found in 59% [158] to 86% [79] of patients with Ph^+ B-ALL. Furthermore, enforced overexpression of BCR-ABL1 in Ph- B-ALL was sufficient to induce AID expression and inhibiting BCR-ABL1 in Ph^+ B-ALL was sufficient to repress AID expression [79]. Thus, there seems to be a causal relationship between the BCR-ABL1 signaling cascade and AID expression, although taken together with the results in the CML system this seems to also require a B cell context [153]. It is important to note that AID expression does not need to be higher than normal to contribute to disease progression, as observed in CML-LBC blasts where AID expression is 5-10 fold lower than observed in germinal center B-cells from human tonsils.

The Role of AID in TKIs Resistance

AID can confer imatinib resistance in CML cell lines *in vitro* by introducing mutations in *BCR-ABL1* [48, 153]. Direct targeting of *BCR-ABL1* by AID *in vivo* is not surprising given the large number of genes AID can mutate with detectable frequency in normal B-cells [51, 55]. Thus, in the cases of CML or B-ALL, the chronic availability of AID, even if expressed at lower levels than in normal B-cells [153], could produce a pathologically relevant mutation frequency. Interestingly, the *BCR-ABL1* fusion gene is close to the human immunoglobulin λ locus, which contains sequence elements within the enhancer region that attracts AID-dependent SHM [59, 159]. The evidence that AID targets and mutates BCR-ABL1 in leukemic cells *in vivo* during CML has been obtained necessarily through correlation but it is convincing. Firstly, a comparison of the number of mutations found on *BCR-ABL1* between CML-CP, which express low levels of AID, and CML-LBC or Ph^+ B-ALL, in which AID is expressed at higher levels, showed more frequent *BCR-ABL1* mutations in the latter groups. Secondly, analysis of a large database of clinical *BCR-ABL1* mutations show 66% of G:C transitions in CML-LBC/Ph^+ B-ALL versus only 20% in CML-CP [153], in keeping with AID deaminating dC. For instance, the E255K replacement produced by a G>A mutation, which is one of the major imatinib resistance mutations found in the clinic, was found 5 times more frequently in the CML-LBC/Ph^+ B-ALL than in the CML-CP group. Thirdly, mutations on BCR-ABL1 tend to occur more often at dC within the favored AID sequence motif WRC [153]. Together with the data obtained *in vitro* and animal models, these findings strongly suggest that AID is a pathologically relevant causal factor of TKIs resistance in CML. Although the absence of a chronic stage in B-ALL might limit the contribution of AID to the generation of mutations leading to drug resistance, it could still contribute to disease progression by driving clonal evolution [82].

EXPLOITING AID REGULATION FOR POSSIBLE THERAPEUTIC TARGETING

Given the probable role of AID in the progression of chronic leukemias like CML and CLL, its pharmacological

inhibition could be useful as an adjunctive therapy. Small-molecule inhibitors are currently being developed against the AID paralog APOBEC3G [160, 161]. Given the chemical and architectural similarities of the active site of APOBECs and AID [162] some of APOBEC3G inhibitors might serve as lead compounds for AID-specific inhibitors. Unfortunately, difficulties in producing sufficient quantities of recombinant enzyme due to its tendency to aggregation and lack of solubility [163, 164] and personal observations.) have hampered the development of small molecule inhibitors for AID. Nevertheless, there is no doubt that developing such an inhibitor should be the long term goal, despite it will probably take several years to go through the validation and approval for clinical use. Meanwhile, the accumulated knowledge on AID regulation provides a few opportunities to indirectly reduce AID levels to some extent, some of which could be applicable in the clinic in a shorter term.

Since the mutagenic activity of AID, either physiological or pathological, is almost linearly correlated to its total protein level [20, 34, 48], partial reductions in the cellular AID content can have significant results in decreasing its pathological side effects. The prediction is that reducing AID levels would reduce the frequency of mutations and thereby delay disease progression. Possible strategies would include drugs that specifically inhibit AID nuclear import or that induce AID degradation either directly or by interfering with the stabilization pathways that maintain AID cellular levels. The reliance of AID on the Hsp90 molecular chaperoning pathway has already suggested a couple of possibilities. An early target in this pathway is the cochaperone DnaJa1, which links AID to the Hsp90 pathway on the condition of being farnesylated [165]. Inhibiting DnajA1 farnesylation using farnesyl transferase inhibitors (FTIs) in B-cells leads to a reduction in AID protein levels [165]. Unfortunately, clinical trials using FTIs have shown little success so far [166, 167]. On the other hand, Hsp90 inhibitors show great promise and might be appropriate.

Multiple Hsp90 inhibitors have been developed, most deriving from the benzoquinone ansamycin antibiotic geldanamycin (GA) [168, 169]. One of these, 17-allylamino-17-demethoxygeldanamycin (17-AAG), entered human trials in 1999 (reviewed in [170]) hoping that the high dependency of different cancers on Hsp90 could be exploited. Although 17-AAG has shown somewhat promising results *in vitro* in a CLL model and in patients with breast cancer [171] toxicity attributed to the DMSO vehicle [170] has prompted the development of a number of other analogues including the water-soluble 17-demethoxygeldanamycin (17-DMAG). 17-DMAG, of high bioavailability *in vivo* [172], has shown antitumour activity in melanoma and carcinoma models [173]. As an example of the early successes of Hsp90 inhibitors in cancer models there are now some 17 different compounds that have entered clinical trials including tanespimycin, alvespimycin and ganetestib to name but a few (reviewed in [174, 175]).

Hsp90 regulates numerous signaling proteins and pathways helping the cancer survive environmental stresses. AID is a relatively novel Hsp90 client and Hsp90 inhibitors cause AID destabilization with the consequent reduction in its protein levels and activity *in vitro* [48] and *in vivo* (DMS and JMDN unpublished results). Due to the chronic nature of CML and the relevance of AID in the progression to blast crisis, it seems like an appropriate model to test the efficacy of pharmacologically targeting AID. The available data suggest that targeting AID, for the moment through Hsp90 inhibitors, in conjuncture with TKIs, could at least significantly delay the rise of TKIs resistance by decreasing the frequency of *BCR-ABL1* mutation [165, 176]. If AID in fact drives the blast crisis as proposed [153], the benefits s of targeting AID would be even greater. Since the presence of a small cell population expressing AID in the CLL clone correlates with progressive disease [119, 122], should AID have a causative role in this, one could speculate that similar benefits might be obtained by targeting AID in these CLL patients. Alternative methods, such as inhibiting the homologous recombination repair pathways, which protects the genome from widespread AID-induced DNA breaks, have been more recently proposed to eliminate CLL cells that express AID through DNA damage initiated apoptosis (REF). Thus, further research on other AID-associated factors and pathways modulating its activity, will surely uncover new potential drug targets to reduce AID oncogenic activity.

FINAL REMARKS

The evidence accumulated so far strongly indicates that AID plays a role in the etiology and/or disease progression of lymphomas and leukemias. The former is most likely through initiating oncogenic chromosomal translocations as a side effect of isotype switching, as is probably the case of *IgH/c-Myc* in Burkitt's lymphoma. AID can also drive clonal evolution more subtly, by point mutations that produce selectable advantages, which can contribute to the cancer progression. AID expression sustained in time in chronic indolent malignancies can thus be pathologically relevant, as it seems to be the case for CLL and CML. Targeting AID in these diseases, which tend to affect older adults, might be beneficial if it can prevent progression or drug resistance. While a specific, small molecule AID inhibitor would be the ideal therapy; identifying factors that interact with and modulate AID could also be exploited to this end.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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