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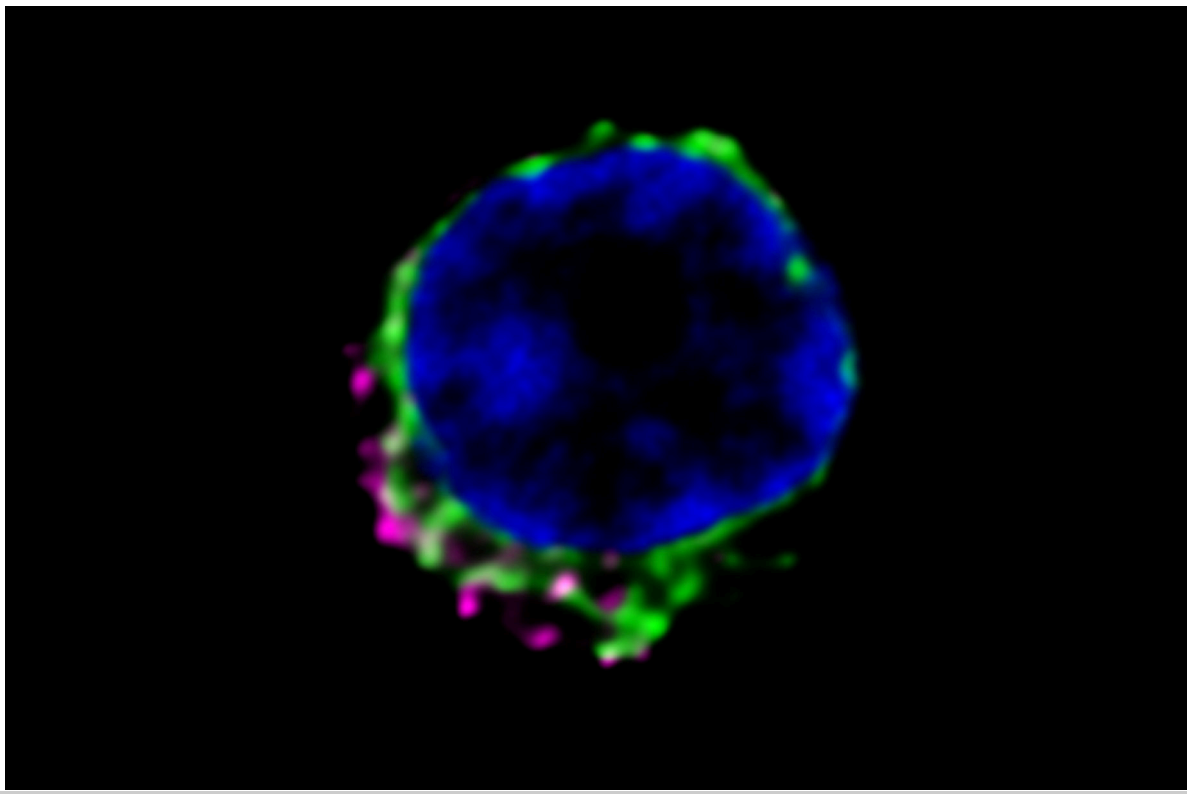

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Caracterización de diferentes marcadores moleculares del microambiente tumoral asociados a la progresión de la Leucemia Linfoide Crónica

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Tesis de Doctorado presentada al Programa de Posgrado en Ciencias Biológicas, Programa de Desarrollo de las Ciencias Básicas de la Universidad de la República, como parte de los requisitos necesarios para la obtención del título de Doctor en Ciencias Biológicas.

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Índice general

Prefacio

Resumen para público general

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 requiere tratamiento, presenta una vida prolongada, y su muerte, generalmente no se encuentra relacionada con la leucemia. Otro tercio de los pacientes comienza con una fase indolente seguida por una progresión de la enfermedad mientras que el resto presenta una enfermedad agresiva desde el debut, que requiere rápidamente de tratamiento. Debido a la heterogénea evolución clínica de los pacientes con LLC, los marcadores pronósticos que permiten predecir la evolución de la enfermedad son sumamente relevantes en ayudar a la decisión clínica de dar o no tratamiento a los pacientes padeciendo esta leucemia. En el presente trabajo de tesis se analizan varios marcadores asociados al contexto celular y molecular en el que se encuentran las células tumorales, el microambiente tumoral, que puede influenciar la expansión o el control del tumor. En particular, se tratan nuestros hallazgos realizados en torno a los exosomas de la LLC, las proteínas que llevan como carga y sus cambios durante el avance de la enfermedad. También se analiza la distribución y función de la proteína lipoprotein lipasa (LPL) en la célula tumoral de LLC, y el desarrollo de un método para predecir la evolución de la enfermedad utilizando esta información.

Resumen

La leucemia linfoide crónica es la más frecuente en el adulto en poblaciones occidentales. Se caracteriza por una acumulación lenta y sostenida de linfocitos B clonales en sangre periférica y órganos linfoides secundarios. Actualmente se entiende que, al igual que otros tumores, implica más que una masa de células malignas que simplemente se acumulan. El intercambio de señales entre las células de LLC y distintos tipos de células en los tejidos linfoides es crucial para la liberacion de señales que promuevan el crecimiento de la masa tumoral, la evasión del sistema inmunitario, la quimiorresistencia, y la remodelación tisular que construyen el microambiente tumoral. Este microambiente es un participante activo en los procesos de progresión del cáncer y metástasis, por lo que su estudio constituye no solo un importante desafío para la ciencia básica, sino además una nueva esperanza de desarrollo terapéutico.
Los exosomas son pequeñas vesículas de origen endocítico secretadas por las células, y su papel en el progreso de la enfermedad tumoral, la resistencia a la quimio y radioterapia, así como el acondicionamiento del nicho metastásico a través de la transferencia de ARNs pequeños o proteínas efectoras han despertado gran interés en los últimos tiempos. Sin embargo, en LLC aun hay muy poca información referente a las cargas proteicas de los mismos. Por este motivo hemos explorado los cambios en el perfil proteómico de los exosomas plasmáticos en la LLC durante la progresión de la enfermedad. Hemos descubierto que los perfiles de los exosomas de pacientes con LLC indolente y progresiva son diferentes, y que los mismos cambian con el avance de la enfermedad. También hemos identificado la proteína S100-A9 como una proteína firma del progreso de la LLC, y la presencia de su receptor EMMPRIN en el clon maligno. Hemos descubierto que los exosomas cargados con S100-A9 son capaces de activar la vía canónica de NF-κB, y promover la proliferación del clon tumoral. Una pregunta para la que aun no poseemos respuesta es si los exosomas cargados con S100-A9 aparecen en el plasma de los pacientes antes o sólo durante la progresión. Esto constituye una importante interrogante, ya que la LLC posee un curso clínico heterogéneo y la identificación temprana de los pacientes cuya enfermedad sera progresiva es crucial para brindarles el tratamiento oportunamente.
Numerosos esfuerzos en el área clínico/básica han sido realizados con el objetivo de encontrar un marcador pronóstico de fácil aplicación en la clínica hospitalaria y que sea lo más fiable posible en predecir la evolución de la enfermedad. Hasta el momento los tres marcadores pronósticos más utilizados en LLC tienen cada uno de ellos diferentes problemas, que restringen su

utilización como método de pronóstico único. Luego del trabajo de nuestro grupo sobre los genes *LPL* y *ADAM-29* otros artículos han confirmado, que la expresión del ARNm del gen *LPL* está asociada a un mal pronóstico y que es un excelente marcador de progresión en la LLC. A pesar de los estudios existentes y que el valor pronóstico de la expresión de LPL está consolidado, el rol funcional de su sobreexpresión en la patogénesis de la LLC aún no ha sido dilucidado, así como tampoco los mecanismos moleculares que regulan su expresión. En el presente trabajo indagamos algunos aspectos, hasta el momento desconocidos, de la biología de la proteína LPL en la célula de LLC, como su distribución subcelular y la capacidad de los linfocitos B de endocitar LPL, y describimos además el desarrollo de un método pronóstico para LLC basado en la detección de la proteína LPL mediante citometría de flujo.

Lista de artículos

Esta tesis ha generado los siguientes artículos:

I. **Prieto, D.**; Sotelo, N.; Seija, N.; Sernbo, S.; Abreu, C.; Durán, R.; Gil, M.; Sicco, E.; Irigoin, V.; Oliver, C.; Landoni, A. I.; Gabus, R.; Dighiero, G.; Oppezzo, P. (2017)

S100-A9 protein in exosomes from chronic lymphocytic leukemia cells promotes NF-κB activity during disease progression.

Blood, 130:777–788

II. **Prieto, D.**; Uriepero, A.; Seija, N.; Ortega, C.; Oliver, C.; Irigoin, V.; Landoni, A.I.; Souto-Padron, T.; Gabús, R.; Oppezzo, P. (2018)

Unmutated IgHV CLL cells express more LPL protein than mutated cells and is associated with a clinical poor outcome

Manuscrito en preparación

III. **Prieto, D.**; Oppezzo, P. (2017)

Lipoprotein lipase expression in chronic lymphocytic leukemia: new insights into leukemic progression

Molecules, 22(12):2083

IV. Palacios, F.; Abreu, C.; **Prieto, D.**; Morande, P.; Ruiz, S.; Fernandez-Calero, T.; Naya, H.; Libisch, G.; Robello, C.; Landoni, A.I.; Gabus, R.; Dighiero, G.; Oppezzo, P. (2015)

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

cell proliferation

Leukemia, 29(1):115-125 ANEXO

V. Palacios, F.; **Prieto, D.**; Abreu, C.; Ruiz, S.; Morande, P.; Fernandez-Calero, T.; Libisch, G.; Landoni, A.I.; Oppezzo, P. (2015)

Dissecting chronic lymphocytic leukemia microenvironment signals in patients with unmutated disease: microRNA-22 regulates phosphatase and tensin homolog/AKT/FOXO1 pathway in proliferative leukemic cells

Leukemia & Lymphoma, 56(5):1560-1565 ANEXO

Introducción

Leucemia linfoide crónica: Generalidades

La Leucemia linfoide crónica (LLC) es la leucemia más frecuente del adulto en poblaciones de origen caucásico (Hallek 2015). En Uruguay, su incidencia es de 5.2 pacientes cada 100.000 habitantes por año(Bianchi et al. 2010), aunque su prevalencia es mucho mayor. La LLC es una enfermedad de linfocitos B maduros, clonales, que se acumulan en la sangre periférica, médula osea y otros tejidos linfoides, y es diagnosticada por la presencia de más de 5000 linfocitos B por microlitro de sangre periférica persistente por más de 3 meses (Fabbri y Dalla-Favera 2016). Esta leucemia se caracteriza por la acumulación de células B clonales circulantes de larga vida, como resultado de un desbalance entre la proliferación celular y la muerte por apoptosis. Cada vez más evidencia sugiere que las células B de LLC en los ganglios linfáticos y la médula osea, que interaccionan con células estromales, reciben señales de proliferación y son protegidas de la muerte celular. Estos datos han llevado a ver a la LLC como un proceso dinámico con células que proliferan y también mueren, a veces a niveles apreciables (Caligaris-Cappio y Ghia 2008). Esta interacción entre células accesorias en microambientes de tejidos especializados favorece la progresión de la enfermedad, promoviendo el crecimiento de las células B malignas, y el surgimiento de nuevas alteraciones genéticas que favorecen la resistencia a los tratamientos (Burger et al. 2009). El pronóstico de la LLC y su curso clínico heterogéneo probablemente se deben, al menos en parte, a esta señalización del microambiente, y aunque los tratamientos existentes a veces producen la remisión de la enfermedad, la LLC sigue siendo incurable en algunos casos (Oppezzo y Dighiero 2013). El debut y la evolución de la LLC son heterogéneos, con pacientes cuya sobrevida oscila entre meses y décadas (Vasconcelos et al. 2003). Un tercio de los pacientes de LLC presenta una enfermedad indolente con una larga sobrevida y nunca requiere tratamiento, otro tercio presenta una enfermedad agresiva desde el comienzo y debe ser tratado inmediatamente, y un último tercio debuta con una enfermedad indolente que puede mantenerse así por años, pero que luego progresa invariablemente hacia una enfermedad agresiva (Dighiero 2003).

El microambiente tumoral

Una visión clásica define al microambiente como el conjunto de células accesorias y matriz extracelular que provee el soporte estructural y molecular, el estroma, para las células parenquimales en un órgano. En el cáncer

ocurren complejas interacciones de naturaleza similar entre las células tumorales y el tejido estromal no tumoral que lo circunda (Ten Hacken y Burger 2014). El intercambio de señales entre las células de LLC y distintos tipos de células estromales, como las células estromales mesenquimales, nurse-like cells derivadas de monocitos, y células T, ocurre en la médula ósea y tejidos linfoides secundarios (Burger y Gribben 2014). Estos tejidos especializados proveen un microambiente favorable para la célula tumoral, promoviendo su proliferación y desarrollo de quimioresistencia en sitios microanatómicos denominados centros proliferantes o seudofoliculos(Burger 2013). Recientemente se ha demostrado mediante la administración de agua deuterada a los pacientes, que el principal sitio de proliferación de las células de LLC es el ganglio linfático, y que la tasa de proliferación en el ganglio se correlaciona inversamente con el tiempo de duplicación linfocitaria y el tiempo al primer tratamiento (Herndon et al. 2017). Tanto las células tumorales como las células accesorias son capaces de liberar vesículas extracelulares al medio tumoral y los fluidos corporales, que funcionan como vehículos de transferencia de información. Así, una visión más moderna del microambiente tumoral debería incluir en su definición también a las vesículas extracelulares (Whiteside 2017a).

Vesículas extracelulares: generalidades

Las vesículas extracelulares constituyen un sistema de comunicación intercelular, dependiente de la dinámica de membranas, que ha recibido una gran atención en las últimas décadas por su capacidad de mediar comunicación entre células a distancia dentro de un organismo. Aunque la terminología en la literatura es confusa en cuanto a su categorización, se ha aceptado que existen al menos tres tipos de vesículas extracelulares exosomas, microvesículas o ectosomas y cuerpos apoptóticos, y se las categoriza de acuerdo a su origen intracelular, tamaño y propiedades (Kosaka et al. 2013; Cocucci y Meldolesi 2015; Urabe et al. 2017). Los exosomas son vesículas cuyo diámetro medio oscila entre 30-100 nm, y tienen su origen en vesículas intraluminales de endosomas tardíos que se convierten en cuerpos multivesiculares (Raposo y Stoorvogel 2013; Cocucci y Meldolesi 2015). Las microvesículas (MV), en cambio, son de mayor tamaño con diámetros que oscilan entre 100 y 500 o 1000nm, y brotan o se desprenden directamente de la membrana plasmática en respuesta a un estimulo (Raposo y Stoorvogel 2013; Urabe et al. 2017). Los cuerpos apoptóticos tienen varias micras de diámetro (800-5000 nm), y se originan cuando las células mueren por apoptosis (Urabe et al. 2017).

Exosomas y microvesículas: origen y tipos de carga

Los exosomas y MV transportan moléculas de microARN, Y-ARN (Haderk et al. 2017), ARNm que pueden ser traducidos a proteína en sus células blanco (Ratajczak et al. 2006; Valadi et al. 2007), lípidos bioactivos, ADN y proteínas(Pando et al. 2018). Su composición refleja su origen intracelular, y permite su identificación. Los exosomas se forman por un proceso de invaginación de los endosomas para formar cuerpos multivesiculares (MVB). Los MVB se fusionan luego con la membrana plasmática liberando así los exosomas al medio extracelular. Así, los exosomas poseen como carga general, miembros de la familia de las tetraspaninas (CD9, CD63 y CD81), miembros de el complejo (endosomal-sorting complex required for transport) ESCRT (TSG101, Alix), proteínas de heat shock (Hsp60, Hsp70, Hsp90), proteínas Rab (Rab27A/B) y proteínas de endosoma tardío/lisosoma (LAMP-1/2) (Iero et al. 2008; Urabe et al. 2017; Hessvik y Llorente 2017). El proceso de formación de las MV es todavía poco conocido, pero se sabe qué ocurre directamente en la membrana plasmática y es dependiente de una vía regulada por calcio. Se requiere la formación de microdominios de membrana tipo lipid rafts, a partir de los cuales ocurre el brotamiento. Como se han identificado también tetraspaninas en las MV (CD9, CD63 y CD81), se ha sugerido que podrían jugar un papel en el brotamiento y fusión de membranas, y en su direccionamiento posterior (Rana y Zöller 2011). También poseen en su superficie externa fosfatidilserina, que normalmente se encuentra en la hemicapa interna de la membrana plasmática, pero durante el brotamiento y fusión de membranas se pierde la asimetría de membrana mediante un proceso de flip-flop. Las microvesículas circulantes forman una población aun más heterogénea y poseen en su superficie algunos marcadores de superficie de sus células de origen (Lawson et al. 2017).

Vesículas extracelulares en el cáncer

Los primeros reportes de vesículas extracelulares fueron realizados a finales de la década de 1970 por dos grupos independientes que trabajaban en neoplasias linfoides, y casi inmediatamente después fueron identificadas en otros tipos de cáncer (Pando et al. 2018). Sin embargo, no fue hasta el ano 2002, que se identificó su rol en la modulación del microambiente tumoral promoviendo la angiogenesis (Kim et al. 2002). Los exosomas y microvesículas presentes en la sangre de los pacientes con tumores constituyen

una población heterogénea compuesta por exosomas y MV originadas en células sanas, células patológicas por condiciones de comorbilidad, y células tumorales. Los exosomas y microvesículas de origen tumoral poseen características moleculares diferentes que las de otras fuentes (Zhang y Grizzle 2014), y entre sus funciones biológicas se incluyen la presentación de antígenos, regulación de la muerte celular programada, angiogenesis, inflamación y coagulación (Théry, Zitvogel y Amigorena 2002).

Las vesículas extracelulares como inmunomoduladoras

Las células estromales mesenquimales, son también llamadas células madre mesenquimales (MSCs), porque son células capaces de autorenovar su población, y de diferenciarse en distintos tipos celulares. Las MSCs poseen baja inmunogenicidad ya que no expresan antígenos HLA de clase II, y son capaces de suprimir la actividad de células efectoras y de promover la actividad de células reguladoras del sistema inmunitario. En el cáncer, las MSC proveen de anclaje a las células tumorales en forma de estroma tumoral, y pueden transdiferenciar en macrófagos M2, células mieloides supresoras (MDSCs), o micrófagos M2 bajo la influencia de quimioquinas o citoquinas (Whiteside 2017a). Las células tumorales pueden ejercer actividades autócrinas, yuxtácrinas, parácrinas, y acaso endocrinas, ya que pueden diseminarse por medio de la circulación y ejercer actividades en sitios del organismo lejanos a su origen. Así, pueden actuar directamente sobre células efectoras, o mediante células de relé inmunomodulatoria como las MSCs. Estas funciones has sido cuidadosamente revisadas recientemente en (Whiteside 2017b). Asimismo, el flujo de información mediado por exosomas o MV puede ser bidireccional, tanto con las MSCs, o con células diferenciadas del sistema inmunitario innato como mastocitos, neutrófilos o células NK (Benito-Martin et al. 2015). Hay evidencias también de que los exosomas derivados de tumores pueden inhibir directamente la proliferación de T CD4+ en respuesta a IL2, que se acompaña de una imposibilidad de up-regular CD25 y de una capacidad supresora mayor de las células T regulatorias posiblemente debido al TGFβ1 asociado a los exosomas. Pueden también afectar a las células T induciendo apoptosis mediada por FAS, y por actividad enzimática, llevando a la producción de adenosina extracelular influenciando así negativamente a los leucocitos infiltrantes del tumor (Thuma y Zöller 2014).

Regulación de la proliferación tumoral y quimioresistencia

La adquisición de resistencia de novo a la quimioterapia, radioterapia, y tratamientos dirigidos representan un obstáculo mayor en la terapéutica del cáncer. Se trata de un proceso multifactorial multifactorial que involucra cambios hacia rutas bioquímicas secundarias cuando la primaria ha sido afectada, supresión epigenética de proteínas supresoras de tumores activadas por microARNs (Rovira et al. 2010), la presencia de células madre tumorales altamente resistentes con capacidad plástica para realizar transiciones epitelio-mesenquimales, o reacciones dermoplásticas que llevan a baja penetración de drogas en el microambiente tumoral, entre otros fenómenos (Azmi, Bao y Sarkar 2013). Se ha reportado que los exosomas pueden jugar un papel en la modulación de estos procesos a través de la transferencia de proteínas como Survivina, que suprime la eficacia de la irradiación por protones en modelos celulares (Khan et al. 2009). Asimismo, los exosomas son capaces de inducir la proliferación de células tumorales, como se ha demostrado en lineas de adenocarcinoma (Xiao et al. 2014) con exosomas derivados de mastocitos, o en linfocitos B con exosomas derivados de lineas celulares de linfoma de Burkitt (Gutzeit et al. 2014), o con exosomas de plasma de pacientes con LLC (Prieto et al. 2017).

Metástasis

La enfermedad metastásica diseminada es responsable de más del 90 % de las muertes relacionadas al cáncer, y representa un desafío científico, médico y tecnológico ya que es difícil de tratar desde la cirugía, la quimioterapia convencional o la radioterapia. La metástasis es un proceso microevolutivo complejo y altamente dinámico en donde algunas células tumorales son capaces de migrar y diseminarse desde su tejido de origen, sobrevivir en microambientes tisulares diferentes, y proliferar a distancia de su origen (Rankin y Giaccia 2016). Una serie de elementos como células de distintos linajes, moléculas de señalización, las matrices extracelulares, y la hipoxia están involucrados en la generación y mantenimiento del microambiente tumoral. Es generalmente aceptada la idea de que estos mismos elementos proveen constantemente al nicho metastásico de sus señales que educan a las células tumorales para volverse agresivas y quimiorresistentes (Azmi, Bao y Sarkar 2013). En los últimos años se han identificado algunos tipos de señalización mediada por exosomas como responsables del condicionamiento del nicho

pre-metastásico (Kaplan et al. 2005; Peinado et al. 2012; Thuma y Zöller 2014), como las proteínas de la familia S100 con roles diversos revisadas en (Bresnick, Weber y Zimmer 2015), así como las integrinas presentes en los exosomas serian responsables del organotropismo metastásico (Hoshino et al. 2015). De este modo, los exosomas derivados de tumores han convocado la atención de una parte de la comunidad científica que ha abordado el desafío de descifrar y controlar esta importante frontera en la biología del cáncer.

Vesículas extracelulares en la LLC

El plasma de pacientes de LLC presenta un numero elevado de microvesículas (MV) y exosomas, y estos modifican vías de señalización intracelular en células estromales de médula osea (Ghosh et al. 2010; Haderk et al. 2013). Como resultado de esta estimulación mediada por MV se alteraría el funcionamiento celular normal, reprogramando vías de señalización que podrían modular el microambiente favoreciendo la progresión tumoral. Según el análisis de su inmunofenotipo, en etapas tempranas de la enfermedad las MV parecen derivar principalmente de plaquetas, mientras que en estadios avanzados son más abundantes (De Luca et al. 2017), y derivarían principalmente de linfocitos B (Ghosh et al. 2010). Recientemente, Crompot y colaboradores han mostrado que una mezcla de exosomas y microvesículas derivadas de células estromales mesenquimales de médula osea en cultivo es capaz de modificar el patrón de expresión génica de las células B de LLC in vitro, y de protegerlas de la apoptosis espontanea. Este tratamiento también mostró que podía estimular la capacidad migratoria de las células B de LLC en ensayos de migración transwell, y la quimioresistencia a 8 drogas, entre las que se encontraba la quimioterapia tradicional (fludarabina, bortezomib) e inhibidores moleculares de última generación (ibrutinib, venetoclax, idelalisib) (Crompot et al. 2017). Las células de LLC liberan más exosomas que los linfocitos B normales, como ocurre en otros tipos de cáncer (Yeh et al. 2015; Wolfers et al. 2001). Los exosomas de LLC son capaces de provocar un fenotipo inflamatorio, modulando una transición fenotípica de células estromales a fibroblastos asociados al cáncer (CAF) a través de la activación de vías de señalización como AKT y NF-κB (Paggetti et al. 2015). Los perfiles de carga de ARNs pequeños y proteínas de los exosomas de la LLC han sido caracterizados (Paggetti et al. 2015; Yeh et al. 2015; Prieto et al. 2017; Haderk et al. 2017). El análisis de estas cargas exosómicas ha permitido comprender parte de la interacción entre las células B de LLC y el micro-

ambiente tumoral, a través de la modulación de células estromales (Paggetti et al. 2015), monocitos (Haderk et al. 2017), o del propio linfocito B (Prieto et al. 2017) que llevan a la generación de un medio inmunotolerante en el que tanto la respuesta a patógenos o a neoantígenos expresados por células malignas esta disminuida (van Attekum, Eldering y Kater 2017).

Marcadores con valor pronóstico en la LLC

La existencia de un grupo de pacientes cuya enfermedad debuta en forma indolente, y que luego progresa hacia una forma agresiva ha hecho que la búsqueda de marcadores con valor pronóstico en la LLC haya sido de importancia capital. En la clínica se utilizan para el pronóstico marcadores clásicos, que surgen de un examen físico o del laboratorio clínico, como la estratificación clínica con los criterios de Rai y Binet, el recuento linfocitario, la morfología linfocitaria en sangre periférica, el tiempo de duplicación linfocitaria, o el grado de infiltración en la médula osea en aspirado o biopsia (Moreno y Montserrat 2008). Se han desarrollado además varios marcadores moleculares con grados variables de implementación en la practica clínica, aunque el más confiable y universal sigue siendo el estado mutacional de los genes de las cadenas pesadas de inmunoglobulina (IgVH) (Damle et al. 1999; Hamblin et al. 1999). Aquellos pacientes portadores de hipermutación somática en sus genes IgVH (IgVH mutados) poseen un mejor pronóstico que aquellos con genes IgVH no mutados. El estado mutacional de los genes IgVH forma parte de un subgrupo de marcadores de tipo genético, junto con las aberraciones cromosómicas y las mutaciones que las preceden en el tiempo (Hurtado et al. 2015). Varias aberraciones cromosómicas han sido identificadas en pacientes de LLC y vinculadas a su evolución(Döhner et al. 2000), de las cuales dos ha sido descritas como marcadores fundamentales de agresividad, las deleciones TP53 en 17p y ATM en 11q (Zenz, Döhner y Stilgenbauer 2007). Otro subgrupo importante de marcadores pronósticos es el de los reporteros plasmáticos de la carga tumoral, β2-microglobulina, CD23 soluble, lactato deshidrogenasa y en algunos casos timidina quinasa (Zenz, Fulda y Stilgenbauer 2010). Un tercer subgrupo de marcadores moleculares son los marcadores celulares, como las proteínas ZAP-70, CD38, y la integrina CD49d (Bulian et al. 2014).

Vesículas extracelulares como marcadores con valor pronóstico en LLC

Como la cantidad de exosomas y MV circulantes aumenta con el progreso de la LLC, se ha sugerido el uso de la cuenta absoluta de MV por microlitro de sangre como factor pronóstico en la LLC (De Luca et al. 2017). También se ha propuesto el uso de algunos biomarcadores derivados de exosomas, principalmente ARNs pequeños con valor pronóstico en la LLC, revisados en (Mirzaei et al. 2018). Es posible que de los resultados de proteómica tipo shotgun (Paggetti et al. 2015; Prieto et al. 2017), así como de los perfiles obtenidos de microarreglos de anticuerpos (Belov et al. 2016) de los exosomas de LLC, aparezcan en el futuro cercano marcadores proteicos con valor pronóstico derivados de exosomas circulantes.

Lipoprotein lipasa (LPL) como marcador pronóstico en la LLC

En la primera década de este siglo, se realizaron análisis de perfil de expresión génica comparando grupos de pacientes IgVH mutados y no mutados. Mediante este tipo de análisis, el nuestro y otros grupos demostraron que el gen LPL, que codifica la lipoprotein lipasa, es expresado de forma diferencial en pacientes mutados y no mutados (Klein et al. 2001; Rosenwald et al. 2001; Vasconcelos et al. 2005). Este resultado motivó el estudio y validación de la expresión de dos genes, LPL en pacientes IgVH no mutados y ADAM29 en pacientes IgVH mutados, como candidatos para proponer un nuevo método pronóstico basado en ARN. El método fue puesto a prueba en una cohorte de 127 pacientes de LLC, y correlacionado con el estado mutacional de los genes IgVH y la evolución clínica. Finalmente, nuestro grupo mostró que la cuantificación de la relación entre la expresión de LPL/ADAM29 es un marcador pronóstico fuerte en la LLC, y que provee mejor discernimiento de la evolución clínica que los marcadores serológicos en estadios avanzados de la enfermedad (Oppezzo et al. 2005). Posteriormente, un gran cuerpo de evidencia ha probado que la presencia del ARNm de LPL esta asociado a un mal pronóstico, y que es el marcador molecular más robusto en la LLC (Heintel et al. 2005; van't Veer et al. 2006; Nückel et al. 2006; Mansouri et al. 2010b; Kaderi et al. 2011; Porpaczy et al. 2013; Mátrai et al. 2017). La expresión incrementada del gen LPL en células B de pacientes IgVH no mutados es una observación destacable, ya que no

hay expresión de LPL en las células B normales. Esta expresión especifica y ectópica constituye no solamente un marcador pronóstico adecuado para estudiar la evolución de la enfermedad, sino que también podría ser de utilidad para entender el curso proliferativo heterogéneo de la LLC. A pesar de que el valor pronóstico de la expresión de LPL esta ya bien consolidado, el papel funcional de la expresión de LPL en la patogénesis de la LLC y los mecanismos que controlan su expresión siguen siendo materia de debate (Prieto y Oppezzo 2017). A pesar de estar bien consolidado como factor pronóstico, hasta el momento no se ha implantado el uso del ARNm de LPL en la practica clínica, dado que requiere análisis de biología molecular al igual que el perfil mutacional de genes IgVH, y muchos servicios médicos aun no proveen de rutina tales análisis. Aunque desde hace tiempo se sabe que la proteína LPL se expresa en algunos pacientes de LLC, y se ha descrito que posee una distribución puntiforme en la célula, se ha intentado infructuosamente determinar la utilidad de la proteína LPL como marcador, ya que los niveles de proteína parecen no reflejar los niveles de ARNm (Heintel et al. 2005), lo que ha minado la posibilidad de utilizarlo como marcador en citología o citometría de flujo, que son las técnicas de rutina normalmente empleadas en la mayoría de los laboratorios clínicos. Esta posibilidad presenta un importante atractivo, ya que los marcadores actualmente disponibles para pronóstico de LLC mediante citometría de flujo como ZAP-70 y CD38 presentan desventajas técnicas que han impedido su universalización. En el caso de ZAP-70, el problema biológico radica en que la proteína es expresada en otras células de la sangre aparte del clon tumoral, lo que plantea problemas técnicos a la hora de establecer los controles internos en las células T o NK, o el origen de las muestras en sangre periférica o médula ósea (Rassenti y Kipps 2006); y en el caso de CD38 el problema surge del cambio que sufren sus niveles de expresión con la activación de las células de LLC (Brachtl et al. 2014). Respecto a las funciones de LPL en las células de LLC, cada vez más evidencia sugiere que su expresión podría ayudar al clon leucémico a incrementar su sobrevida y señalización proliferativa, llevando al progreso de la enfermedad.

Nuestro grupo ha mostrado que las señales del microambiente pueden inducir la expresión de LPL y un fenotipo proliferante en cultivos primarios de células B de LLC (Abreu et al. 2013; Moreno et al. 2013). En el mismo sentido, Rozovski, Grgurevic, et al. Mostraron que LPL confiere una ventaja en la sobrevida a las células de LLC, puesto que el silenciamiento de LPL con shRNA incrementa la muerte apoptótica de las mismas (Rozovski et al. 2015). Asimismo, recientemente se ha reportado que las mutaciones en el gen NOTCH1, que están asociadas a la progresión de la enfermedad

y refractariedad al tratamiento (Sportoletti et al. 2010) están directamente relacionadas a la expresión de LPL en la LLC (Kristensen et al. 2016). Respecto de los mecanismos que regulan la expresión de LPL, nuestro grupo ha demostrado que la expresión anormal del gen LPL en pacientes de LLC IgVH no mutado es producto de la desmetilación en la isla CpG que abarca todo el exón 1 y los primeros nucleótidos del primer intrón de LPL (Abreu et al. 2013). Este mecanismo epigenético parece ser disparado principalmente por señales dependientes de células T, y en algunos pacientes a través del cross-linking del receptor de la célula B (BCR). Por el contrario, la señalización a través de las vías de TLR9 o TLR1/2 no son capacees de inducir la desmetilación de la isla CpG, la expresión de LPL ni la proliferación de las células B (Moreno et al. 2013). Rozovski, Grgurevic, et al. mostraron que la expresión de LPL también puede ser regulada transcripcionalmente por la fosforilación de STAT3 y su translocación al núcleo, donde puede unirse al promotor de LPL (Rozovski et al. 2015). Además, la expresión de LPL puede ser regulada postranstripcionalmente a través del micro ARN miR-29 (Chen et al. 2011; Bouvy-Liivrand et al. 2014), suya expresión se sabe que esta disminuida en pacientes de alto riesgo con IgVH no mutado (Calin et al. 2004; Fulci et al. 2007; Marton et al. 2008; Stamatopoulos et al. 2009).

Funciones metabólicas de LPL en el linfocito B de LLC

LPL es capaz de mediar procesos de lipólisis y consecuentemente alimentar la proliferación celular mediada por ácidos grasos en varios tipos de tumores sólidos(Kuemmerle et al. 2011), y recientemente se ha reportado que las lipoproteínas de baja densidad pueden potenciar respuestas proliferativas de las células de LLC en respuesta a señales pro-inflamatorias(McCaw et al. 2017). Los niveles de la proteína PPARα en linfocitos B de LLC se han correlacionado con la leucocitosis y con la estratificación clínica de Rai, lo que sugiere un cambio metabólico hacia la oxidación de ácidos grasos vía PPARα(Spaner et al. 2013) y PPARδ (McCaw et al. 2017). A estos hallazgos se suma la observación de que los linfocitos B tumorales de LLC tienen estructuras vacuolares en su citoplasma, y que la incubación con ácidos grasos libres (FFAs) incrementa su tasa metabólica en función del consumo de oxígeno (Rozovski et al. 2015). Además se ha encontrado que la incidencia de la hiperlipidemia es mayor en pacientes de LLC, y que el tratamiento de la misma con estatinas retrasa el tiempo al tratamiento de la LLC(Chow, Buckstein y Spaner 2015). El mismo grupo expandió su estudio inicial a

una cohorte de >2000 pacientes de LLC en retrospectiva y encontró que tanto el tratamiento con drogas que disminuyen los lípidos, como el tratamiento con estatinas, prolongaba la sobrevida total de los pacientes en 3,7 años (Mozessohn et al. 2017). Estos hallazgos sugieren que un segundo mecanismo mediado por LDL podría convergir en la fosforilación de STAT3, generando una activación de los linfocitos B en la LLC (McCaw et al. 2017). El estudio de perfiles transcripcionales ha permitido identificar un cambio metabólico hacia una estrategia tipo muscular o adiposa, con oxidación de lípidos en células B de pacientes de mal pronóstico IgVH no mutado(Bilban et al. 2006). Cómo es que esta reprogramación metabólica termina repercutiendo en una mala evolución de la enfermedad, es algo que recién se esta comenzando a entender. Los ácidos grasos de cadena larga, el colesterol libre y la vitamina E aumentan la fosforilación de STAT3 mediada por IL-10, interferon-α o esteres de forbol en células de LLC(McCaw et al. 2017). La fosforilación de STAT3 a su vez dirige directamente la expresión de LPL, a través de la unión a un elemento tipo GAS 280pb rio arriba del sitio de inicio de la transcripción de LPL, y activando su transcripción(Rozovski et al. 2015). LPL favorece la oxidación de ácidos grasos, y esto parece resulta en una mayor sobrevida celular, puesto que tanto el silenciamiento de LPL como su inhibición química reducen la viabilidad de las células de LLC(Pallasch et al. 2008; Rozovski et al. 2015), lo que podría explicarse en parte por una respuesta transcripcional(Porpaczy et al. 2013). Asimismo, la inducción de la expresión de LPL por señales del microambiente estimula la proliferación celular(Moreno et al. 2013). Estos hallazgos indican que la expresión de LPL puede ser regulada por el microambiente, sea por señales autócrinas o parácrinas, y reflejan un cambio metabólico en las células B de LLC que les confiere una ventaja adaptativa. Una retroalimentación positiva podría mantener la expresión de LPL y empeorar el escenario para los pacientes IgVH no mutado. En la LLC STAT3 esta constitutivamente activado, lo que a su vez activa la expresión de LPL(Rozovski et al. 2015). LPL degrada lipoproteínas de muy baja densidad (VLDLs) y quilomicrones, liberando ácidos grasos libres(FFAs), y generando un estado pro-inflamatorio que a su vez activa STAT3(Spaner et al. 2013) y más activación de la transcripción de LPL. Esto a su vez incrementaría los niveles de FFAs, exagerando la respuesta de las celulas de LLC a la señalización por citoquinas. Los aspectos más generales de las vías metabólicas en LLC han sido recientemente revisados(Rozovski et al. 2016).

Lipoprotein lipasa

Funciones no metabólicas de LPL en el linfocito B de LLC En muchos estudios se ha encontrado una expresión aumentada de LPL en LLC de mal pronóstico, y varias vías metabólicas podrían estar involucradas en la progresión tumoral, como se ha indicado. Sin embargo, los intentos por determinar la actividad metabólica de LPL directamente no han podido correlacionar mayor expresión con mayor actividad metabólica. Un trabajo fermental con 33 pacientes de LLC reporto menor actividad catalítica en pacientes con IgVH no mutado respecto de aquellos con IgVH mutado(Nückel et al. 2006). Otro estudio analizando datos de 42 pacientes no logro hallar diferencias entre grupos de LLC, y reporto que la actividad de LPL era comparable a la de los individuos sanos(Porpaczy et al. 2013). LPL puede mediar la internalización de lipoproteínas por parte de las células(Merkel et al. 1998), el anclaje de quilomicrones a la superficie celular a través del receptor relacionado a LDL(Beisiegel, Weber y Bengtsson-Olivecrona 1991), y la marginación de lipoproteínas en vasos sanguíneos pequeños, uniéndose por un lado a la superficie extracelular del endotelio vía GPIHBP1, y por el otro a las lipoproteínas ricas en triglicéridos(Goulbourne et al. 2014). Además de su rol canónico en el metabolismo de lípidos, una interesante e inexplorada función no metabólica de LPL se conoce desde hace 20 años. LPL puede actuar como molécula de conexión (bridging) entre células, como ocurre en la adhesión de monocitos a las células endoteliales mediada por proteoglicanos de heparán sulfato (HSPGs) y LPL(Mamputu, Desfaits y Renier 1997), cuya interacción es dinámica, según se ha reportado recientemente(Allan et al. 2017). Como las células de LLC poseen HSPGs en si superficie(Van Bockstaele et al. 2007), y LPL forma homodímeros, podría ocurrir una unión entre las células B leucémicas y otras células que posean en su superficie HSPGs o GPIHBP1, como las células endoteliales, y que esto ocurra a través de un puente de LPL. Varios grupos han especulado respecto de la función de un puente intercelular mediado por LPL en la patogénesis de la LLC(Mansouri et al. 2010a; Moreno et al. 2013; Rombout, Verhasselt y Philippé 2016), aunque aun nadie lo ha demostrado. Si tal puente ocurriera, LPL estaría entonces pivotando entre los HSPGs en la superficie del linfocito leucémico, y HSPGs o GPIHBP1 en otra célula. Rombout et al. Han descrito que dos polimorfismos de nucleótido único (SNPs) encontradas comúnmente en LPL, rs328 (codón stop prematuro) y rs13702 estaban significativamente asociadas con el curso de la LLC(Rombout et al. 2015). Aunque ambos SNPs son mutaciones de ganancia de función conocidas(Deo et al. 2009; Richardson et al. 2013), los autores reportaron no haber podido detectar diferencias

significativas en LPL ni en ARNm, ni en niveles proteicos, ni en actividad enzimática en los pacientes que portaban los SNPs(Rombout et al. 2015). Cómo es que estas mutaciones afectan el curso clínico de la LLC no esta claro aun, pero tampoco se ha explorado aun si estos SNPs podrían tener una función no metabólica en la LLC. Además, se han descrito al menos nueve isoformas de LPL con diferentes puntos isoeléctricos en la sangre de individuos sanos(Badia-Villanueva et al. 2014), lo que abre un nuevo abanico de estudios posibles para las funciones de LPL en la LLC y otras patologías. LPL podría contribuir así a la progresión leucémica por si misma, a través de la reprogramación metabólica, o a través de una contribución en sinergia con un microambiente activador, en el que el clon leucémico es estimulado constantemente (Figura 1).

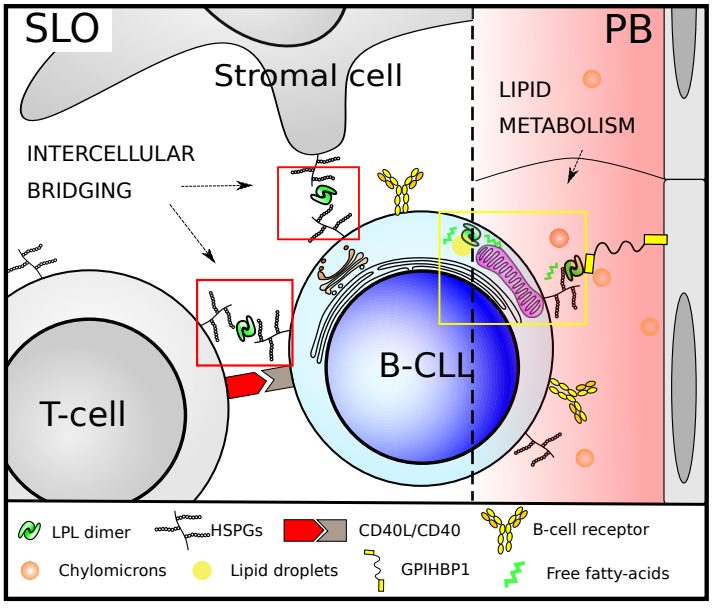

Figura 1: Modelo hipotético de las funciones de LPL en las células B de LLC en órganos linfoides secundarios (SLO, izquierda) y sangre periférica (PB, derecha). Las moléculas de LPL ancladas a HSPGs en la superficie del linfocito B de LLC pueden unirse a lipoproteínas de muy baja densidad y quilomicrones contribuyendo al metabolismo oxidativo y la señalización mediada por ácidos grasos. Se ha propuesto que LPL podría tener un papel similar en el compartimiento intracelular, liberando FFAs desde gotas lipídicas en el citosol(Rozovski et al. 2015, 2016). Un papel no canónico para LPL en la superficie del linfocito leucémico podría contribuir a la señalización microambiental. LPL funcionaría como una molécula puente entre células capaces de unirla en su superficie, sea a través de HSPGs o GPIHBP1, facilitando interacciones modulatorias aquí ejemplificadas con una activación T-dependiente a través de la unión CD40L/CD40.

Objetivos

Objetivo general

Identificar nuevos marcadores moleculares de la progresión de la LLC.

Objetivos específicos

1. Caracterizar el perfil proteómico de los exosomas plasmáticos de LLC en proceso de progresión, y comparar el perfil proteómico de exosomas plasmáticos de la progresión de LLC con el de pacientes indolentes.
2. Evaluar el potencial de alguna de las proteínas exclusivas de los tiempos de progresión como biomarcador.
3. Explorar la capacidad de afectar la proliferación del linfocito B leucémico por las proteínas identificadas como potenciales biomarcadores exosómicos de progresión de LLC.
4. Caracterizar el patrón subcelular de expresión de la proteína LPL en la célula B de LLC.
5. Determinar si la proteína LPL en el linfocito B leucémico se corresponde con el patrón de expresión de su ARN mensajero, y por qué no ha sido posible utilizar la proteína LPL como marcador con valor pronóstico en la LLC hasta el momento.
6. Desarrollar un protocolo basado en citometría de flujo para evaluar la expresión de LPL en la LLC, para evaluar si la expresión de la proteína lPL puede convertirse en un marcador pronóstico para la LLC.

Resultados

I. S100-A9 protein in exosomes from chronic lymphocytic leukemia cells promotes NF-κB activity during disease progression

En este artículo realizamos una caracterización proteómica de los exosomas obtenidos del plasma de pacientes con leucemia linfoide crónica (LLC) progresiva o indolente, al inicio de la enfermedad y durante su evolución. Investigamos si existía un perfil proteómico diferencial de los exosomas a lo largo de la evolución clínica de la LLC, y nos enfocamos en las vías y proteínas asociadas a los exosomas que podrían estar implicadas en la progresión de la enfermedad. Para ello, realizamos un estudio prospectivo en el que purificamos exosomas a partir de plasma de pacientes de LLC con enfermedad indolente y progresiva al momento del debut clínico, y al momento de la progresión, o en el caso de los pacientes con LLC indolente, luego de 4 años de sobrevida. En las LLC progresivas identificamos 138 proteínas, de las cuales 99 (71 Comparando los dos tiempos de los pacientes con enfer-

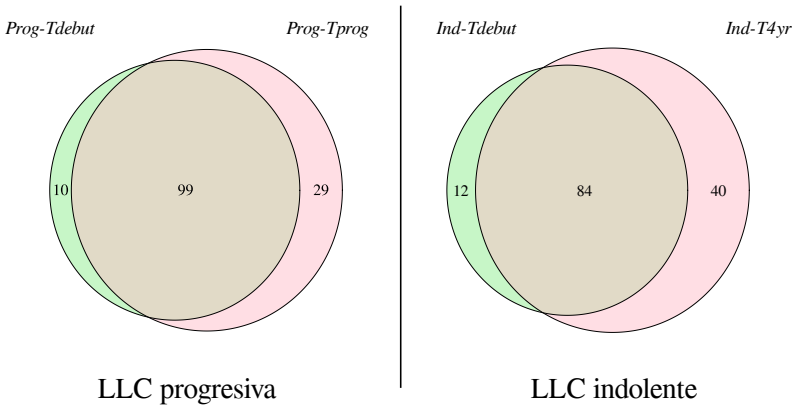

Figura 2: Proteínas identificadas en los exosomas plasmáticos de LLC en pacientes con enfermedad progresiva o indolente al tiempo del debut de la enfermedad (T*debut*), de la progresión (T*prog*), o de 4 años de sobrevida libre de progresión en el caso de los pacientes con LLC indolente(T*4yr*)

medad progresiva y los indolentes identificamos un perfil proteómico propio de los exosomas plasmáticos durante la evolución de la LLC. Considerando aquellas proteínas que se encontraban diferencialmente en al menos tres pacientes, como medida de consistencia, identificamos 10 proteínas que se

encontraban unicamente en durante la progresión de la enfermedad, de las cuales seleccionamos dos para continuar trabajando. Descubrimos que los exosomas de pacientes con enfermedad progresiva, poseen sobrerepresentadas las proteínas S100-A9 y plakoglobina de uniones (JUP), asociadas a progresión tumoral, como activadoras de las vías de señalización NF-κB y Wnt respectivamente. Demostramos además que los exosomas cargados de S100-A9 pueden ser liberados *ex vivo* por células B de pacientes cuya enfermedad progresa, al estimular sus inmunoglobulinas de superficie (BCR) con anticuerpos anti IgM. También probamos que las células B de LLC expresan uno de los receptores de S100A9, EMMPRIN (CD147), y que éste es capaz de unir *ex vivo* a su ligando en la superficie celular. Asimismo, describimos que las células B de LLC expresan S100-A9, que su expresión es mayor en los pacientes con enfermedad progresiva respecto de los indolentes, y que los niveles máximos de expresión se encuentran en los ganglios linfáticos respecto de las células circulantes. Esta elevada expresión de S100-A9 coincide con un perfil de activación celular, ya que las células de los ganglios que expresan S100-A9, presentan además elevados niveles del activador de la proliferación celular Ki-67, y de la proteína antiapoptótica survivina. También en este artículo probamos que las células B de LLC con elevada expresión de S100-A9 presentan la vía NF-κB activada. Finalmente, demostramos que los exosomas plasmáticos de LLC progresiva cargados de S100-A9 son capaces de activar NF-κB de forma dosis-dependiente en células B de LLC *ex vivo*, y que esta activación de NF-κB ocurre unicamente por la vía canónica.

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LYMPHOID NEOPLASIA

S100-A9 protein in exosomes from chronic lymphocytic leukemia cells promotes NF-κB activity during disease progression

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Key Points

- Plasma-derived exosomes from patients with CLL exhibit different protein cargo compositions depending on disease status and progression.
- S100-A9 protein is overexpressed and S100-A9 cargo in exosomes activates NF-κB pathway in patients with CLL during disease progression.

Chronic lymphocytic leukemia (CLL) is an incurable disease characterized by accumulation of clonal B lymphocytes, resulting from a complex balance between cell proliferation and apoptotic death. Continuous crosstalk between cancer cells and local/distant host environment is required for effective tumor growth. Among the main actors of this dynamic interplay between tumoral cells and their microenvironment are the nano-sized vesicles called exosomes. Emerging evidence indicates that secretion, composition, and functional capacity of exosomes are altered as tumors progress to an aggressive phenotype. In CLL, no data exist exploring the specific changes in the proteomic profile of plasma-derived exosomes from patients during disease evolution. We hereby report for the first time different proteomic profiles of plasma exosomes, both between indolent and progressive CLLs as well as within the individual patients at the onset of disease and during its progression. Next, we focus on the changes of the exosome protein cargoes, which are found exclusively in patients with progressive CLL after disease progression. The alterations in the proteomic cargoes underline different networks specific for leukemia progression related to inflammation, oxidative stress, and NF-κB and phosphatidylinositol 3-kinase/AKT pathway activation. Finally, our results suggest a preponderant role for the protein S100-A9 as an activator of the NFκB pathway during CLL progression and suggest that the leukemic clone can generate an autoactivation loop through S100-A9 expression, NF-κB activation, and exosome secretion. Collectively, our data propose a new pathway for NF-κB activation in CLL and highlight the importance of exosomes as extracellular mediators promoting tumor progression in CLL. (*Blood.* 2017;130(6):777-788)

Introduction

Chronic lymphocytic leukemia (CLL) develops through accumulation of malignant B cells that circulate in the blood and are continuously supported by microenvironment signals within the bone marrow and secondary lymphoid organs.[1] Exchange between primary tumor cells and their microenvironment represents bidirectional crosstalk, in which the tumor clone is not only being supported by the microenvironment but also modulating it to maintain and promote its own survival.[2,3]

Among the main actors in the dynamic and reciprocal interplay between tumor cells and their microenvironment are extracellular vesicles (EVs).[4] They represent an important mode of intercellular communication, serving as vehicles for transfer between cellular membranes, proteins, lipids, and RNA. EVs include microvesicles (MVs), which shed directly from the plasma membrane with a diameter of 100 to 1000 nm,[5] and exosomes, which are conformed by nano-sized vesicles of endosomal origin of 40 to 100 nm in diameter.[6] Similar to MVs, exosomes are directly associated with oncogenic reprogramming,[7] tumor progression,[8] metastasis,[9] and therapy refractoriness.[10]

In CLL, the total number of MVs in plasma is greater than in healthy donors, and in vitro studies have demonstrated that MVs could mediate AKT activation in bone marrow stromal cells.[11] Regarding CLL exosomes, recent work has demonstrated that they could induce an inflammatory phenotype through a transition of stromal cells into cancer-associated fibroblasts and activation of key signaling pathways such as AKT and/or NF-κB.[12] Although microRNAs and proteomic profiles of exosomes have been described,[12,13] proteomic analysis of primary exosomes throughout CLL evolution has not been fully performed. Furthermore, the direct implications of changes occurring in

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exosome protein cargoes throughout CLL evolution and their role during disease progression remain unknown.

We hereby report a comprehensive analysis involving the proteomic characterization of exosomes from patients with indolent or progressive CLL at the onset of disease and during disease evolution. We examined whether there is a differential proteomic profile from exosomes throughout CLL evolution and focused on the different pathways and proteins that could be implicated in disease progression. We found that S100-A9 protein is only present in exosomes of patients with progressive disease at the time of progression and that this protein is specifically overexpressed by leukemic cells at this stage. Finally, we demonstrate that exosomes carrying S100-A9 are able to activate the NF-κB pathway in primary CLL cells, suggesting that the leukemic clone could generate an autoactivation loop through S100-A9 expression, exosome secretion, and NF-κB activation.

Together, these results show the importance of exosomes in cell-to-cell communication during CLL evolution and suggest that exosomes with S100-A9 protein cargo could be one of the mechanisms responsible for the activation of the NF-κB pathway during disease progression.

Materials and methods

Clinical samples

Plasma and peripheral blood mononuclear cells (PBMCs) were collected in a prospective study from 34 patients with typical CLL diagnoses. Five age-matched healthy donors (HDs) were included as control samples. Patients were segregated into 2 groups: (1) progressive disease, defined by lymphocyte doubling time <6 months, treatment required within 3 years, activation-induced cytidine deaminase/lipoprotein lipase expression, and >2% of CLL B cells with ongoing class-switch recombination process in peripheral blood (PB), as described by Palacios et al,[14] and (2) indolent disease, defined by lymphocyte doubling time >4 years, no treatment required after 4 years, absence in PB (<1%) of CLL B cells with ongoing class-switch recombination process to immunoglobulin G (IgG), and absence of activation-induced cytidine deaminase expression. All samples were obtained at disease onset and after 4 years during disease evolution or after treatment in those with progressive disease. All patients were observed at Hospital Maciel and at the Haematology Department of Clinical Hospital in Montevideo. They provided informed consent according to the ethical regulations of Uruguay and the Helsinki Declaration.

Exosome isolation

Patients' plasmas were diluted 1:2 in phosphate-buffered saline and centrifuged (10 minutes at 400 g, then 20 minutes at 2000 g) to remove cell debris and larger vesicles. Next, centrifugation at 10 000 g for 60 minutes at 4C° was performed to separate MVs. Exosomes were isolated by ultracentrifugation (120 minutes at 100 000 g at 4°C) followed by floatation on Optiprep cushion (Axis-Shield; 17%) for 120 minutes at 100 000 g at 4°C to remove nonexosomal protein complexes. After washing, exosomes were suspended in phosphate-buffered saline and subjected to NanoSight analysis (Malvern Instruments, Inc., Westborough, MA).

Proteomic analysis

Peptides were separated by nano–high-performance liquid chromatography (EASY 1000; Thermo Fisher) fitted with a reverse-phase column (50 cm × 75 μm ID, PepMap RSLC C18; Thermo Fisher) using a linear gradient (acetonitrile, 0.1%; formic acid, 5%-55% in 75 minutes). Peptide analysis was carried out via an LTQ Velos (Thermo Fisher) in a data-dependent acquisition mode (top 10) with a dynamic exclusion list of 45 seconds. Data analysis was performed using PatternLab for proteomics software (version 3.2.0.3).[15,16] Raw files were searched against a *Homo sapiens* target-decoy database using the Comet search engine (PatternLab). The approximate area proportional Venn diagram module was used to perform comparisons between different progression times and determine proteins uniquely identified in each situation.[16] Exosomal protein data sets were subjected to functional enrichment analyses using FunRich software.[17] The mass spectrometry proteomic data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD005941.

Flow cytometry, western blot, confocal microscopy, and reverse-transcription polymerase chain reaction

Isolation of PBMCs from patients with CLL and HDs, as well as cytometry analysis (flow cytometry) and immunoblot, was performed as described.[18,19] Antibodies used in this work are described in supplemental Table 1, available on the *Blood* Web site. Flow cytometry analysis was performed with R/Bioconductor[20] packages or FlowJo (Treestar, Ashland, OR) or Summit software (Beckman Coulter, CA). Microscopy analysis was performed as previously described,[21] with antibodies listed in supplemental Table 1. RNA isolation and reverse-transcription polymerase chain reaction were carried out as described by Oppezzo et al.[22] Primers used for the amplification of extracellular matrix metalloprotease inducer (EMMPRIN) isoforms and the full-length S100-A9 are provided in supplemental Table 2.

In vitro analysis

Purification of $CD19^+$ cells from patients with CLL and activation with anti-IgM (Jackson ImmunoResearch) were performed as described by Moreno et al.[23] Exosome activation on CLL cells was performed with PBMCs (5×10^6) cultured in RPMI 1640, 10% fetal bovine serum and incubated for 90 minutes with exosomes (30 μg/mL total protein). For blocking experiments, exosomes were preincubated with 1 μg/mL of anti-human S100-A9 (R&D Systems, Minneapolis, MN) or Human IgG Isotype Control (Thermo Fisher) for 1 hour at 4°C. Recombinant human S100-A9 protein (rhS100-A9; MyBiosource, San Diego, CA) was incubated with CLL PBMCs as described in supplemental Table 1.

Statistical analysis

Results are expressed as the mean ± standard error of the mean of at least 3 independent experiments. Differences between groups were analyzed using the Mann-Whitney test or 1-way analysis of variance (Student *t* test); $P < .05$ was considered statistically significant. Analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). For proteomic assays, false-discovery rate correction was performed using GeneSpring GX11.0.2 software (Agilent Technologies, Santa Clara, CA); cutoff of enrichment analyses in FunRich software was kept as default ($P < .05$) after Bonferroni correction.

Results

Characterization of patients with CLL and plasma-derived exosomes

To determine the clinical relevance of circulating exosomes in patients with CLL, we isolated and characterized exosomes from the plasma of a working cohort composed of: (1) group A, comprising 5 patients with progressive disease, with samples obtained at the time of diagnosis during stable disease (Prog-dt) and later during disease progression (Prog-ddp) but before treatment, and (2) group B, consisting of 5 patients with indolent disease, with samples obtained at diagnosis (Ind-dt) and after 4 years of follow-up without disease evolution (Ind-4years). Rationale of the study, clinical statement, and molecular characterization of patients with CLL are provided in Table 1.

Isolated particles presented bona fide characteristics of exosomes, with size ranging from 40 to 100 nm and a typical profile confirmed by NanoSight and electron microscopy (Figure 1A). Additionally, different protein migration patterns were visualized between exosomes

Table 1. Clinical and molecular characterization of patients with CLL

		CLL samples at time of disease onset (subgroups Prog-dt and Ind-dt)							CLL samples during disease progression or after 4 y of follow-up (subgroups Prog-ddp and Ind-4years)						
Study rationale	Patient No.	Binet stage	LC, × 10^3/μl	Clinical disease status	FISH	AID/LPL*	CSR, %†	Binet stage	LC, × 10^3/μl	Clinical disease status	FISH	AID/LPL*	CSR, %†	TFT, months	IgVH status‡
Study cohort (progressive patients)	1	A	19.0	Ind.	Normal	(+/+)	2.5	B	74.00	Prog.	del17p	(+/+)	2.0	18	UM
	2	A	28.0	Ind.	del11q	(+/+)	2.1	B	162.0	Prog.	del11q	(+/+)	2.9	28	UM
	3	A	23.00	Ind.	del13q/del11q	(+/+)	2.8	C	120.0	Prog.	del13q/del11q	(+/+)	3.1	32	UM
	4	A	35.00	Ind.	del11q	(+/+)	4.1	C	164.0	Prog.	del11q	(+/+)	5.5	14	UM
	5	A	15.90	Ind.	Normal	(+/+)	3.8	C	220.0	Prog.	Normal	(+/+)	4.2	9	UM
Study cohort (indolent patients)	6	A	27.70	Ind.	Normal	(−/−)	1.2	A	19.64	Ind.	Normal	(−/−)	0.9	N'T	Mut
	7	A	24.60	Ind.	Normal	(−/−)	0.02	A	34.55	Ind.	Normal	(−/−)	0.5	N'T	Mut
	8	A	14.70	Ind.	del13q	(−/−)	1.1	A	12.50	Ind.	del13q	(+/−)	1.2	N'T	Mut
	9	A	8.25	Ind.	del13q	(−/−)	0.8	A	14 490	Ind.	del13q	(−/−)	0.3	N'T	Mut
	10	A	18.00	Ind.	Normal	(−/−)	1.5	A	20.00	Ind.	Normal	(−/−)	1.8	N'T	Mut
Validation cohort (progressive patients)	11	A	26.20	Ind.	del13q	(+/−)	1.5	C	183.0	Prog.	del13q	(+/−)	2.2	33	UM
	12	A	19.45	Ind.	Normal	(−/+)	0.5	B	112.0	Prog.	del17p	(+/+)	2.5	35	UM
	13	A	40.20	Ind.	del13q	(+/+)	1.2	B	98.00	Prog.	del11q	(+/+)	2.2	31	UM
	14	A	18.30	Ind.	del13q/del11q	(+/+)	1.6	C	178.00	Prog.	del13q/del11q	(+/+)	3.1	6	UM
	15	A	20.26	Ind.	del17p	(−/+)	1.8	B	154.00	Prog.	del17p	(+/+)	2.3	40	UM
	16	A	10.58	Ind.	del11q	(+/+)	0.5	B	120.0	Prog.	del11q	(+/+)	1.8	10	UM
	17	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	B	77.00	Prog.	del11q	(+/+)	3.6	9	UM
	18	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	C	161.00	Prog.	del13q	(+/−)	2.9	11	UM
	19	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	B	72.0	Prog.	del11q	(+/+)	4.4	38	UM
	20	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	C	900.0	Prog.	Normal	(+/+)	1.2	8	UM
	21	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	B	110.0	Prog.	del13q	(−/+)	4.4	12	UM
	22	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	C	97.00	Prog.	Normal	(+/+)	2.5	26	UM
	23	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	N/A$_{(1)}$	B	65.00	Prog.	Normal	(+/+)	1.5	24	UM
Validation cohort (indolent patients)	24	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	15.60	Ind.	Normal	(−/+)	0.6	130	Mut
	25	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	8.90	Ind.	tris12	(−/−)	0.5	N'T	Mut
	26	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	9.92	Ind.	Normal	(−/−)	0.07	N'T	Mut
	27	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	15.00	Ind.	Normal	(−/−)	0.05	N'T	Mut
	28	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	7.00	Ind.	del13q	(−/+)	1.5	N'T	Mut
	29	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	11.60	Ind.	del13q	(+/−)	1.1	N'T	Mut
	30	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	14.70	Ind.	tri12	(−/−)	2.1	126	Mut
	31	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	22.20	Ind.	Normal	(−/+)	1.8	N'T	Mut
	32	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	11.60	Ind.	Normal	(−/−)	0.6	N'T	Mut
	33	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	17.50	Ind.	Normal	(−/−)	0.7	N'T	Mut
	34	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	N/A$_{(2)}$	A	19.90	Ind.	del13q	(−/−)	1.1	N'T	Mut

AID, activation-induced cytidine deaminase; CSR, class-switch recombination; FISH, fluorescence in situ hybridization; LC, lymphocyte count; LPL, lipoprotein lipase; Mut, mutated; N/A$_{(1)}$, not applicable (refers to patients with progressive disease whose samples were not collected at disease onset); N/A$_{(2)}$, not applicable (refers to analyzed samples collected from indolent patients after 4 years of follow-up); N'T, not treated (refers to patients who did not receive any treatment at 4-year follow-up); TFT, time from initial diagnosis to first treatment for clinical progression; UM, unmutated.

*AID and LPL expression were assessed by quantitative polymerase chain reaction, as described by Prieto et al.[21]

†Percentage of proliferative fraction expressing IgM$^+$ /IgG$^+$ evaluated by cytometry studies, as described by Palacios et al.[14]

‡+- 2% difference from germline gene defined UM patients; = 2% difference defined Mut patients.

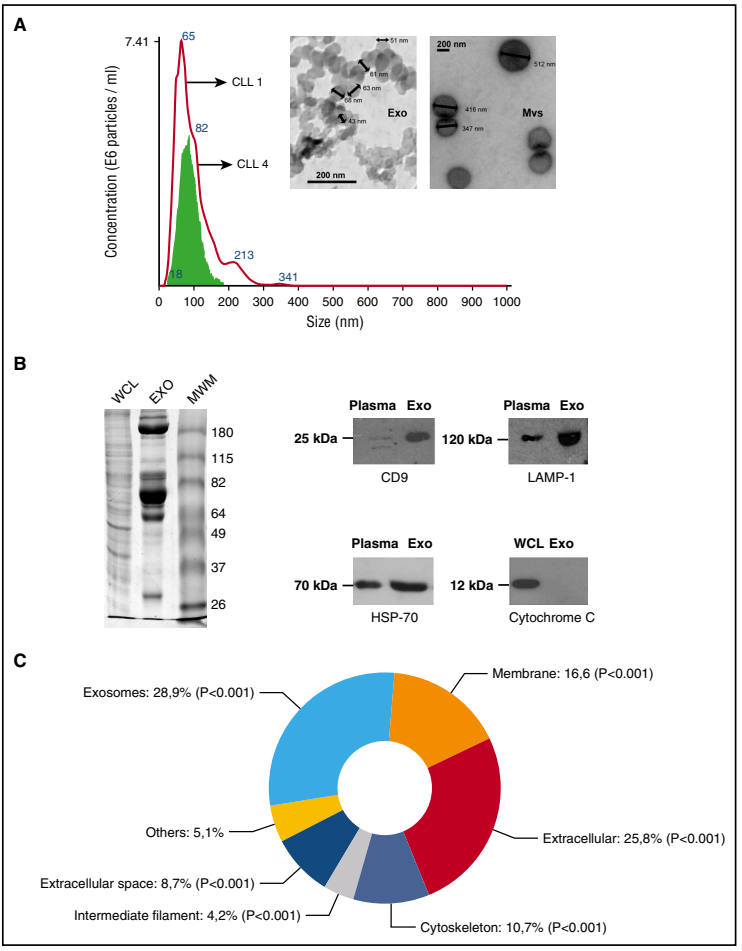

Figure 1. Characterization and proteomic analysis of CLL plasma-derived exosomes. (A) Electron microscopy and NanoSight analysis of exosomes (Exo) purified from CLL plasmas. Comparative electron micrographs of MVs and exosomes from the same patient are depicted. Blue numbers indicate size of main peaks. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot characterization of plasma-derived exosomes. After protein quantification, exosomes were lysed in Laemmli buffer to perform SDS-PAGE and immunoblot. Different migration profiles in SDS-PAGE of exosome fractions and immunoblot with specific monoclonal antibodies against typical exosome proteins were used to validate the quality of our isolation technique. The proteins identified were tetraspanin CD9, heat shock protein HSP-70, and lysosomal marker LAMP-1. These were compared with the plasma of the same patient at the same disease time. Cytochrome C was also evaluated comparing whole-cell lysate (WCL) with plasma-derived exosomes of the same patient. (C) Subcellular localization of the proteins present in the plasma-derived exosomes purified of the different subgroups was analyzed by FunRich software. MWM, molecular-weight marker.

and whole-cell lysate of the same patients, whereas classical exosome markers such as CD9, HSP-70, and Lamp-1[24] were enriched in the exosome fraction. In turn, cytochrome C was absent, excluding contamination with apoptotic bodies (Figure 1B). Component enrichment analysis of the different exosome fractions showed mainly exosome proteins, which indicates that the

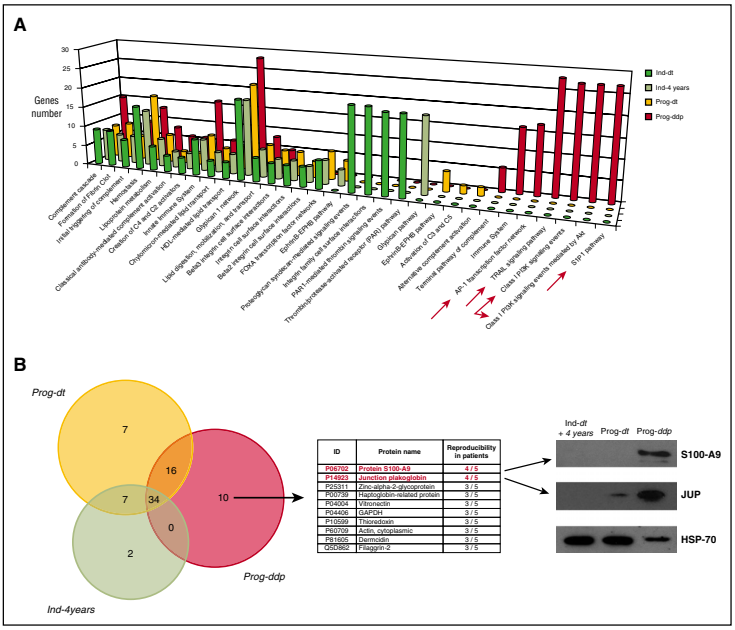

Figure 2. Proteomic analysis of plasma-derived exosomes during CLL evolution. (A) Molecular networks associated with proteins identified in plasma-derived exosomes during CLL evolution. The PPI networks were elucidated using the interaction model of FunRich software with the Vesiclepedia database, which focuses on statistically significant enriched genes and particular biological pathways in each subgroup. Different and specific networks according to the number of genes associated with the functions in the Prog-ddp subgroup are marked by red arrows: (1) Leukemic cell infiltration of secondary lymphoid organs, highlighted by genes related to the receptor sphingosine 1-phosphate (S1P1) pathway ($P = 2.24e{-}4$); (2) tumor proliferation network, involving genes of phosphatidylinositol 3-kinase (PI3K)/AKT kinase pathway ($P = 2.24e{-}4$); (3) survival and apoptosis network, involving genes implicated in the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) pathway; and (4) inflammation and oxidative stress networks, with genes involved in the AP-1 transcription factor pathway ($P = 1.43e{-}4$). (B) Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of CLL exosome proteins. LC-MS/MS data analysis was performed in accordance with the recently published PatternLab for proteomics 4.0 software (http://www.patternlabforproteomics.org) protocol for data analysis.[16] The PatternLab approximately area-proportional Venn diagram module was used for pinpointing proteins exclusively identified in 1 biological condition. For the exosome protein-enriched sample, the analysis only considered proteins present in at least 3 of 5 patients for each biological condition. Junction plakoglobin (JUP) and S100-A9 (proteins in red font) were confirmed by specific monoclonal antibodies in immunoblot analysis. Plasma-derived exosomes were obtained from 10 patients (20 samples) corresponding to the 4 different subgroups (5 patients each) and subjected to 12% polyacrylamide gel (SDS-PAGE) electrophoresis. Representative immunoblots of both proteins at the different disease times are depicted. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein.

procedures used for isolation and purification are reproducible and reliable (Figure 1C).

Proteomic profiling of plasma-derived exosomes during CLL evolution

Considering all patients and conditions, liquid chromatography–tandem mass spectrometry analysis of CLL exosomes resulted in the identification of 138 proteins in group A and 136 proteins in group B, with at least 2 mapping peptides per sequence. Among the 138 proteins in the progressive group, 99 (62%) were shared by the 2 subgroups (Prog-dt and Prog-ddp), whereas 39 proteins were differentially expressed: 10 in Prog-dt and 29 in Prog-ddp. Regarding indolent CLLs, among the 136 proteins, 84 (61%) were shared between both subgroups (Ind-dt and Ind-4years), and 52 proteins were differentially identified: 12 in Ind-dt and 40 in Ind-4years (supplemental Data 1, including tables and Venn diagram). The protein-protein interaction (PPI) network offers a conceptual framework to better understand the functional organization of the proteome and, in this case, to deepen the insight into the protein cargoes of plasma exosomes during CLL evolution. Selection of the 20 most significant PPI networks from each subgroup (Ind-dt, Ind-4years, Prog-dt, and Prog-ddp) and further comparison among them allowed us to highlight a total of 32 different biological pathways (Figure 2A; supplemental Data 2). Many of these pathways were shared among progressive and indolent CLLs, but some of them were found exclusively within a determined subgroup (Figure 2A). Focusing on the PPI networks derived from the proteins present in exosomes during disease progression (Prog-ddp subgroup), we found interesting pathways associated with inflammation and cancer progression, such as S1P1 pathway, class I PI3K

signaling events, tumor necrosis factor–related apoptosis-inducing ligand signaling pathway, and AP-1 transcription factor network (Figure 2A).

To select the proteins relevant to disease progression, we focused on the proteins that remained differentially present in the Prog-ddp subgroup. To this aim, we performed a comparative analysis between Ind-4years, Prog-dt, and Prog-ddp. The PatternLab for Proteomics software Venn diagram module pinpointed proteins uniquely identified in each subgroup. Considering proteins present in at least 3 patients per class, only 34 proteins were shared among the 3 subgroups; 2 were only found in the Ind-4years patients, 7 in Prog-dt, and 10 in Prog-ddp (Figure 2B; supplemental Data 3). Among the 10 proteins detected exclusively in the Prog-ddp subgroup, the 2 most represented (4 of 5 patients) were S100 calcium binding protein A9 (S100-A9) and JUP (Figure 2B). To confirm these results, we performed immunoblot analysis in the CLL exosomes of the 10 patients at the different disease points included in the proteomic analysis. Our results confirmed the presence of these 2 proteins in 5 of 5 patients for S100-A9 and 4 of 5 patients for JUP, whereas none or very low presence of these proteins was detected in the samples before progression or in indolent CLLs. A representative case for each protein is depicted in Figure 2C. Interestingly, both proteins are involved in cancer progression. S100-A9 is an activator of the NF-κB pathway[25] and has been associated with inflammatory networks,[25] and JUP is implicated in the activation of Wnt pathway.[26,27]

Plasma-derived exosomes with increased levels of S100-A9 are found in patients with progressive disease after disease progression and released by CLL cells

S100-A9 and the complex S100-A8/A9 are key factors leading to inflammatory cell recruitment, tumor growth, and metastasis.[4,28] Our proteomic and immunoblotting analysis identified S100-A9 as 1 of the proteins specifically present in the plasma exosomes of the Prog-ddp subgroup. To confirm these results, we performed immunoblot analysis in the validation cohort (Table 1). As depicted in Figure 3A, 7 of the 8 samples showed a significant increase of S100-A9 cargo in CLL exosomes at the time of disease progression (mean, 1.12 and 0.51, respectively; Mann-Whitney test, $P = .010$; n = 8). In contrast, no significant differences were found within the indolent group, (Ind-dt: mean, 0.44; Ind-4years: mean, 0.33; Mann-Whitney test, $P = .657$; n = 4). In turn, S100-A9 levels from HDs (mean, 0.31) seemed similar to those observed in indolent and Prog-dt CLL samples. Representative cases of CLL patients from the different subgroups at both evolution times and from HDs are depicted in Figure 3B. To evaluate whether CLL cells are able to release S100-A9$^+$ exosomes, we performed in vitro assays with CD19$^+$ cells from patients with progressive disease during disease progression. Our results showed that after anti-IgM incubation, CLL cells are able to release exosomes carrying S100-A9, as evidenced by immunoblot analysis (Figure 3C). Overall, these findings validate proteomic analysis concerning the specific presence of S100-A9 during disease progression and suggest that these exosomes originate at least in part from CLL cells.

S100-A9 receptor EMMPRIN is expressed in leukemic cells and binds S100-A9 in vitro

EMMPRIN is a multifunctional transmembrane protein that regulates cell proliferation and migration and promotes cancer progression.[29] Because it has been shown that S100-A9 is able to bind EMMPRIN, thus mediating inflammation and cancer proliferation,[28] we investigated the expression levels of this protein and its ability to bind S100-A9 protein in CLL cells. Our results showed that EMMPRIN messenger RNA (mRNA) isoform 2 but not isoform 1 was expressed in the leukemic clone, whereas low mRNA levels of isoforms 3 and 4 were found (Figure 4A). Concerning EMMPRIN protein levels,

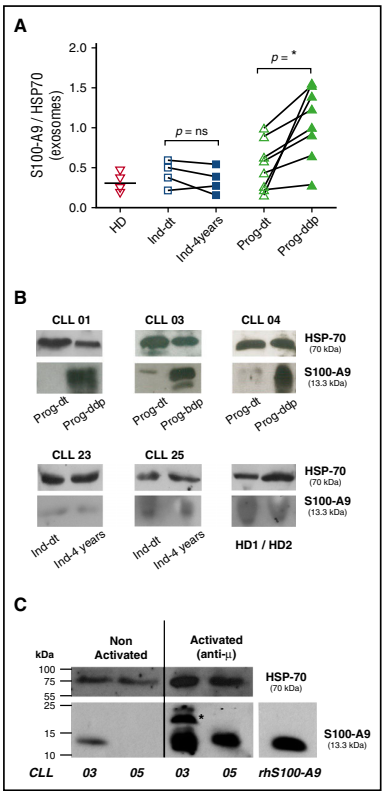

Figure 3. CLL plasma-derived exosomes show increased levels of S100-A9 protein and are released by CLL cells. (A-B) S100-A9 quantification from plasma-derived exosomes between the different subgroups; immunoblot analysis was performed after exosome protein separation. A total of 50 μg of exosome protein from the different samples was transferred onto nitrocellulose and developed with specific anti–S100-A9 monoclonal antibody. HSP-70 protein was visualized as internal charge control. Values of S100-A9 were normalized to those of HSP-70 accordingly. Results showed that S100-A9 protein was significantly higher in the plasma exosomes at the time of disease progression, whereas no or low presence of this protein was found in the exosomes of the Prog-dt subgroup, the indolent group, or the matched HDs. *$P < .05$. (C) Release of S100-A9$^+$ exosomes by CLL cells; in vitro analysis was performed with CD19$^+$ cells (100 exp^{10} lymphocytes incubated O.N. with anti-IgM 15 μg/ml). AIM V medium 10 mL was collected and exosomes purified as previously described in Material and Methods. Results showed that CLL cells release exosomes carrying S100-A9 protein to the culture medium after incubation with anti-IgM. *S100-A9 homodimer. Immunoblot analysis was performed as described. ns, not significant.

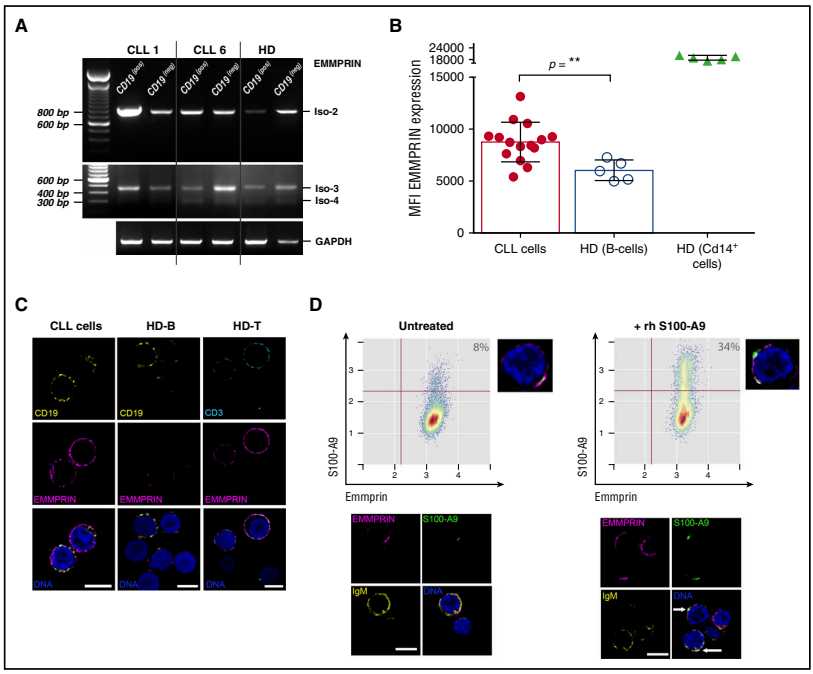

Figure 4. EMMPRIN expression in CLL cells and S100-A9 binding in vitro. (A) Reverse-transcription polymerase chain reaction analysis of EMMPRIN mRNA isoforms in CLL. *EMMPRIN* mRNA isoforms 1 to 4 expression was evaluated in MACS-sorted B lymphocytes ($CD19^+$) and non-B cells ($CD19^-$) as described by Oppezzo et al.[18] *EMMPRIN* isoform 2 is expressed in leukemic B cells at higher levels than in normal B cells, whereas isoforms 3 and 4 are also expressed but at basal levels. (B) Flow cytometry analysis of EMMPRIN protein levels in B lymphocytes. Median fluorescence intensity analysis of EMMPRIN staining in CLL and HD B cells reveals higher levels of EMMPRIN staining in CLL B cells (mean, 8.751; n = 15) compared with normal B cells (mean, 5.964; n = 5; Mann-Whitney test, $P = .0037$). MFI, mean fluorescence intensity. (C) EMMPRIN in situ immunostaining. Direct in situ immunolocalization of EMMPRIN (magenta) shows higher staining intensities in CLL B cells compared with their normal counterparts ($CD19^+$ in yellow). EMMPRIN staining in normal T cells ($CD3^+$ in cyan) was assessed as positive control. Single confocal planes are shown; deconvolution was performed with Huygens Essential 4.5 (Scientific Volume Imaging, Hilversum, The Netherlands; http://svi.nl). Scale bar, 5 μm. (D) rhS100-A9 B-cell CLL in vitro binding assay. The binding ability of rhS100-A9 (green) on B-cell CLL cells (IgM^+ in yellow) expressing EMMPRIN (magenta) was evaluated on incubation of 10^6 cells with 5 μg/mL for 1 hour at 37°C. An increase from basal 8% to 34% of $S100\text{-}A9^+$ cells was found. Flow cytometry analysis was performed with R/Bioconductor. Confocal microscopy analysis showed an increase in S100-A9 immunostaining and suggested colocalization of rhS100-A9 with EMMPRIN receptor, shown in white in the overlaid images (arrows). Deconvolved single confocal planes are shown. Scale bar, 5 μm.

flow cytometry analysis showed a significantly higher expression in CLL cells compared with normal B cells (Figure 4B). In contrast, no significant differences were found after comparison of patients with progressive and indolent disease (data not shown). EMMPRIN expression was also characterized in T cells and macrophage/monocyte cells from HDs and patients with CLL. Different expression levels are shown in supplemental Figure 1. Confocal microscopy analysis confirmed the higher expression of EMMPRIN in CLL cells compared with those of HDs (Figure 4C). To elucidate whether EMMPRIN receptor is able to bind S100-A9 in CLL cells, we performed in vitro analysis, incubating PBMCs from progressive CLLs with rhS100-A9. Our results showed a percentage of leukemic cells expressing EMMPRIN that was able to bind rhS100-A9 (Figure 4D). These results were confirmed by confocal microscopy showing colocalization of rhS100-A9 with EMMPRIN receptor (Figure 4D, arrows).

S100-A9 is overexpressed in CLL cells, increases during disease progression, and correlates with NF-κB pathway activation

On the basis of our previous results, we evaluated S100-A9 expression at mRNA and protein levels in the leukemic cells from patients with indolent disease and from the Prog-ddp subgroup. Analysis of S100-A9 mRNA expression demonstrated that gene transcription is increased in CLL cells from patients with progressive disease (supplemental

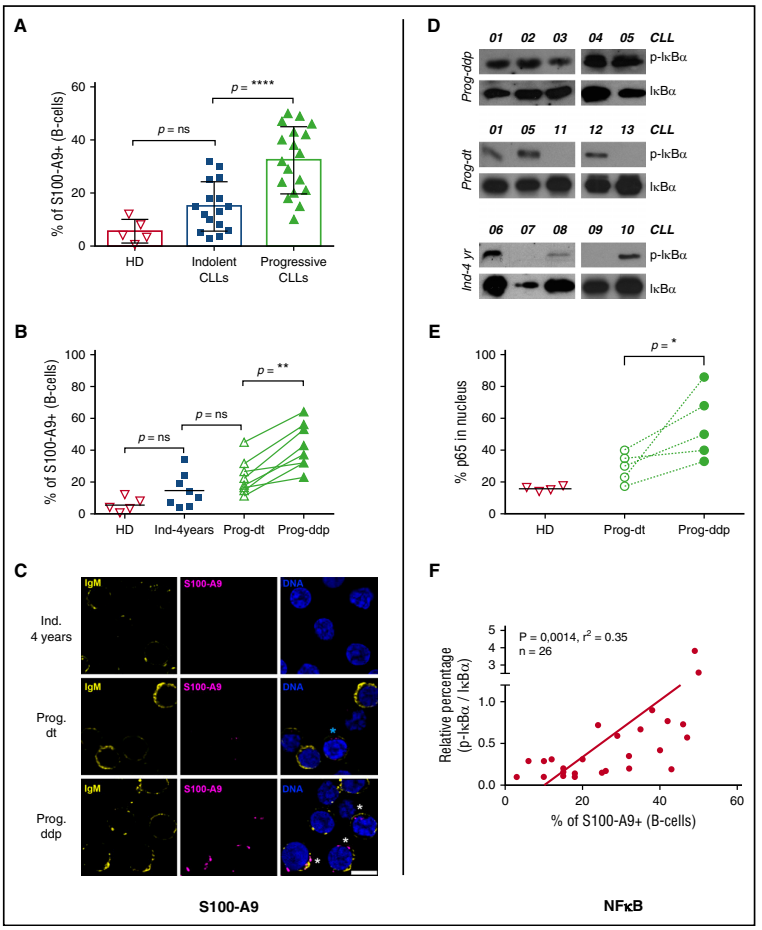

Figure 5. Quantification of S100-A9 expression in the leukemic clone. Flow cytometry analysis of PBMCs was performed evaluating IgM, CD5, and S100-A9$^+$ cells between HDs and the different CLL subgroups. CD19$^+$ cells of age-matched HDs were used to obtain reference values, and control isotypes were used for IgM and S100-A9 antibodies. (A) S100-A9 expression in indolent and progressive CLL samples during disease progression. Flow cytometry analysis was performed with specific antibodies, as described in supplemental Table 1. Five age-matched HDs to determine the physiological levels of S100-A9 in B lymphocytes and a total of 16 indolent CLLs and 18 progressive CLLs at the time of progression were evaluated. A significant increase of S100-A9 levels was found in the samples obtained from Prog-ddp CLLs (mean, 32.44) compared with samples from indolent CLLs (mean, 14.98; 1-way analysis of variance [ANOVA], $P < .01$). No significant changes were observed between HD samples compared with indolent CLLs (mean, 5.55 and 14.98, respectively). ns, not significant. (B) S100-A9 protein expression levels in B lymphocytes at different times of disease evolution. Five HDs, 8 Ind-4years, and 8 progressive CLLs at the time of onset and 8 during disease progression were evaluated. A significantly higher level of S100-A9 protein in the leukemic cells from the Prog-ddp samples (mean, 42.50) compared with Prog-dt (mean, 23.24) and with Ind-4years (mean, 14.64) was demonstrated (1-way ANOVA, $P < .01$). No significant differences were visualized comparing the expression of S100-A9 in B lymphocytes (mean, 1.38) with Ind-4years (mean, 14.64) or Prog-dt (mean, 23.24). Microscopy analysis was performed with specific anti-IgM, anti-CD5, and anti–S100-A9 (supplemental Table 1). (C) S100-A9 expression in the leukemic clone was confirmed as a discrete cytoplasmic pattern shown in red color. DNA staining was performed with methyl green as described by Oppezzo et al.[22] Gray and white asterisks indicate low and high S100-A9 expression levels, respectively. Scale bar, 5μm (lower right). (D) NF-κB pathway is more activated in the samples of progressive CLLs during disease progression. Characterization of NF-κB pathway activation by immunoblot assays evaluating IκB-α and inhibitory IκB-α phosphorylation in PBMCs of the different

Figure 2A). At the protein level, our results showed a significant increase in the percentage of cells expressing S100-A9 in samples from patients with progressive disease when compared with those from patients with indolent disease or CD19$^+$ cells from HDs (Figure 5A). As found with exosomes, there was an increased expression of S100-A9 at the time of progression in Prog-ddp samples compared with the same samples at disease onset (Figure 5B). These results were confirmed by confocal microscopy of PBMCs from the different subgroups (Figure 5C). Representative histograms with different S100-A9 protein expression levels in the different subgroups and in a HD are depicted in supplemental Figure 2. Supporting the hypothesis that S100-A9 is overexpressed in activated CLL cells from patients with progressive disease, we found an increased percentage of S100-A9$^+$ B cells in lymph nodes (LNs) compared with their tumoral counterparts in the PB of the same patient (supplemental Figure 3). Interestingly, CLL cells from LNs expressing S100-A9 also showed an activated molecular profile, indicated by a higher expression of Ki-67 and survivin proteins when compared with S100-A9$^-$ CLL cells (supplemental Figure 4).

Because S100-A9 has been reported to increase NF-κB activity,[26,28] we evaluated the activation of the NF-κB pathway in leukemic cells with increased expression of S100-A9. To this aim, we analyzed the Ser32/36 phosphorylation of IκB-α and the nuclear/cytoplasmic rate of the transcription factor p65 between Prog-dt and Prog-ddp samples. As shown in Figure 5D, differences in IκB-α phosphorylation at different disease points were found, indicating the release of NF-κB from inhibitory IκB-α during disease progression. Moreover, a comparison of p65 localization between Prog-dt and Prog-ddp samples of the same patient demonstrated a significant increase of this transcription factor in the nucleus of the Prog-ddp sample (Figure 5E; supplemental Figure 1D). Finally, a significant and positive correlation was found between IκB-α phosphorylation and S100-A9 expression, which suggests a link between this protein and NF-κB activity. Together, these results show that leukemic cells derived from patients with progressive disease express high levels of S100-A9 when compared with indolent CLLs and that this expression is correlated with canonical NF-κB pathway activation.

Plasma exosomes with S100-A9 cargo promote NF-κB pathway activation in leukemic cells

S100-A9 is able to activate the NFκB pathway in carcinoma and melanoma cells,[26,30] but little evidence exists regarding its role in CLL. Taking our results into account, we hypothesized that specific expression of S100-A9 in the leukemic clone during disease progression as well as its significant increase in plasma exosomes could be one of the mechanisms triggering NF-κB activation during CLL progression. To evaluate the role of plasma exosomes with S100-A9 cargo regarding the NF-κB pathway, we obtained plasma exosomes from 3 patients with progressive disease (01, 02, and 04) at disease onset (S100-A9$^-$) and during disease progression (S100-A9$^+$). We incubated PBMCs of 2 patients with no mutation and 1 patient with mutation at time of disease onset and evaluated IκB-α phosphorylation and p65 nuclear translocation as regards activation of the canonical NF-κB pathway and the inducible processing of p100/p52 proteins as regards activation of the noncanonical pathway. Our results showed a clear activation of the canonical NF-κB pathway in at least 2 of the 3 plasma exosomes carrying S100-A9 in the 3 tested patients. As depicted in Figure 6A, a significant increase of IκB-α phosphorylation was observed after incubation of the exosomes extracted from the Prog-ddp subgroup (S100-A9$^+$) but not with plasma-derived exosomes extracted from the Prog-dt subgroup (S100-A9$^-$). The canonical NF-κB pathway activation was also confirmed by nuclear localization of p65 in the leukemic cells (Figure 6B). Interestingly, this activation seemed to be specific to the canonical NF-κB pathway but not to the noncanonical pathway (p100/p52 immunoblot analysis in supplemental Figure 5).

To better understand the molecular mechanism of this activation, we also performed in vitro analysis, treating CLL PBMCs at the time of disease progression with different amounts of plasma-derived exosomes carrying S100-A9. Our results suggest that phosphorylation of IκB-α is dependent on S100-A9 plasma exosome dose (Figure 6C). Finally, we preincubated these exosomes with anti–S100-A9 antibody to demonstrate the specificity of NF-κB activation in the leukemic clone. Immunoblot assays showed a decrease of IκB-α phosphorylation after incubation of PBMCs with S100-A9$^+$ exosomes that were previously incubated with anti–S100-A9 antibody, which suggests that the interaction between cells and exosomes is blocked by specific anti–S100-A9 antibody. In contrast, high levels of IκB-α phosphorylation were maintained after incubation of plasma exosomes carrying S100-A9 or with these exosomes previously incubated with an irrelevant isotype control (Figure 6D). Together these results confirm that plasma exosomes carrying S100-A9 protein are able to promote activation of the NF-κB pathway, suggesting a specific role for these exosomes and this protein in CLL progression.

Discussion

The dynamic interplay between the tumor and its microenvironment orchestrates critical events that contribute to tumor progression and drug resistance.[31] Microenvironment signals are provided not only by cell-to-cell interactions but also by different molecules such as soluble factors (cytokines, chemokines) or EVs, which play a key role in tumor-host crosstalk.[32] Specifically, the functional capacity of exosomes is altered as tumors progress to an aggressive phenotype.[33] We hereby report that plasma-derived exosomes of progressive and indolent CLLs throughout disease evolution display different proteomic profiles. Particularly during disease progression, we found different PPI networks associated with inflammation, tumor progression, leukemic cell infiltration, and cell survival. The PPI network AP-1 transcription factor is activated by inflammation and oxidative stress.[34] In CLL, AP-1 family members have been described as being associated with NF-κB induction after CD40L activation.[35] The other major network highlighted during disease progression is the pathway of class I PI3K signaling events mediated by AKT. This network is associated not only with PI3K/AKT pathway activation but also with canonical NF-κB pathway activation.

Figure 5 (continued) subgroups (Prog-dt and Prog-ddp). Representative cases (10 of 20 analyzed patients) as depicted after immunoblot reaction. (E) Increased NF-κB activation in the Prog-ddp subgroup was evidenced after quantification of p65 nucleus/cytoplasm stain ratio from a total of 100 CLL cells (IgM$^+$/CD5$^+$) in 5 samples from each subgroup (Prog-dt and Prog-ddp; $n = 10$). Prog-dt samples display a low percentage of cells with the transcription factor p65 in the nucleus (mean, 30) compared with Prog-ddp samples of the same patient (mean, 50; Mann-Whitney test, $P = .019$). Immunofluorescent staining was performed with specific human anti-p65 transcription factor, specific anti-IgM, and anti-CD5 antibodies. (F) Positive correlation of S100-A9 expression with IκB-α phosphorylation in patients with CLL. The relationship between S100-A9 protein expression and IκB-α phosphorylation was investigated. A significant positive correlation was detected ($r^2 = 0.35$; Spearman's rank test, $P < .001$).

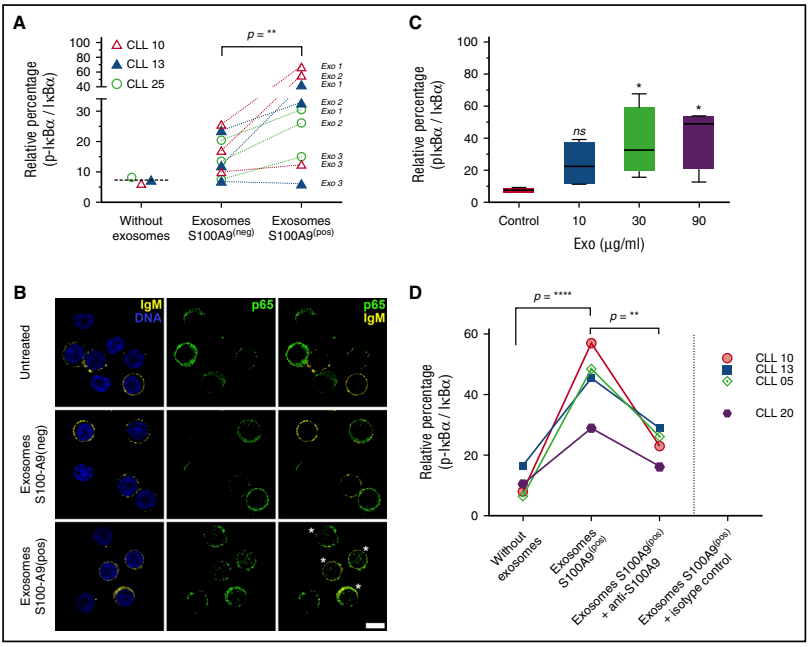

Figure 6. Plasma-derived exosomes with S100-A9 cargo promote NF-κB pathway activation in leukemic cells. (A-B) NF-κB pathway is activated after in vitro incubation with plasma-derived exosomes (Exo) carrying S100-A9. PBMCs of unmutated and mutated CLLs were incubated in RPMI 1640 and fetal bovine serum with plasma-derived exosomes (S100-A9$^+$) extracted from Prog-ddp and plasma-derived exosomes without S100-A9 extracted from Prog-dt samples (90 minutes; 60 μg/ml of total protein). Negative and positive exosomes were categorized by immunoblot. NF-κB activation was determined as the relative percentage by densitometry of the corresponding signal from phosphorylated IκB-α/total IκB-α. "Without exosomes" indicates basal phosphorylation of IκB-α in the PBMCs of CLLs. Significant changes were observed in the rate of IκB-α phosphorylation after incubation with S100-A9$^+$ plasma-derived exosomes compared with their counterparts (S100-A9$^-$ plasma-derived exosomes; Wilcoxon signed rank test, $P = .0078$). In this experiment, PBMCs at disease onset from 3 different patients, 2 with unmutated and 1 with mutated CLL (CLLs 10 and 13 and CLL 25, respectively) were incubated with plasma-derived exosomes from 3 different CLLs (Exo1, CLL 02; Exo2, CLL 05; Exo3, CLL 07) extracted at disease onset (S100-A9$^-$) and during disease progression (S100-A9$^+$). NF-κB pathway activation after incubation with plasma-derived exosomes carrying S100-A9 was also evidenced by p65 nuclear translocation. After 2 hours of incubation with plasma-derived exosomes (with or without S100-A9), PBMCs of the 3 CLLs were collected and stained with anti-IgM, anti-CD5, and anti-p65 transcription factor. A representative figure of the 3 patients with CLL studied is provided in Figure 5B. Green color indicates p65, and yellow staining corresponds to IgM$^+$ cells. DNA staining with methyl green was performed. More than 95% of IgM cells for CLL 10 and 13 and 72% for CLL 25 were positive for CD5 marker (estimated by flow cytometry analysis and confocal microscopy; data not shown). Leukemic cells after incubation with exosomes carrying S100-A9 with increased nuclear localization of p65 transcription factor are highlighted by white asterisks. Scale bar, 5μm (lower right). (C) Phosphorylation of IκB-α in PBMCs of CLL after 90-minute incubation with increasing concentrations of plasma-derived exosomes carrying S100-A9. Data are reported as relative changes of phosphorylated IκB-α/total IκB-α. (D) Monoclonal antibody against S100-A9 was used to block the interaction of S100-A9$^+$ exosomes with leukemic cells. To confirm the specificity of NF-κB activation, 60 μg of CLL plasma-derived exosomes (S100-A9$^+$) were extracted from patient 01 and preincubated for 60 minutes with polyclonal antibody anti–S100-A9 at 6°C or with isotype control. Next, PBMCs from 4 different patients were incubated for 90 minutes at 37°C with these cocktails (S100-A9$^+$ exosomes plus anti–S100-A9 antibody, S100-A9$^+$ exosomes, and S100-A9$^+$ exosomes plus isotype control antibody). Low levels of IκB-α phosphorylation were found after PBMC incubation with anti–S100-A9 (data not shown). This unspecific activation was subtracted to obtain the values corresponding to the "exosomes S100-A9$^+$ plus anti–S100-A9 group. Our results showed a significant reduction in the 4 samples in the ratio *p*-IκB-α / IκB-α after CLL PBMCs were incubated with S100-A9$^+$ exosomes plus anti–S100-A9 antibody (mean, 22) compared with CLL PBMCs incubated only with S100-A9$^+$ exosomes (mean, 44.95; 1-way analysis of variance $P < .01$). Significant differences were found between CLL PBMCs incubated only with S100-A9$^+$ exosomes compared with untreated CLLs (mean, 10.40; without exosomes).

The importance of synergism between NF-κB and PI3K in CLL[36] and other lymphoid neoplasms[37] has been extensively demonstrated.

To identify the most relevant proteins present in CLL plasma exosomes from patients with progressive disease during disease progression, we focused on the proteins that constantly seemed to be over-represented in progressive samples analyzed by liquid chromatography–tandem mass spectrometry. Among the 10 proteins exclusively present in this subgroup, we focused on S100-A9 protein, which was also found in exosomes secreted by an unmutated CLL, as described by Pagetti et al,[12] whose study proposes that exosomes could induce an inflammatory phenotype in CLL and that the NF-κB pathway is activated in stromal cells. Taking these results into account and our proteomic characterization, we decided to investigate the role of S100-A9 during CLL progression. S100-A9 belongs to a family of

25 homologous intracellular calcium-binding proteins. Although S100-A9 is mainly found in heterodimers with S100-A8 (S100A8/A9, also known as calprotectin), it also exists as a homodimer, with its own functions.[38] Calprotectin and S100-A9 have been largely associated with chronic inflammation and tumor promotion.[4,39] Both molecules are responsible for NF-κB pathway activation, but different receptors are implicated in this process. Calprotectin activates NF-κB through TLR4,[40,41] whereas S100-A9 does so through EMMPRIN receptor.[25,28] Considering that CLL exosomes engage PPI networks associated with PI3K/NF-κB pathways during disease progression, that S100-A9 is 1 of the most representative proteins in exosomes of all progressive samples, and that we did not find S100-A8, we decided to evaluate the correlation between S100-A9 expression and NF-κB activity in the leukemic clone of the different subgroups. Our results showed that the elevated expression of S100-A9 in B cells of patients with disease progression is accompanied by increased activation of the NF-κB pathway. In addition, our results on EMMPRIN expression in CLL suggest that it could be 1 of the possible receptors for S100-A9. Our results showed that EMMPRIN isoform 2 is the main mRNA expressed in CLL cells, which agrees with similar results reported in other cancer cells.[42] High expression of EMMPRIN in CLL cells and its interaction with S100-A9 might implicate a new activation axis for the leukemic clone during disease progression. In this line, we must take into account that EMMPRIN is able to induce MMP production in tumor cells.[4,43] Interestingly, recent studies describe that overexpression of MMP-9 occurs in CLL and that elevated intracellular levels of (pro)MMP-9 correlate with advanced CLL stage and poor patient survival.[4,44,45]

Previous work has shown that S100-A9 is crucial in establishing the premetastatic niche, chemotherapy resistance, and subsequent metastasis in breast cancer[25] and melanoma.[4] The presence of S100-A9 in CLL plasma exosomes, their overexpression during leukemic progression, and the role of this protein in tumor development suggest a role in leukemia promotion and generate new and interesting questions in the pathogenesis of CLL. S100-A9 is required for the recruitment of myeloid-derived suppressive cells (MDSCs) and for suppression of an immune antitumor response.[46] Specifically in CLL, Jitschin et al[47] describe that through a still unknown mechanism, CLL cells are able to induce MDSCs with high expression of indoleamine-2,3-dioxygenase, which in turn becomes responsible for suppression of T-cell activation and induces suppressive regulatory T cells. Our results suggest that this unknown mechanism responsible for MDSC accumulation could originate in CLL cells expressing S100-A9 and/or exosomes carrying this protein. Another interesting question concerns the role that S100-A9 protein expressed in CLL cells could have in interacting with β2 integrins. It has been demonstrated that S100-A9 mediates neutrophil adhesion to fibronectin through activation of β2 integrins[48] and that in CLL β2 integrins are essential for survival and apoptosis resistance.[49] Thus, CLL cells expressing S100-A9 could interact through β2-integrins with endothelial cells to gain new survival and proliferative signals, as proposed by Maffei et al.[49]

Tumor-released exosomes become an efficient platform for the in vivo transfer of soluble crosstalk factors.[50] The function of exosomes seems to be dependent on their protein cargoes and, in turn, dependent on the cell types from which they originate.[6] Our proteomic approach demonstrates that CLL exosomes specifically contain S100-A9 during disease progression and that these exosomes activate the canonical NF-κB pathway. The fact that NF-κB activity is reduced by in vitro incubation of plasma exosomes carrying S100-A9 with specific anti–S100-A9 antibodies confirms these results. Regarding exosome function in CLL, our results support and extend the findings of Paggetti et al,[12] suggesting that exosomes extracted from MEC-1 cells or, as in our case, from the plasma of patients during disease progression are able to activate the NF-κB pathway, not only in stromal cells, as described by Paggetti et al, but also in the leukemic clone. Recently, Peinado et al[4] reported that melanoma-derived exosomes are able to induce the expression of S100-A8/S100-A9 in premetastatic niches after NF-κB activation, which in turn becomes activated by calprotectin and/or S100-A9.[25] According to our results, an analogous loop could be envisaged in CLL when disease progression occurs. Microenvironment signals in the leukemic clone could upregulate S100-A9 expression in CLL cells, which in turn could activate the canonical NF-κB pathway, leading to an increase of proliferation and survival signaling, as suggested by the results analyzing CLL cells in LNs of patients with progressive disease, depicted in supplemental Figures 3 and 4.

In this work, we report for the first time different proteomic profiles of plasma exosomes among indolent and progressive CLLs, as well as within individual patients at the time of disease onset and during disease evolution. We describe the presence of the protein S100-A9 in CLL exosomes as well as an increased expression of this protein in leukemic cells from patients experiencing disease progression. Finally, we demonstrate that exosomes with S100-A9 cargo are able to increase NF-κB activity in CLL cells of patients with typical nonprogressive disease. This report proposes a new origin of NF-κB activation in CLL and highlights the importance of exosomes as extracellular mediators in the promotion of CLL progression.

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Authorship

Contribution: D.P., N. Sotelo, and N. Seija performed experiments, collected chronic lymphocytic leukemia (CLL) samples, prepared figures, and wrote the paper; S.S. and C.A. performed experiments and collected CLL samples; R.D. and M.G. performed mass spectrometry and proteomic analysis; E.S. performed electronic microscopy analysis; V.I., C.O., A.I.L., R.G., and G.D. performed clinical activities and data collection for patients with CLL; and P.O. designed research, coordinated the study and data analysis, and wrote the paper.

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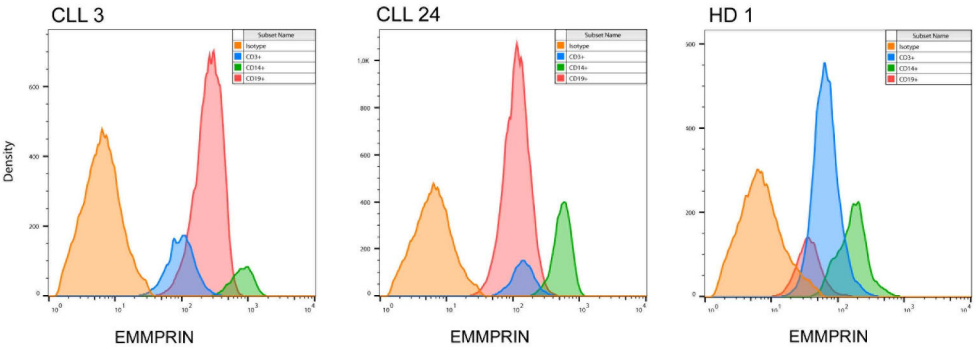
Supplemental Figure 1



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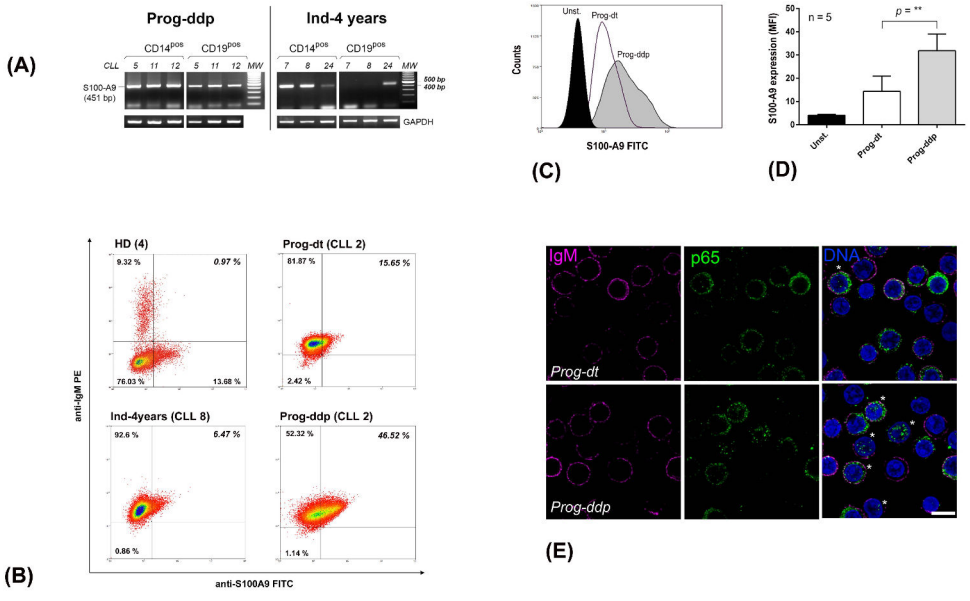

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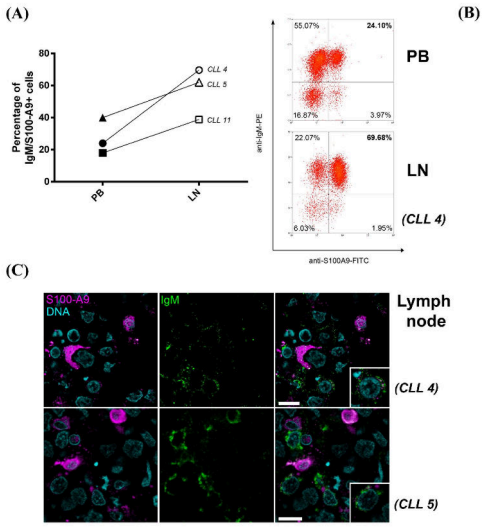

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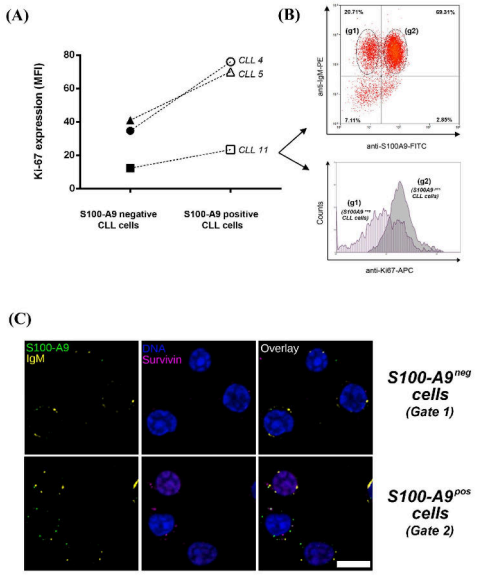
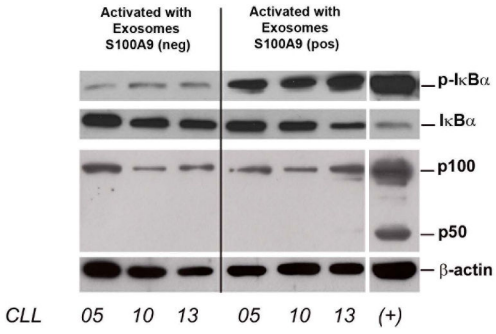


Supplemental Figure 5



Supplemental Table 1. Antibodies and reagents. Usage conditions per application.

Antibody and reagents	*Supplier*	*Microscopy*	*Cytometry*	*Western Blot*	*In-vitro analysis*
anti-human CD14	BD Pharmingen	1/10 [a]	20µl/10^6 cells [a]	-	-
anti- human CD19	BioLegend	1µg/mL [a]	0.5µg/10^6 cells [a]	-	-
anti- human CD5	BioLegend	1µg/mL [a]	0.5µg/10^6 cells [a]	-	-
anti- human CD9	Santa Cruz Biotechnology	-	-	1/300 [c]	-
anti- human HSP-70 (4G4)	Santa Cruz Biotechnology	-	-	1/100 [c]	-
anti- human IgM Fc5µ F(ab') 2	Jackson Immunoresearch	1/50 [a]	1/50 [a]	-	15µg/ml
anti-human IκBα (L35A5)	Cell Signaling	-	-	1/1000 [c]	1/1000 [c]
anti-human phospho IκBα (Ser32/36)	Cell Signaling	-	-	1/1000 [c]	1/1000 [c]
anti-NF-κB p65 (D14E12)	Cell Signaling	1/400 [a]	-	-	1/400 [a]
anti-p100/52	Abcam	-	-	1/10000 [c]	-
anti-Ki-67	Santa Cruz Biotechnology	-	5µl/10^6 cells [a]	-	-
anti-Survivin	Santa Cruz Biotechnology	1/50 [a]	-	-	-
anti-LAMP-1 (1D4B)	Abcam	-	-	1/100	-
anti- Cytochrome C (7H8.2C12)	Abcam			3 µg/ml [b]	-
anti-human CD147 (EMMPRIN) - HIM6	BioLegend	1/400 [a]	1µg/10^6 cells [a]	-	-
anti-Mouse Igs	Life Technologies	1/1000 [a]	1/1000 [a]	-	-
anti-Mouse Igs	Dako Cytomation	-	-	1/10000 [a]	-
anti-Rabbit Igs	Santa Cruz Biotechnology	-	-	1/10000 [a]	-
Human IgG Isotype	Thermo Fisher	-	-	-	1µg/mL [a]
anti-S100-A9	MyBioSource	1/50 [a]	1µg/10^6 cells [a]	1/100 [b]	-
anti-human S100-A9	R&D Systems	-	-	0.5µg/mL [c]	1µg/mL [a]
anti-human MRP-14 (S100-A9)	BioLegend	-	5µl/10^6 cells [a]	-	-
Recombinant human protein S100-A9	MyBioSource	-	-	-	0.5µg/mL [a]

a: 1 hour incubation.
b: 2 hours incubation.
c: overnight incubation.

Supplemental Table 2.
List of primers for RT-PCR (5' > 3')

Oligo	*Sequence*	*Source*	*Expected size (bp)*
EMMPRIN1/2-F	AGGAATAGGAATCATGGCGGC		Isoform 1 = 1135
EMMPRIN1/2-R	GTCATTCTGGTGCTGCCCGC		Isoform 2 = 787
EMMPRIN3/4-F	CGGCTTAGTCTGCGGTCC	Belton *et al.*, 2008	Isoform 3 = 458
EMMPRIN3/4-R	GGGAGGAAGACGCAGGAGTA	Belton *et al.*, 2008	Isoform 4 = 300
S100A9-For	TCGGCTTTGACAGAGTGCAA		S100A9-Full Length = 451
S100A9-Rev	GGCCTCCTGATTAGTGGCTG		

Supplemental Table 2.
List of primers for RT-PCR (5' > 3')

Oligo	*Sequence*	*Source*	*Expected size (bp)*
EMMPRIN1/2-F	AGGAATAGGAATCATGGCGGC		Isoform 1 = 1135
EMMPRIN1/2-R	GTCATTCTGGTGCTGCCCGC		Isoform 2 = 787
EMMPRIN3/4-F	CGGCTTAGTCTGCGGTCC	Belton *et al.*, 2008	Isoform 3 = 458
EMMPRIN3/4-R	GGGAGGAAGACGCAGGAGTA	Belton *et al.*, 2008	Isoform 4 = 300
S100A9-For	TCGGCTTTGACAGAGTGCAA		S100A9-Full Length = 451
S100A9-Rev	GGCCTCCTGATTAGTGGCTG		

SUPPLEMENTAL FIGURE LEGENDS:

Supplemental Figure 1: Expression of EMMPRIN receptor in different cell types from CLL patients and HDs. Surface staining with conjugated anti-EMMPRIN/APC, anti-CD3/FITC and anti-CD14/PE (Supplementary Table 1) was performed in different CLL patients and HD. T-cells, macrophage/monocyte and B-cells were gated in order to evaluate EMMPRIN expression in each one of the cell subsets.

Supplemental Figure 2: Expression levels of S100-A9 in CLL cells of the different subgroups. RT-PCR from CD19 and CD14 positive cells as well as cytometry analysis were performed in order to identify S100-A9 expression at mRNA and protein levels. Specific oligos and specific antibodies anti-IgM, anti CD5 and anti S100-A9 were used (Supplemental table 1 and 2). Representative patients showing amplification of S100-A9 full length (451 bp) and expression differences between CD19 positive cells from Prog-ddp and Indolent CLLs are depicted. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as constitutive control gene as described in[14] (Supplemental Figure 1A). S100-A9 expression at protein levels in CLL samples was analyzed after gating IgM/CD5 positive cells. Specific anti-CD19 antibody was used to analyze B-cells of HD. Given that the antibody recognizing S100-A9 is a polyclonal rabbit immunoglobulin we used a FcR Blocking Reagent to block unwanted binding to human Fc receptor of the leukemic cells. Representative patients are depicted in the Supplemental Figure 1B and C, whereas figure 1D shows S100-A9 MFI of different patients at the onset of the disease and during disease progression. A significant increase of S100-A9 MFI was found in the Prog-ddp subgroup (mean = 29.80) compared with Prog-dt (mean = 18.37, p = 0,0079, two-tailed, Mann-Whitney test, n=5). Figure 1E. Representative picture confocal micrograph comparing nuclear/cytosolic localization of p65 transcription factor between Prog-dt and Prog-ddp subgroups. A higher percentage of leukemic cells after disease progression with increased nuclear localization of this transcription factor is highlighted by white asterisks. Scale bar 100μm (right lower)

Supplemental Figure 3: Expression of S100-A9 protein in the lymph nodes of progressive CLL patients. (A) Comparison of the percentage of S100-A9 positive B cells in the PB and in the corresponding LN of 3 different progressive CLL patients. **(B)** A portion of extracted LNs were disaggregated and subjected to FICOLL Histopaque in order to isolate mononuclear cells to be analyzed by Flow Cytometry with anti-IgM, anti-CD5 and anti-S100-A9 conjugated antibodies (Supplemental table 1). **(C)** Microscopy analysis of LN tissue was performed on 15 μm-thick cryosections. Briefly, fresh LN from biopsied tissue were cut into pieces, fixed in 4% PFA, cryoprotected in 20% sucrose and embedded in Tissue-Tek OCT™ compound and sectioned in a cryostat. For staining, sections were washed three times with PBS, blocked 30 min with 3% BSA in

PBS-T (0.1% Triton X-100 in PBS), and incubated with antibodies as described in (Supplemental table 1) and counterstained with methyl green. Stained tissues were then mounted in 80% glycerol pH=8.8 and images were developed with Huygens Essential. Scale bar: 15 µm

Supplemental Figure 4: Ki-67 and Survivin expression in CLL cells expressing S100-A9. A portion of extracted LNs were disaggregated and subjected to FICOLL Histopaque in order to isolate mononuclear cells to be analyzed by Flow Cytometry **(A) Expression levels of Ki-67 proliferative marker in positive or negative S100-A9 CLL cells**. Values are mean SE of mean fluorescence intensity (MFI) of Ki-67 expression from disaggregated lymph nodes, (n=3). **(B) Representative lymph node of patient 11.** g1 and g2 correspond to the gates with CLL cells whether negative or positive for S100-A9 respectively. These gates were selected to evaluate Ki-67 MFI expression and to perform cell sorter isolation. **(C) Survivin microscopy analysis in CLL cells expressing or not S100-A9**. Direct *in situ* immunolocalization of IgM (yellow), S100-A9 (green) and Survivin (magenta). The result shows higher staining intensities of survivin in S100-A9 positives CLL B-cells compared to CLL cells that do not express S100-A9. Scale bar: 5 µm.

Supplemental Figure 5: Activation of canonical and non-canonical NF-κB **pathways after incubation with exosomes with or without S100-A9.** CLL cells from progressive patients at the debut time were treated with exosomes positive or negative for S100-A9 protein and then migrated in SDS-PAGE 10% and transferred onto nitrocellulose. Anti-NF-κB p100/p52 antibody (Abcam) to evaluate non-canonical NF-κB pathway and anti-phospho-IκB alpha and total anti-IκB alpha antibodies (Cell Signalling) to evaluate the canonical pathway, were used in the immunoblot. Positive controls used in this experiment were CLL cells activated with PHA or CD40L for canonical and non-canonical NF-κB pathway, respectively.

II. Unmutated IgHV CLL cells express more LPL protein than mutated cells and is associated with a clinical poor outcome

En este artículo exploramos la localización subcelular de la proteína lipoprotein lipasa en linfocitos de pacientes con leucemia linfoide crónica que expresan el ARNm de *LPL* y en aquellos que no lo expresan. Reportamos que las células B de ambos grupos de pacientes presentan inmunorreactividad contra LPL, y describimos un patrón diferencial de distribución subcelular de la proteína LPL en células B de aquellos pacientes de LLC que expresan el ARNm de *LPL* y aquellos que no. Mediante el análisis de marcadores de organelos y microscopia de barrido láser confocal, demostramos que hay dos poblaciones subcelulares de LPL en los pacientes que expresan el ARNm de *LPL*, una en las cisternas del retículo endoplásmico, y otra en endosomas tardíos o lisosomas. Los pacientes que no expresan el ARNm de *LPL* presentan únicamente la población vesicular. Posteriormente, verificamos estos resultados mediante inmunomicroscopía electrónica, describiendo, hasta donde sabemos por primera vez, la localización ultraestructural de la proteína LPL en una célula B. La identificación de LPL en vesículas de origen endocítico en células de pacientes que no expresan el ARNm de *LPL* nos sugirió, que la inmunorreactividad positiva en estas células podía deberse a una endocitosis de LPL de origen extracelular. Por este motivo, incubamos *ex vivo* células de LLC con un medio químicamente definido (libre de suero), o con suero fetal bovino (un componente de la mayoría de las formulas de medios de cultivo completos, y el principal componente del medio de congelación de las células al ingreso al banco celular), o bien con plasma autólogo. De este modo, demostramos que la proteína LPL de origen extracelular puede ser internalizada desde el plasma por las células de LLC, independientemente de si expresan o no el ARNm de *LPL*. También reportamos que al incubarlas suero fetal bovino las células de algunos pacientes son capaces de internalizar LPL del mismo, mientras que las células de otros pacientes no. Además, en este artículo probamos que la limpieza de la proteína LPL de la superficie de los linfocitos B de LLC con heparina mejora la detección de la proteína LPL endógena permitiendo discriminar entre pacientes que expresan el ARNm de *LPL* y aquellos que no, y describimos un sencillo protocolo de rutina para realizar esta limpieza de LPL superficial. Uno de los hallazgos más sorprendentes es que la discriminación entre los grupos LPL positivo o negativo es máxima cuando se utilizan células obtenidas de sangre fresca en lugar de las congeladas y descongeladas de un

biobanco, lo que aumenta la posibilidad de su implementación en la clínica. Es posible que éste fenómeno se deba a la capacidad que hemos descubierto del linfocito de LLC de internalizar LPL del medio, ya que el medio de congelación contiene suero fetal bovino.
Reportamos también que la cuantificación de los niveles endógenos de la proteína LPL reflejan los niveles de su ARNm, y describimos un método pronóstico utilizando citometría de flujo. Finalmente, investigamos la capacidad del método que aquí describimos de predecir la evolución clínica, medida como el tiempo al primer tratamiento, y encontramos que en nuestra cohorte de pacientes la medición directa del porcentaje de células LPL positivas permite estratificar a los IgVH mutados y no mutados con soporte estadístico, y aparece como una prometedora alternativa, luego de validarse en una cohorte mayor, como un predictor del tiempo al primer tratamiento. Las nuevas terapias con inhibidores moleculares selectivos o anticuerpos monoclonales permiten lograr remisiones completas de la LLC. Es así que la identificación temprana de aquellos pacientes cuya enfermedad se manifiesta de forma asintomática pero que luego progresara es crucial para ofrecerles el tratamiento a tiempo. El método que aquí describimos presenta una alternativa de fácil implementación en la rutina clínica, que permite identificar estos pacientes mediante una biopsia líquida, y que no requiere mayor infraestructura que la habitual de citometría de flujo analítica presente en la mayoría de los servicios de hematología, incluso en países en vías de desarrollo.

LPL protein in Chronic Lymphocytic Leukemia have different origins in Mutated and Unmutated patients. Advances for a new prognostic marker in CLL

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ABSTRACT

Among the different prognostic factors in Chronic Lymphocytic Leukemia (CLL), it has been demonstrated that lipoprotein lipase (LPL) is associated to an unmutated (Um) immunoglobulin profile and poor clinical outcome. The expression of LPL gene in Um CLL B cells is a very interesting observation because there is no expression of LPL in normal B cells. This anomalous expression, constitutes not only a suitable prognostic marker to study disease prognosis but could also be helpful to understand the heterogeneous proliferative behavior in the CLL disease. To date, the functional role of LPL overexpression in CLL pathogenesis and/or tumor progression are still open issues. Among these unsolved matters, one additional question is why LPL protein levels in CLL appear not to be correlated to LPL expression at the mRNA level. In this work we evaluated the subcellular localization of LPL protein in CLL cells expressing LPL mRNA and compared this protein levels with negative CLL cells for LPL mRNA. Our results show that LPL protein inside CLL cells could have different origins depending on the *IgVH* mutational status. Whereas LPL protein in Um cases is principally found associated with markers of endoplasmic reticulum, mutated cases, which also show LPL protein being negative for LPL mRNA, depict that this protein is incorporated from the extracellular medium and remains associated with endosome/lysosome vesicles. Specific quantification of endogenous LPL in CLL cells correlates to mRNA expression levels and reveal that LPL protein could become a new, easy and reliable prognostic marker in this leukemia.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is a low-grade, B-cell tumor with monoclonal CD5 positive cells that relentlessly accumulate in lymphoid organs (LO) and bone marrow and flow into peripheral blood (PB). Lack of proliferative properties makes these cells inherently quiescent. However, a proliferating pool of cells has been described in LO that might feed the accumulation pool in the PB. (1);(2). Microenvironmental interactions regulate this proliferative/quiescent signaling and probably is one of the major causes of the biological and clinical CLL heterogeneity (3). The advent of new treatments such as tyrosine kinase inhibitors and monoclonal antibodies able to induce complete remissions may allow early treatment for asymptomatic patients whose disease is likely to progress (3). Accurate identification of these patients is therefore increasingly important. The mutational status of immunoglobulin heavy chain variable (*IgVH*) genes has been considered the best prognostic marker in CLL (4). However, many other surrogates have been proposed with the main goal of recognizing biomarkers that can predict therapy response (5). We previously described that Unmutated (Um) patients could be differentiated from mutated (Mut) ones by the expression of lipoprotein

lipase (*LPL*) gene (6) and proposed the prognostic value of *LPL*/*ADAM-29* ratio as a surrogate of the mutational status (7). Since then, a body of evidence has confirmed that the expression of *LPL* mRNA is associated to bad prognosis, (8, 9) and that it is the most robust molecular marker in CLL (10).

Besides the potential relevance of *LPL* as a prognostic marker in CLL, there are still several unsolved questions regarding the authentic role of this enzyme in the pathogenesis of this leukemia. LPL is a protein located on the luminal side of the vessel wall, and is anchored to heparan sulfate proteoglycans which contains binding sites for both heparan sulfate chains and apoproteins(11). Their main role is related to overall lipid metabolism and transport. In this line, it has recently been shown that low-density lipoproteins may enhance proliferative responses of CLL cells to inflammatory signals (12) and that *LPL* expression confers a survival advantage to CLL cells (13). However, other works suggest that LPL protein in CLL is catalytically inactive (14) , thus it does not appear to significantly influence CLL cell survival on its own (15).

To confirm the different roles proposed for *LPL* expression in the leukemic clone a clear identification and characterization of LPL protein inside the tumor lymphocyte became an essential issue. Presently, few data about LPL protein expression in CLL cells is available and their correlation with disease progression as well as their prognosis value is controversial (16). Why LPL expression on cell surface does not correlate to its mRNA level is greatly unknown up to now. In this work we provide deep insight into this question analyzing the sub-cellular localization of LPL protein in CLL cells expressing *LPL* mRNA ($LPL^{mRNA/pos}$) and comparing with negative CLL cells for *LPL* mRNA ($LPL^{mRNA/neg}$). The results obtained led us to study the possibility whether measuring LPL protein content by flow cytometry could become a reliable *IgVH* surrogate. Our results show that LPL protein inside CLL cells could have different origins depending on the *IgVH* mutational status and that LPL protein expression correlates to mRNA expression levels. Whereas LPL protein in Um cases is principally found associated with markers of endoplasmic reticulum (ER), Mut cases, which also show LPL protein but are negative for *LPL* mRNA, depict that this protein is incorporated from the extracellular medium and remain associated with endosome/lysosome vesicles. This different subcellular localization of endogenous LPL in CLL cells reveal that it is possible to set up a specific method directed to quantify endogenous LPL protein levels to be used as a prognostic marker in this leukemia.

Materials and Methods

Patients, sample collection and molecular processing

Peripheral blood was obtained from 42 patients with a typical diagnosis of CLL All patients were followed at the Hospital Maciel and Hospital de Clínicas in Montevideo, and provided an informed consent according to local ethical regulations and the Helsinki Declaration. Mononuclear cells were isolated by Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) layer separation according to the manufacturer. Mutational status was performed as previously by (17) and LPL mRNA expression as described in (7, 18). Briefly, RT-PCR and Real-time (qRT-PCR) were performed with RNA previously extracted with TriZol. First-strand cDNA was synthesized from 500 ng total RNA with MMLV-RT (Thermo Scientific, Waltham, MA, USA) using an oligo dT18–20 primer. Real-time qRT-PCR reactions were performed using Kapa SYBR Fast qPCR kit (Kapa Biosystems,Wilmington, NC, USA) according to the manufacturer in a Corbett 6000 (Qiagen, Venlo, Netherlands) thermal cycler. Clinical and molecular characterization of CLL patients used in this work as depicted in Table 1.

Surface LPL removal and culture of PBMC CLLs

PBMCs culture of different CLL samples were performed in AIM-V serum free medium (Thermo-Fisher, Whaltham, MA), Fetal Bovine Serum (FBS, Thermo-Fisher, Whaltham, MA), or autologous plasma. Different conditions of heparin treatment such as concentrations (25, 50, 100U/ml) temperatures (6, 20 and 37°C) and times (5, 10 and 30 min) were evaluated. The best condition found to remove mostly cell surface-attached LPL in CLL was: incubation of 5 million PBMCs with 50 U/ml sodium heparin in RPMI/3% BSA for 5 min at 37°C. Next, samples were centrifuged at 400g for 5 min, and washed once with RPMI.

Flow cytometry
Cells were fixed with 10% formaldehyde from PFA for 15 min. Fixative was washed away with 2 washes with 10 ml PBS, and centrifugation at 400g for 5 min at 4°C. Cells were then blocked and permeabilized with 3% BSA in PBS-T (0.1% Triton X-100 in PBS) 10 min at room-temperature, and incubated overnight with 1 µg/ml anti-LPL monoclonal antibody (Mab) 5D2, (kind gift of Dr John D. Brunzell) or mouse IgG1 isotype control (Thermo-Fisher, Waltham, MA). After two washes PBS, cells were incubated with anti-mouse alexa 488 (Life Technologies, Eugene, OR, USA) 2 µg/mL in 3% BSA in PBS-T for 45 min at room-temperature (RT), washed twice with PBS and acquired data with a CyAN ADP flow cytometer (DakoCytomation, Glostrup, Denmark). Data were analyzed in R/Bioconductor with the packages flowCore, flowStats and flowViz.

Confocal microscopy
Cells were fixed with 10% formaldehyde from PFA, attached to poly-L-lysine-coated slides and blocked for 15 min with PBS-T/BSA (0.1% Triton-X100/3% BSA). Immunostaining was performed with mouse anti-LPL (5D2) 1 µg/ml overnight at 4°C and goat anti-mouse Alexa 594 1:1000 (Life Technologies, Eugene, OR, USA), donkey anti human-IgM PE 1:50 (Jackson ImmunoResearch, West Grove, PA, USA), 0.1 µg/ml DiO C18 (Sigma-Aldrich, St. Louis, MO, USA), and methyl green as previously described (19) Golgi cisternae staining was performed in vivo with Bodipy TR ceramide (Thermo Fisher, Waltham, MA, USA) and fixed afterwards. Cells were then mounted in 70% glycerol pH=8.8, and imaged in a Leica TCS SP5 confocal microscope. Three-dimensional deconvolution was performed using Huygens Essential 4.5 (Scientific Volume Imaging B.V., Hilversum, The Netherlands).

Electron microscopy
Cells were fixed in 0.01% glutaraldehyde, 4% formaldehyde from PFA in 0.1M cacodylate buffer pH=7.2/5mM CaCl2/0.1M sucrose, dehydrated in a series of 30-50-70-90-100% cold ethanol, and embedded in Lowycryl K4M (Sigma-Aldrich, St. Louis, MO). Blocks were sectioned at 70 nm and tranferred onto non-coated 400 mesh nickel grids. Immunostaining was performed by incubating the grids with 50 mM ammonium chloride in PBS (pH=7.2) for 20 min at RT, washing in PBS/2% BSA/0.01% Tween-20 (pH=8) for 20 min at RT, incubating with mouse anti-LPL (5D2) 1:500 overnight at 4°C, washing (3x) PBS/2% BSA/0.01% Tween-20 (pH=8), incubated with donkey anti-mouse 10 nm colloidal gold (Sigma-Aldrich, St. Louis, MO, USA) 1:100 1 hour at RT, and washing with water before air drying. Dried sections were contrasted with uranyl acetate and lead citrate before imaging in a Morgagni F268 Transmission Electron Microscope (FEI, Eindhoven, The Netherlands), operating at 80 kV.

Statistical analyses
Analyses were done using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA) and R/Bioconductor. Normality of data was assessed using the Shapiro-Wilk's normality test. Kendall's rank correlation test was performed using Kendall package, and null hypothesis was rejected at $p<0.05$. Optimal cut-off determination of continous LPL-FC data was analyzed using the OptimalCutpoints (20) package with MaxDOR and Youden's J statistic method at a 95% confidence interval. *IgVH* mutational status was used as a categorical covariate for calculation. Survival rates based on adjusted Kaplan-Meier estimates were calculated for 36 patients with clinical follow-up with the packages survival and survminer.

RESULTS

LPL protein is present in Mut and Um CLL cells but depict a different sub-cellular localization

LPL protein is located both on the surface and cytoplasm of CLL B-cells as it has been previously shown (8). However, a detailed characterization of the cellular LPL compartmentalization in Mut and Um CLL cells it is missing. With this purpose we performed 3D image deconvolution and electron microscopy revealing the existence of a different subcellular localization pattern of LPL protein in CLL cells LPL$^{mRNA/pos}$ compared with CLL LPL$^{mRNA/neg}$ cells (Figure 1). Interestingly, in CLL cells from LPL$^{mRNA/pos}$ patients a perinuclear sub-localization is evidenced by higher immunomarcation (figure 1A, white arrows), whereas it does not appear to be the case for LPL$^{mRNA/neg}$ patients. In this latter sub-group LPL protein shows lower intensity and maintains a peripheral localization (second panel, whit arrows, figure 1A). A similar staining pattern is found in B cells from healthy donors (data not shown). To further address this observations, we performed co-immunostaining of LPL with the ER marker calnexin (CLX), a classical marker of the ER cisternae, and with anti-Lamp1 used as a marker of late endosome/lysosome vesicles. Our results show that in LPL$^{mRNA/pos}$ cases, an association of LPL with ER is mainly evidenced by overlapping of LPL and Calnexin signals (figure 1B, arrows in first panel), whereas low quantities of LPL protein are found associated with Lamp-1. In contrast, in LPL$^{mRNA/neg}$ cases lower co-localization of LPL protein with Calnexin is found, whereas the majority of LPL overlaps with Lamp-1 (figure 1C, white arrows in second panel). These results were confirmed by immuno electron-microscopy in CLL patients in which LPL immunogold labeling corresponding to LPL$^{mRNA/pos}$ cases is mainly observed in cisternae of ER whereas this is not the case for LPL$^{mRNA/neg}$ patients in which labeling is mainly found in vesicles, (Figure 1C, white arrows).

Altogether these results suggest that LPL protein in LPL$^{mRNA/pos}$ CLL cells is mainly produced by a classical transcription/translation mechanism and that this protein follows at least in part a classical ER/Golgi-dependent secretory pathway. Concerning LPL$^{mRNA/neg}$, our results show that LPL is detected associated to cell-surface or intracellular vesicles but interestingly no association with ER nor with Golgi cisternae could be evidenced.

Extracellular LPL protein is internalized from plasma by CLL cells independent of the mutational status

After biosynthesis, LPL is secreted into the blood vessels and associated with the luminal side of capillaries and arteries where it anchors to heparan sulfate proteoglycans (11). The presence of LPL protein inside CLL cells in LPL$^{mRNA/neg}$ patients could only be explained by the external incorporation of this protein into tumour cells, therefore confirmation of this hypothesis could help us to understand the fact that LPL protein does not correlate to mRNA levels as described in (8). To gain insight into this issue we performed in vitro experiments with CLL cells from patients expressing or not LPL mRNA. We incubated these cells by 24hs in serum free medium (SFM) or in autologous plasma and evaluated by flow cytometry and by confocal microscopy the presence of LPL in CLL cells and its localization, respectively. Our results show that after 24hs both CLL cells expressing or not *LPL* mRNA are able to incorporate LPL protein from the plasma. Significant differences showing external incorporation of LPL protein were found when comparing LPL protein from CLL cells incubated for 24hs in medium without soluble LPL or with autologous plasma containing LPL ($p=0.015$, Wilcoxon test, two tailed). Similar results were found using non-autologous plasma (data not shown), whereas a similar but not significant tendency was also observed when CLL cells were incubated 24 hs only with RPMI plus 10% FBS. As depicted in Figure 2A (second panel) some patients appear to be able to incorporate LPL from FBS which is in turn recognized by the anti-LPL 5D2 mAb used in flow cytometry analysis as expected, since this antibody was originally produced against bovine LPL (21). Representative patients confirming these results by microscopy and flow cytometry are depicted in Figure 2B. Subcellular localization analysis of LPL protein from both subgroups after incubation with autologous plasma was evaluated. Our results show that in LPL $^{mRNA/pos}$ subgroup LPL immunostaining colocalizes with CLX in addition to Lamp-1 (Figure 2C, first panel, white and red arrows, respectively), whereas in LPL $^{mRNA/neg}$ patients, the protein internalized from autologous plasma remains excluded of ER and is mainly associated with Lamp-1 vesicles (white arrows in figure 2C, second panel).

Altogether these results suggest that LPL protein in CLL cells could come from two different origins, one produced *de-novo* in LPL$^{mRNA/pos}$ patients or the other, incorporated from plasma in both subgroups (LPL$^{mRNA/neg}$ and LPL$^{mRNA/pos}$ CLLs).

Fresh CLL cells and surface heparin peeling improve the detection of LPL protein between LPL$^{mRNA/neg}$ and LPL$^{mRNA/pos}$ CLL cells.

The different grades of LPL internalization in CLL patients could turn the development of a reliable prognosis method at the protein level very difficult. The fact that CLL cells incorporate either human LPL from plasma or bovine LPL from FBS, represents an additional problem because frozen PBMCs, which is one of the usual forms of preserving CLL cells, could not be used. To further investigate this assumption, and knowing that in the presence of heparin LPL can be released into the blood we optimized the best concentration, temperatures and times of heparin incubation for CLL PBMCs for removal of cell surface attached LPL (data not shown) and we compared this protocol between fresh and frozen PBMCs of the same patients. Our results demonstrate that heparin treatment detaches LPL protein from cell surface in RNA/neg or in RNA/pos CLL cells. However, LPL protein either in intracellular vesicles of LPL $^{mRNA/neg}$ cases or in the ER cells of LPL $^{mRNA/pos}$ patients, remain positive for a LPL specific staining (white arrows in heparin-treated panels, and representative histograms, figure 3A). These results confirm that external LPL could be incorporated by CLL cells, and show that heparin treatment is not enough to totally improve a putative prognostic method in CLL.

With these results in mind, and knowing that frozen cells could be a source of unspecific LPL immunostaining (figure 2A) we tried to found the best conditions in which LPL protein detection displayed the best specificity/sensitivity properties allowing a reliable prognostic method for CLL. To this aim we selected 20 patients, 10 corresponding to the subgroup LPL$^{mRNA/neg}$ and 10 to the subgroup LPL$^{mRNA/pos}$ and evaluated different conditions such as: **a)** frozen/thawed PBMCs; **b)** frozen/thawed PBMCs plus heparin; **c)** fresh PBMCs; and **d)** fresh PBMCs plus heparin. All these conditions were equally tested using the anti-LPL 5D2 mAb in flow cytometry. Our results show that LPL protein detection either in frozen/thawed PBMCs or in frozen/thawed PBMCs plus heparin is not enough to provide a clear discrimination between RNA/neg and RNA/pos CLLs (figure 3B); intead it is improved when LPL protein analysis are performed in fresh PBMCs from CLL samples (figure 3C, first panel). Interestingly, and according to our previous observations, heparin treatment in fresh PBMCs appears to be the best option to improve the detection of LPL protein in CLL (figure 3C, second panel).

Altogether, these results show that LPL protein detection from frozen/thawed may not correspond to the expression of *LPL* at the mRNA level, probably due to the unspecific uptake of human/bovine LPL protein from plasma and FBS, respectively. Instead, LPL protein detection after heparin peeling from fresh CLL PBMCs demonstrates the best difference between LPL$^{mRNA/neg}$ and LPL$^{mRNA/pos}$ groups and led us to evaluate the prognostic prediction power in a larger number of patients.

Quantification of the endogenous LPL protein levels in CLL cells reflect LPL mRNA levels and predict clinical outcome in CLL

Since *LPL* mRNA has been reported to be a strong predictor of CLL outcome, we wondered whether endogenous LPL protein (eLPL) levels evaluated on fresh CLL PBMCs could report mRNA levels, and if this methodology could be proposed as an alternative prognostic method to *LPL* mRNA detection or a complementary methodology for the *IgVH* mutational status. To this aim, we set up a flow cytometry protocol for eLPL evaluation and compared it to *LPL* mRNA expression by qRT-PCR and to *IgVH* status in a validation cohort of 42 CLL samples (21 mutated and 21 unmutated). Finally, we compared the predictive prognostic power of all these different methods using the time to first treatment (TTFT) of a selected cohort. As depicted in figure 4A our results evaluating *LPL* at the mRNA level by qRT-PCR depict that the best segregation between Mut and Um patients is obtained using a threshold of 10 as has been previously described by (8). This cutoff correctly segregates 40 of 42 patients, depicting 95% of accordance rate with the

mutational status. The two discordant cases are CLL 16 and 11, with values of 13.2 and 10.5 respectively. Concerning LPL protein measurement after heparin peeling from fresh CLL PBMCs the best cutoff value necessary to obtain the best concordance rate with *IgVH* mutational status is 25%. Using it, 41 of 42 patients (97%) could be achieved, in this case the only discordant case was the CLL 37 = 21 %. Both methodologies show significant differences between Mut and Um cases. Specifically, for LPL protein detection the Mut cases show a mean = 14,16 ± 1,2 n=21, and Um cases a mean = 44,51 ± 3,1 n=21, (Unpaired t test with Welch's correction, p=0.0001), Figure 4B. The threshold values calculated for both tests showing the best concordance rate with the *IgVH* mutational status were determined using the Youden index as indicated by a horizontal pointed lines in Figure 4 A-B. Both established cutoff values were used for further calculation of odds ratio and clinical outcome. Median TTFT for the evaluated cohort (n = 36) was 25 months in CLLs displaying Um *IgVH* genes (p= 0.001), 20 months in CLLs expressing *LPL* at mRNA level (p = 0.005) and 25 months for CLLs expressing LPL at the protein level, (p = 0.02), whereas median TTFT was not achieved neither for Mut CLLs, or LPL mRNA/negative or LPL protein/negative subgroups (Figure 4 A-C, respectively).

Altogether, these data suggest that measurement of LPL protein could be an interesting prognostic method to be evaluated as a good alternative either for the measurement of *LPL* mRNA or as a surrogate for the mutational *IgVH* status in CLL.

DISCUSSION:

The high expression of *LPL* gene in Um CLL B-cells is a very remarkable observation because there is no expression of LPL in normal B cells. This specific and ectopic expression, constitutes not only a suitable prognostic marker to study disease evolution but could also be helpful to understand the heterogeneous proliferative behavior in CLL disease. To date, the functional role of *LPL* overexpression in CLL pathogenesis and/or tumor progression are still open issues. Different hypothesis have been proposed about the functional capacity of LPL expressed by CLL cells : ***i.*** LPL expression play a role in the lipid metabolism of CLL and supply survival advantages for tumor cells. This hypothesis has been raised by Pallasch and colleages (22) and supported by works of Rozovski et al. showing that survival advantages are driven by LPL-dependent metabolism of intracellular stored lipids for energy production (13, 23, 24). However, this catalytic function of LPL in CLL is questioned by the fact that the levels of catalytically activity of LPL in CLL cells is very low and it could not be correlated to mRNA expression levels or *IgVH* mutational status (14, 15). ***ii.*** LPL expression plays a role through the microenvironmental crosstalk also promoting proliferation, homing and survival. This hypothesis is also supported by results of Pallasch et al. who observed elevated *LPL* mRNA levels in CLL cells after stimulation of the B cell receptor, and is reinforced by our previous observation that *LPL* expression in CLL cells could be increased by specific microenvironmental signaling such as CD40L/IL4 or BCR interaction as well as after autologous T-cell activation, (18, 25). Despite membrane-bound LPL has been proven to enhance cell adhesion (26) and could be associated with migration/spreading of the leukemic clone in proliferative centers, this premise has neither been proven yet (27). Among these unsolved issues concerning LPL expression in CLL, one additional question is why LPL protein levels appear not to correlate with the expression of *LPL* at the mRNA level (8). Heintel et al. described that LPL protein expression is mainly restricted to B cells of CLL patients but interestingly, they found that LPL protein on the surface of CLL cells did not correlate to *LPL* mRNA. In contrast, this correlation appear to be certain when LPL protein is evaluated by indirect immunofluorescence (IFI) in the cytoplasm of leukemic cells (8). Unfortunately, this observation was not deepened probably because IFI practice is complex and not typically used as a quantification methodology to be extrapolated to the clinical routine.

In this work we provide deep insight into the subcellular localization of LPL protein within the different prognostic subgroups of CLL in order to gain new perspectives about the functional role of this protein. We explored the possibility to set up the measurement of LPL protein in flow cytometry as a new and useful prognosis method for CLL. Our result show that despite the fact that LPL protein exists inside and in the membrane of the tumor cells, the sub-cellular localization is different between Mut and Um patients. Whereas

LPL protein in Mut CLL cells remain associated to cell-surface or intracellular vesicles, the LPL of Um cases is mainly associated with classical markers of ER or visualized by electron microscopy in this compartment. Interestingly, when focusing on LPL protein in Mut CLL ($LPL^{mRNA/neg}$ subgroup) our data demonstrates that LPL protein in the leukemic cells remain in the cell membrane even after heparin treatment and could be internalized from plasma either for Mut or Um cases. Our work describes for the first time that LPL protein quantities in the leukemic clone could account for two different origins; ***a)*** an internal source from *LPL* mRNA transcription that mainly occurs in Um CLL, and ***b)*** an external source from plasma from where LPL appears to be uptake either in Um and Mut cases. These findings support and complement the results of Heintel et al. (8) explaining why the expression of mRNA in leukemic cells of Um patients, largely demonstrated in CLL (7-9, 28) does not correlate with a higher expression of LPL protein. This complex scenario about the origin of LPL protein in the leukemic clone as well as the fact that LPL from FBS is also detected by mAb 5D2 inside CLL cells explain the inconsistent data between measurement of total LPL protein and mRNA levels previously described in (15) and (8). Furthermore, these results call attention to the data suggesting a low catalytic activity of LPL and to the assumption that LPL protein expressed by CLL cells exists mostly as a non-active monomer (15). We think that more cellular and biochemical experiments with a purified LPL isolated of Um CLL cells should be carried out to finally understand whether the LPL protein secreted specifically by the leukemic clone is functional or not.

The second goal of this work was to evaluate the possibility that LPL protein assessment could be a reliable prognostic marker in CLL. To this aim, we first confirmed that the best results and the most significant p values were obtained using fresh CLL cells and heparin surface peeling. These data are reinforced by our previous results (Figure 2A) showing that the 5D2 anti-LPL mAb cannot be used on previously cryopreserved CLL samples because bovine LPL is randomly internalized and recognized by the anti-LPL mAb. These results are expected knowing that mAb 5D2 was originally developed by immunization with bovine LPL and later confirmed that it also cross-reacted with human LPL (21, 29). Under these conditions, the measurement of LPL protein expression reported here appears to be a good prognostic method similar to the measurement of *LPL* mRNA expression previously described (7-10, 28). The fact that *LPL* at mRNA (9) and protein level (as demonstrated by our data) could perfectly be performed on fresh PBMC without the need to identify other cells such as T lymphocytes or monocyte/macrophage cells, make this protocol a simple flow cytometry method that could be performed in any flow cytometer meeting minimal requirements. This protocol does not present the classical problems previously described for other flow cytometry-technologies in CLL prognosis concerning the existence of a single cut-off level that should previously be agreed by the laboratories. This is the case for prognostic markers in CLL such as ZAP-70 (30-32) or for CD38 (33). In both cases, problems due that the target protein being expressed in other blood cells than the leukemic clone (32), or that the target protein expression levels changed with the activation of the CLL cells (34), were described . Concerning technical approach issues on the LPL protein protocol described in this work we remark that all the results obtained here were performed with the 5D2 mAb kindly provided by Dr. Brunzell described in (21). None of the commercially available 5D2 mAb were evaluated.

The prognostic methodology here proposed was finally tested in a cohort of 42 non-cryopreserved CLLs and their prognostic value compared with those of the mutational VH status profile and with the *LPL* mRNA levels. Our results show that the percentage of leukemic cells expressing LPL protein is significantly higher than in Mut patients (Figure 4) and that with the proposed cut-off value of 25% we obtained data supporting that expression of LPL in CLL could be a powerful prognostic test. Despite the fact that high concordance rate with the mutational status was achieved (95%) and that TTFT appears to be significant and similar to the mean observed for mutational status and *LPL* mRNA expression in the same CLL cohort we realize that the protocol proposed here should be validated on a bigger cohort.

In summary, our data appear to reinforce the hypothesis that LPL protein in progressive cases could have a catalytic function providing metabolic survival advantages for the tumor clone. In this case LPL protein is generated de novo and appears to be correctly expressed in a classical ER/Golgi-dependent secretory pathway. Instead, the hypothesis suggesting that membrane-bound LPL could support proliferation, migration and

spreading for progressive patients seems rather unlikely, because our results show that both Mut or Um patients are able to bind or internalize LPL from plasma indistinctly. Furthermore, we present here an easy and accessible flow cytometry protocol based on the single immunolabeling of LPL protein on fresh PBMCs of CLL patients. This methodology is related to *LPL* mRNA expression levels as well as the mutational *IgVH* status, and we think that after validation in a larger cohort, could become a useful tool to predict disease evolution in CLL.

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TABLE 1: Clinical and molecular characterization of CLL patients

Table 1. Clinical and molecular characteristics of the patient cohort.

Patient No.	Sex	Binet stage[1]	LC (x10³/µl)	FISH[2]	*IgVH* status	LPL[3]
1	F	A	5.9	N/D	Mutated	Negative
2	F	B	162.0	del13q	Mutated	Negative
3	F	A	12.5	del13q	Mutated	Negative
4	M	A	32.6	N/D	Mutated	Negative
5	M	A	55.8	N/A	Mutated	Negative
6	M	A	18.9	N/A	Mutated	Negative
7	M	A	8.2	N/A	Mutated	Negative
8	M	B	19.0	N/D	Mutated	Negative
9	M	A	7.3	del13q	Mutated	Negative
10	F	A	43.2	N/A	Mutated	Negative
11	M	C	62.3	Tris 12	Mutated	Positive
12	F	A	19.8	N/A	Mutated	Negative
13	F	A	22.2	N/A	Mutated	Negative
14	M	A	19.6	N/D	Mutated	Negative
15	M	B	58.8	N/A	Mutated	Negative
16	M	A	21.6	N/A	Mutated	Positive
17	F	A	10.0	del13q	Mutated	Negative
18	M	B	6.3	N/A	Mutated	Negative
19	F	A	23.6	N/A	Mutated	Negative
20	F	A	11.5	N/D	Mutated	Negative
21	M	A	N/D	N/D	Mutated	Negative
22	F	A	53.0	del17p	Unmutated	Positive
23	F	A	17.9	N/A	Unmutated	Positive
24	M	A	161.0	del13q	Unmutated	Positive
25	F	B	83.8	N/A	Unmutated	Positive
26	M	C	900.0	N/A	Unmutated	Positive
27	M	C	100.0	del17p	Unmutated	Positive
28	F	A	24.9	N/A	Unmutated	Positive
29	F	A	7.9	N/A	Unmutated	Positive
30	M	C	3.8	del11q	Unmutated	Positive
31	M	A	N/D	N/D	Unmutated	Positive
32	F	A	10.0	N/D	Unmutated	Positive
33	F	A	15.6	N/A	Unmutated	Positive
34	M	B	77.8	del13q	Unmutated	Positive
35	M	B	120	N/A	Unmutated	Positive
36	F	C	130.0	del17p	Unmutated	Positive
37	F	A	40.0	N/A	Unmutated	Positive
38	F	B	66.5	N/D	Unmutated	Positive
39	F	B	7.7	N/A	Unmutated	Positive
40	F	A	6.0	N/D	Unmutated	Positive
41	M	C	102.8	N/A	Unmutated	Positive
42	M	B	220	Tris12	Unmutated	Positive

[1] N/D: not determined / [2] N/A: no alterations detected / [3] determined by qRT-PCR

FIGURE LEGENDS

Figure 1. Subcellular localization of LPL in CLL B-cells from peripheral blood.
(A) Subcellular distribution of LPL immunoreactivity in relation to cellular membranes. LPL signal shows a different pattern in cells from $LPL^{mRNA/pos}$ patients than in those from $LPL^{mRNA/neg}$ patients. In cells from $LPL^{mRNA/pos}$ patients a perinuclear membrane localization was found. The inset shows a representative cells with typical LPL distribution in $LPL^{mRNA/pos}$ and $LPL^{mRNA/neg}$ patients cells (white arrows). **(B)** Cells from $LPL^{mRNA/pos}$ patients display two different populations of LPL, one localized in the cisternae of the endoplasmic reticulum (ER) -as shown by calnexin staining (arrowheads, upper panels), and the other punctually excluded from the ER of which some coincided with LAMP-1 positive vesicles (arrowheads, lower panels). In cells from $LPL^{mRNA/neg}$ patients, we were only able to find LPL immunomarcation localized to LAMP-1 positive vesicles (white arrows in second panel B). **(C)** Subcellular distribution of LPL as revealed by immunoelectron microscopy, the inset shows a 1.7x magnification of the rectangle region. Arrows indicate LPL immunogold staining. $LPL^{mRNA/pos}$ cells showed cytoplasmic LPL immunolocalization and within ER cisternae, whereas $LPL^{mRNA/neg}$ cells showed cytoplasmic and mainly vesicular immunoreactivity, but we are not able to found LPL immunogold staining in ER cisternae. N: nucleus, C: cytosol, v: vesicles, NM: nuclear envelope. Scale bar A: 5 um, B-C: 2.5 um, D:500nm(left), 200nm(right).

Figure 2. Plasma LPL internalization by in vitro culture of CLL cells from Mut or Um patients.
(A) Percentage of LPL positive cells measured by flow cytometry after culturing them for 24h in serum-free medium (SFM) or in CLL plasma (left panel). The number of LPL positive cells increased when cultured in CLL plasma ($p=0.015$, $n=6$), independent of the *IgVH* mutational status of the patient. Cells cultured in fetal bovine serum (FBS) shows a trend to internalization, with no statistical significance and independent of the mutational status. **(B)** In situ immunolocalization of LPL (magenta) shows an increase in LPL signal upon culturing cells from the same patient in CLL plasma compared with FBS or SFM. The median intensity of fluorescence obtained by flow cytometry (green line in histograms) is increased when CLL cells were cultured in CLL plasma compared with SFM or FBS. A representative density plot is shown (log scale). **(C)** LPL internalization after culturing $LPL^{mRNA/pos}$ (upper panels) or $LPL^{mRNA/neg}$ (lower panels) cells in CLL plasma. In $LPL^{mRNA/pos}$ LPL labeling (magenta) is mainly visualized into calnexin-positive zones (Blue), depicted with red arrows, whereas a minority of LPL protein localize into LAMP-1 (green) positive vesicles (white arrows). In the second panel $LPL^{mRNA/neg}$ cells display LPL colocalization with LAMP-1 positive vesicles (white arrows) and is excluded from calnexin-positive zones. Scale bar B, C: 5um.

Figure 3. Optimization of the different protocol conditions to develop a reliable methodology for measure LPL protein in CLL.
(A) In situ immunolocalization of LPL (magenta) shows staining of both untreated $LPL^{mRNA/neg}$ and $LPL^{mRNA/pos}$ cells (upper panels). A decrease in LPL staining in both subgroups is observed after heparin treatment, uncovering a punctate pattern with noticeable differences between LPL in both subgroups

(arrowheads, lower panels). LPL signal was excluded from membrane structures stained by DiOC16 (green). Flow cytometry analysis of median fluorescent staining intensities (MFI) reveals an improvement in LPL protein discrimination. Representative density plots depict MFI differences between anti-LPL labeled cells and its isotype control (deltaMFI, right column, log scale). **(B)** Heparin treatment as previously optimized using frozen PBMC appear to be not enough to obtain a clear cut-off that correctly discriminate $LPL^{mRNA/neg}$ and $LPL^{mRNA/pos}$ (p values = 0.34 and 0.03 for frozen/thawed and frozen/thawed plus heparin respectively, n=20). **(C)** Freshly collected cells without heparin treatment improve discrimination of $LPL^{mRNA/neg}$ and $LPL^{mRNA/pos}$ cells (right panel, p=0.01, n=20). However best discrimination is obtained with the combination of both variables (heparin treatment and fresh PBMC), as is depicted in the right panel, (p<0.0001, n=20). Each dot represents the percentage of IgM and LPL positive cells from an individual patient.

Figure 4. Endogenous LPL protein levels reflect mRNA levels, *IgVH* mutational status, and predict time to first treatment. (A) LPL mRNA levels as assessed by qRT-PCR in *IgVH* Mut and Um patients (ΔCq). $LPL^{mRNA/pos}$ is considered when ΔCq > 10 (cut-off indicated by dotted line. **(B)** Endogenous LPL protein levels assessed by flow cytometry in freshly collected heparin-treated CLL cells in an expanded cohort shows significant difference between *IgVH* Mut and Um groups (p=0.0001, n=42). **(C)** LPL mRNA levels predicts TTFT and segregates into two risk groups (low risk $LPL^{mRNA/neg}$, and high risk $LPL^{mRNA/pos}$) confirming previous results (8, 9, 35), n=36. **(D)** Endogenous LPL protein levels predict and can segregate patients into high-risk (LPL positive) and low-risk (LPL negative) groups with statistical significance (p=0.05, n=36) reflecting LPL mRNA levels and *IgVH* mutational status. Cut-off = 25% is indicated by dotted line. **(E)** *IgVH* mutational status was performed and TTFT calculated. ≤ 2% difference from germline gene defined Um patients, ≥ 2% difference define Mut patients.

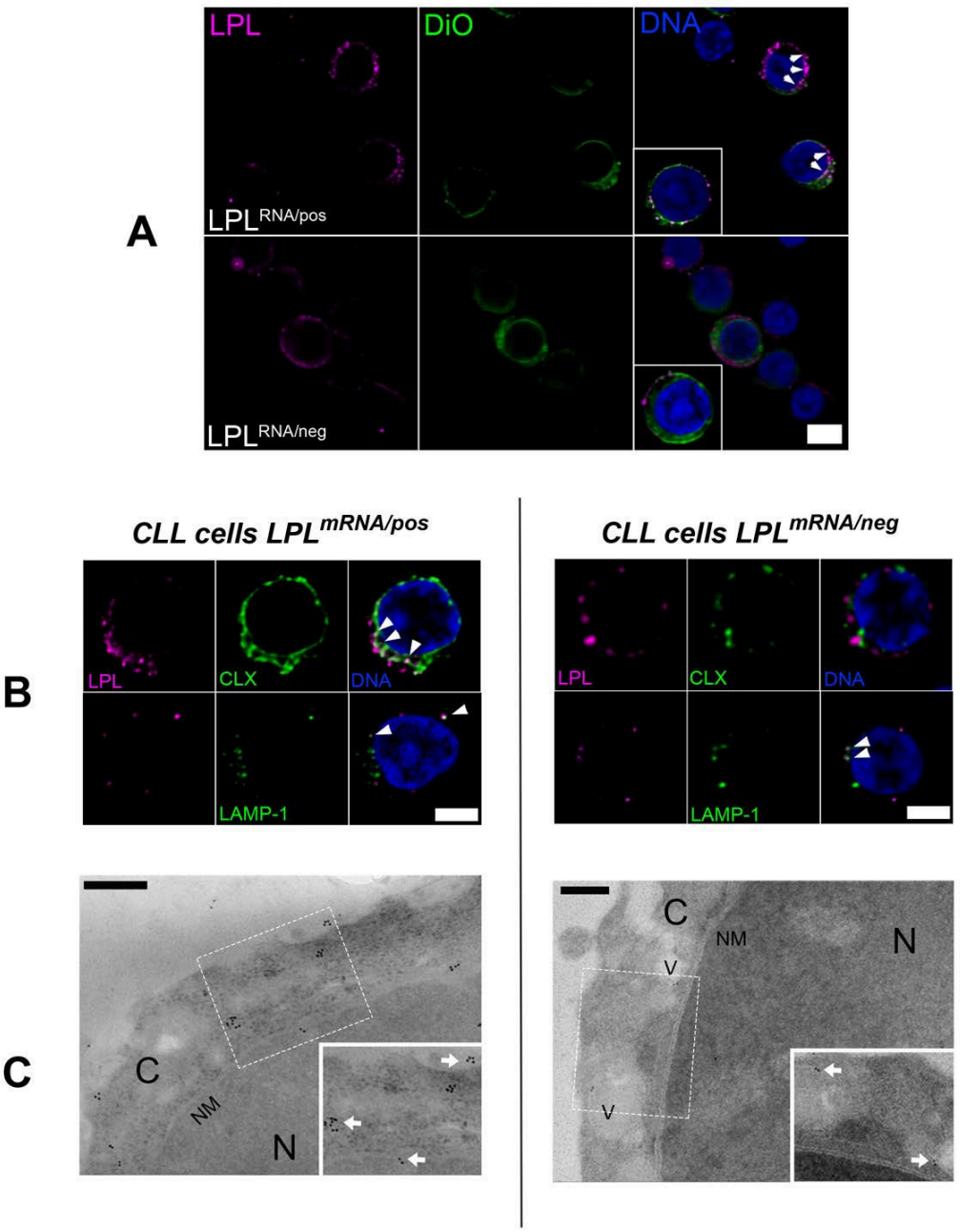
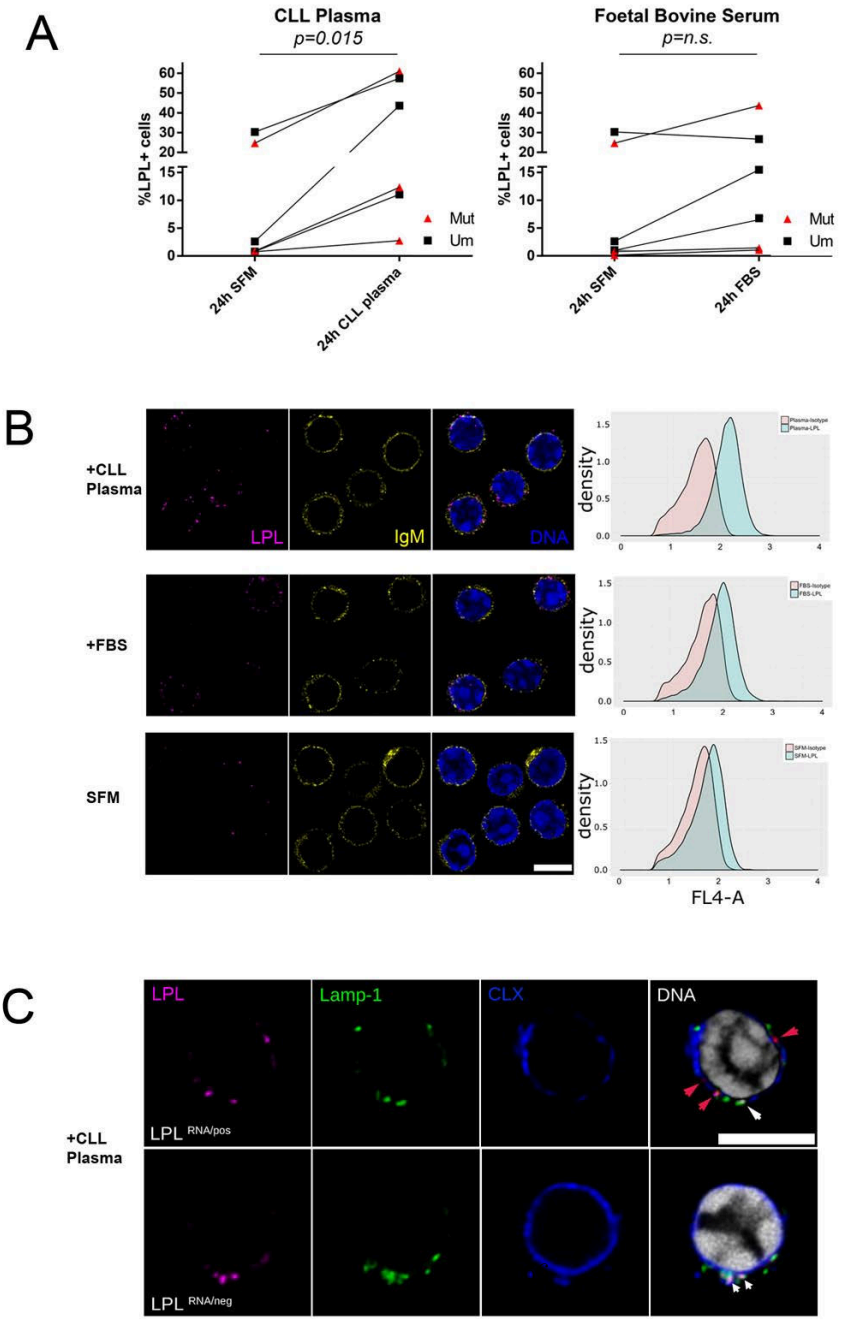

A
LPL
DiO
DNA
LPL RNA/pos
LPL RNA/neg
B
CLL cells LPL mRNA/pos
CLL cells LPL mRNA/neg
LPL
CLX
DNA
LAMP-1
C
C
NM
N
V

Figure 1

Figure 2


A
CLL Plasma
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Foetal Bovine Serum
p=n.s.
%LPL+ cells
Mut
Um
24h SFM
24h CLL plasma
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B
+CLL Plasma
+FBS
SFM
LPL
IgM
DNA
density
FL4-A
C
LPL
Lamp-1
CLX
DNA
LPL RNA/pos
LPL RNA/neg

Fig 3

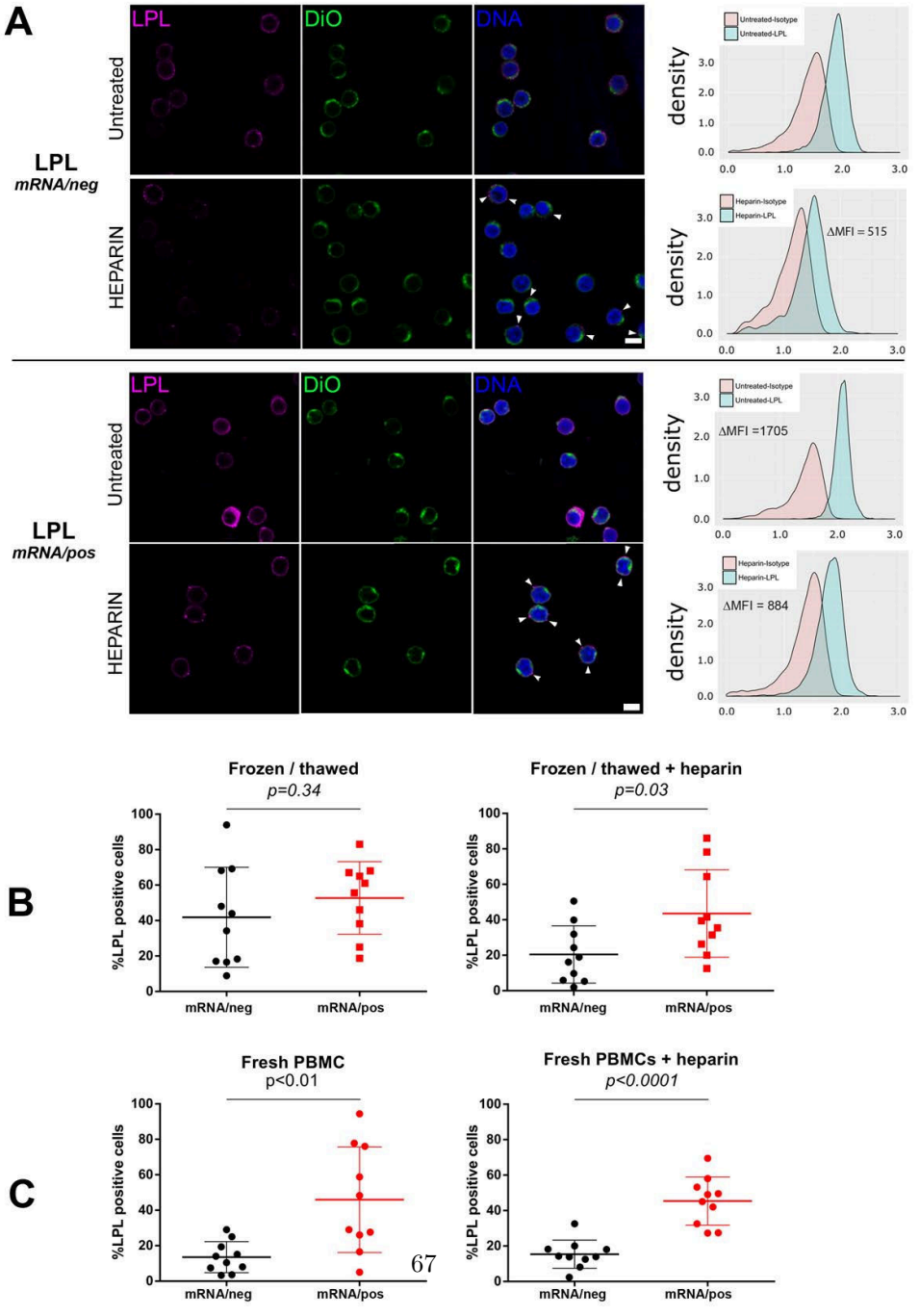
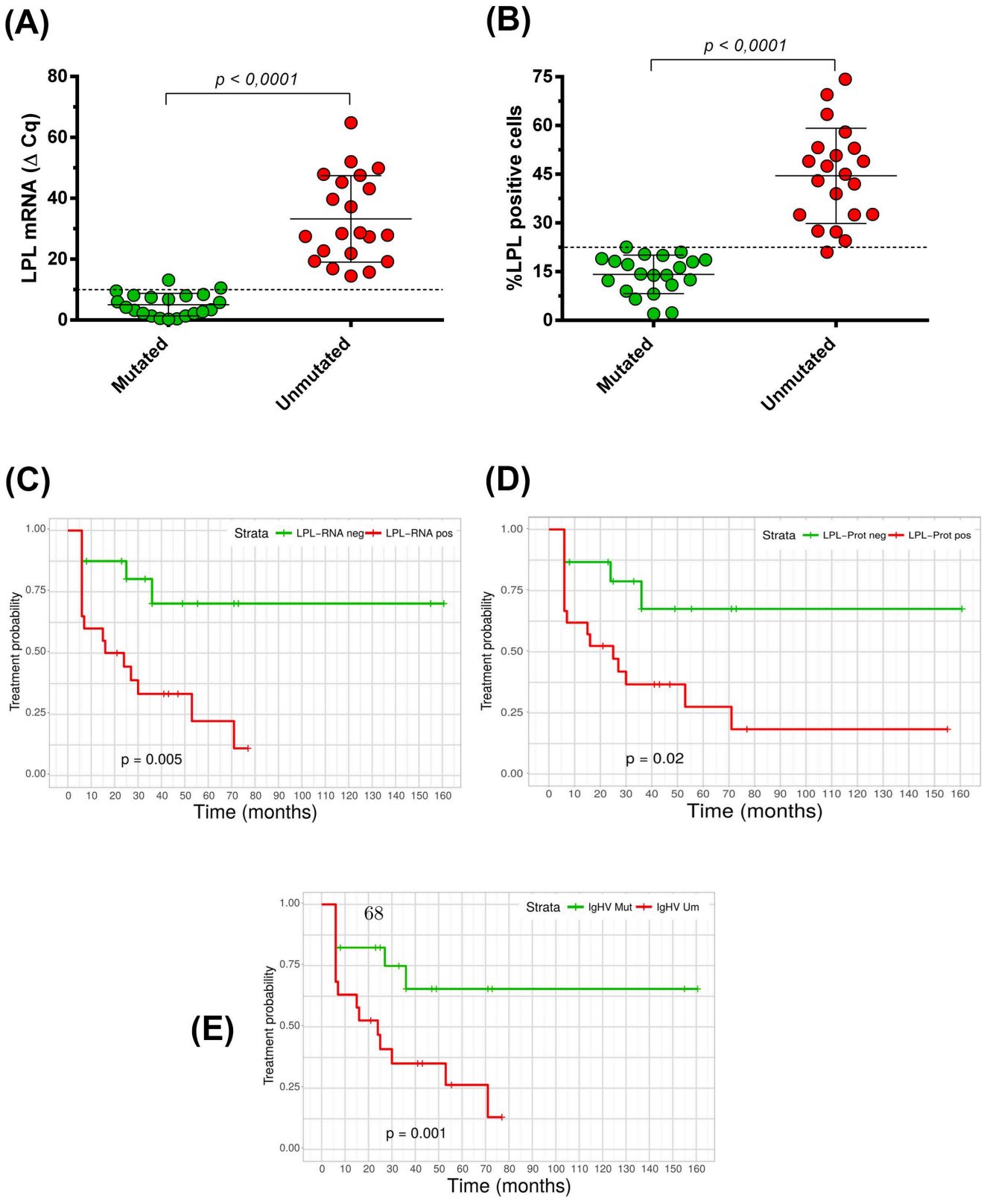

A
LPL
mRNA/neg
LPL
DiO
DNA
Untreated
HEPARIN
density
Untreated-Isotype
Untreated-LPL
Heparin-Isotype
Heparin-LPL
ΔMFI = 515
LPL
mRNA/pos
ΔMFI =1705
ΔMFI = 884
B
Frozen / thawed
p=0.34
Frozen / thawed + heparin
p=0.03
%LPL positive cells
mRNA/neg
mRNA/pos
C
Fresh PBMC
p<0.01
Fresh PBMCs + heparin
p<0.0001

Figure 4


(A)
p < 0,0001
LPL mRNA (Δ Cq)
80
60
40
20
0
Mutated
Unmutated
(B)
p < 0,0001
%LPL positive cells
75
60
45
30
15
0
Mutated
Unmutated
(C)
Strata LPL−RNA neg LPL−RNA pos
Treatment probability
1.00
0.75
0.50
0.25
0.00
p = 0.005
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
Time (months)
(D)
Strata LPL−Prot neg LPL−Prot pos
Treatment probability
1.00
0.75
0.50
0.25
0.00
p = 0.02
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
Time (months)
(E)
68
Strata IgHV Mut IgHV Um
Treatment probability
1.00
0.75
0.50
0.25
0.00
p = 0.001
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
Time (months)

III. Lipoprotein Lipase Expression in Chronic Lymphocytic Leukemia: New Insights into Leukemic Progression

En este artículo realizamos una revisión de la literatura acerca de la lipoprotein lipasa (LPL) en la leucemia linfoide crónica (LLC). Introducimos aspectos generales de la bioquímica y biología de LPL de relevancia para comprender su posible función en la LLC. Analizamos desde una perspectiva histórica la relación del gen *LPL* con la LLC, y el descubrimiento por parte de nuestro grupo del potencial valor pronostico de su expresión, ya que los pacientes de alto riesgo con genes IgVH no mutados expresan *LPL*, mientras que los IgVH mutados de bajo riesgo no. A pesar de tener una función bien caracterizada y ser considerada una enzima central en el metabolismo de los libidos, la expresión anómala en linfocitos B tumorales plantea interrogantes, ya que los linfocitos B normales no la expresan. A pesar de que la expresión del ARNm de *LPL* tiene un valor pronostico ya bien establecido, la función que pueda tener LPL en la patogénesis de la LLC, así como los mecanismos moleculares que regulan su expresión todavía no están bien establecidos. En este artículo revisamos además el conocimiento actual acerca de la regulación de la expresión de LPL en la LLC, y la evidencia que soporta las hipótesis que plantean un rol metabólico de tipo canónico (enzimático) para LPL en el linfocito B leucémico. Asimismo, exploramos el cuerpo de evidencia emergente que apunta a una función no metabólica de LPL, y nos aventuramos a exponer las preguntas que permanecen abiertas a este respecto en la LLC, y las perspectivas que abren a la investigación futura.

molecules

MDPI

Review

Lipoprotein Lipase Expression in Chronic Lymphocytic Leukemia: New Insights into Leukemic Progression

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Abstract: Lipoprotein lipase (LPL) is a central enzyme in lipid metabolism. Due to its catalytic activity, LPL is involved in metabolic pathways exploited by various solid and hematologic malignancies to provide an extra energy source to the tumor cell. We and others described a link between the expression of LPL in the tumor cell and a poor clinical outcome of patients suffering Chronic Lymphocytic Leukemia (CLL). This leukemia is characterized by a slow accumulation of mainly quiescent clonal CD5 positive B cells that infiltrates secondary lymphoid organs, bone marrow and peripheral blood. Despite LPL being found to be a reliable molecular marker for CLL prognosis, its functional role and the molecular mechanisms regulating its expression are still matter of debate. Herein we address some of these questions reviewing the current state of the art of LPL research in CLL and providing some insights into where currently unexplored questions may lead to.

Keywords: lipoprotein lipase; chronic lymphocytic leukemia; cancer; prognostic markers

1. Lipoprotein Lipase

Lipoprotein lipase (LPL, EC 3.1.1.34) is a N-glycosylated protein [1] that forms homodimers and is able to hydrolize triglycerides from chylomicrons [2] and very low-density lipoproteins [3]. The first evidence of its existence was serendipitously found when studying circulating red blood cell mass in dogs. In those experiments, it was found that the administration of heparin as an anticoagulant was able to counteract alimentary lipemia in five minutes or less [4]. LPL plays a central function in lipid metabolism and has been subject of intense and meticulous studies ever since. General aspects of LPL biology have already been reviewed elsewhere [5,6].

LPL Synthesis and Trafficking

LPL active dimer consists of two antiparallel subunits [7] whose formation and trafficking rely on a series of post-translational modifications. Interaction with calcium-dependent chaperones of the N-glycosylated polypeptide chain has been proven essential to the correct folding of LPL [8]. Furthermore, a lipase chaperon—Lipase-maturation factor 1 (Lmf1)—has been suggested to be required for dimer assembly and activity, as mutations in *LMF1* cause lipase deficiency in mice [9]. A mouse model overexpressing *LMF1* has increased LPL activity [10], and LPL has been co-immunoprecipitated with Lmf1 and Sortilin-related receptor (SorLA) [11]. It has been shown that LPL intracellular localization is regulated by SorLA, which directs its trafficking from the trans-Golgi network to endosomes [11]. LPL internalization by receptor-mediated endocytosis has been studied [12] either through LDL receptor-related protein [13] or by an LDL-receptor independent pathway [14].

2. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia among adult populations of Caucasian origin [15]. CLL is a malignancy of mature clonal B lymphocytes that accumulate in the blood, bone marrow and other lymphoid tissues, and is diagnosed upon the presence of $\geq$5000 clonal B lymphocytes per microliter of peripheral blood persisting for more than 3 months [16]. This leukemia is characterized by the accumulation of long-lived circulating clonal leukemic B-cells resulting from a complex balance between cell proliferation and apoptotic death. Increasing evidence suggests that CLL B-cells in lymph nodes (LN) and bone marrow (BM) that interact with stromal cells receive proliferative signals and are protected from cell death. These data led to the view that CLL is a dynamic process composed of cells that also proliferate and die, often at appreciable levels [17]. This crosstalk with accessory cells in specialized tissue microenvironments favors disease progression, by promoting malignant B-cell growth and the emergence of new genetic alterations which will lead to drug resistance [18]. Disease prognosis and the heterogeneous clinical evolution in CLL are probably related at least in part to these microenvironmental signaling, and although available treatments often induce remissions, CLL remains an incurable disease [19].

In CLL one third of the patients have an indolent disease with long survival and never require treatment, another third have an aggressive disease from onset and need to be immediately treated, and the last third have an indolent disease at onset which may last for years but then invariably progress to an aggressive disease [20]. It is because of this latter group that the search for strong prognostic markers in CLL predicting disease evolution has been of capital importance, and a number of them have been developed, the most reliable and universal still being the mutational status of the variable region of the heavy chain of immunoglobulin (IgHV) genes [21,22]. Patients carrying somatic hypermutation in their IgHV genes—mutated CLL (Mut)—display a better prognosis than patients with unmutated (Um) IgHV genes

3. LPL in Chronic Lymphocytic Leukemia

3.1. LPL As a Prognostic Marker of Disease Progression

Gene expression profiling analyses comparing Um and Mut patients were performed during the first decade of the century. We and others have performed these studies and described that *LPL* is differentially overexpressed in Um patients [23–25]. With these results in mind we selected and validated two genes, *LPL* for Um and *ADAM29* for Mut CLLs, as candidates to propose a novel prognostic method. This methodology was tested in a cohort of 127 CLL patients, and correlated to clinical outcome and IgHV mutational status. Finally, we demonstrated that quantification of *LPL* and *ADAM29* gene expression ratio is a strong prognostic indicator in CLL, providing better prognostic assessment than serologic markers in advanced stages of the disease [26]. A body of evidence has confirmed that the expression of *LPL* mRNA is associated to bad prognosis, and that it is the most robust of the molecular markers in CLL [27–33].

The elevated expression of *LPL* gene in Um CLL B-cells is a very remarkable observation, because there is no expression of *LPL* in normal B cells. This specific and ectopic expression constitutes not only a suitable prognostic marker to study disease evolution, but could also be helpful to understand the heterogeneous proliferative behavior in CLL. Despite the prognostic value of *LPL* expression is well established, the functional role of *LPL* overexpression in CLL pathogenesis as well as the molecular mechanisms regulating its expression are still open questions.

Concerning the functional role of LPL in CLL cells, increasing evidence supports the idea that LPL expression could help the leukemic clone to increase survival and proliferative signaling, leading to disease progression. We have also shown that microenvironmental signaling can induce LPL expression and proliferative phenotype in primary CLL B-cells [34,35]. Supporting this idea Rozovski, Grgurevic, et al. demonstrated that LPL confers CLL a survival advantage, since shRNA knockdown of *LPL* increases apoptotic death [36]. Accordingly, it has recently been reported

that *NOTCH1* gene mutations which are associated with disease progression and treatment refractoriness [37] are directly related to *LPL* expression in CLL [38].

Concerning the molecular mechanism that regulates *LPL* expression we previously demonstrated that abnormal expression of *LPL* gene in Um CLL patients results from the lack of methylation in the CpG island involving the whole exon 1 and the first nucleotides of intron 1 of *LPL* [34]. This epigenetic mechanism appears to be mainly triggered by proliferative T-cell-dependent signals and, in some patients, through the cross-linking of the B-cell receptor (BCR). By contrast, signaling through TLR9 or TLR1/2 pathways are unable to induce demethylation of the CpG island, *LPL* expression and B-cell proliferation [35]. Rozovski, Grgurevic, et al. have shown that *LPL* expression can also be transcriptionally regulated by STAT3 phosphorylation, and nuclear translocation where it can bind *LPL* promoter [36]. Additionally, it is necessary to mention that *LPL* expression can be regulated post-transcriptionally by miR-29 [39,40]. It has been reported that miR-29 expression is down-regulated in high-risk Um CLL patients [41–44]. In a more recent study of the microRNAome of a large patient cohort, down-regulation of miR-29c was the feature better related to IgHV Um profile [45]. In fact, Santanam et al. have developed a mouse model of early onset indolent CD5+ B-CLL by targeted overexpression of miR-29 in B-lymphocytes under control of the Eμ enhancer [46]. The authors focused on the effect on leukemogenesis by the interaction of miR-29 and *TCL1* [44,47] and did not evaluate *LPL* expression, which would be expected to be low. Deregulation of miR-29 is known to have important effects in diverse hematological disorders (reviewed in [48]), to respond to cellular signaling processes such as BCR or CD40 stimulation, and to engage NF-κB activation through TCL1 [47]. Linking these microenvironmental signaling to the epigenetic changes described by us in Um patients as well as their correlation with miR-29 and *LPL* expression could be an interesting issue that is still awaiting to be studied in CLL progression.

3.2. LPL in CLL B-Cell Metabolism

LPL has been shown to mediate lipolysis and subsequent fatty acid (FA)-mediated fueling of cell proliferation in several solid tumors [49], and it has recently been shown that low-density lipoproteins may enhance proliferative responses of CLL cells to inflammatory signals [50]. PPARα protein levels in CLL B-cells have been shown to correlate with leukocytosis and clinical Rai stages, which suggests a metabolic switch to oxidation of fatty acids via PPARα [51] and PPARδ [50]. These findings are supported by the observation that CLL B-cells have lipid vacuoles in their cytoplasm, and that incubation with free fatty-acids (FFAs) increased their metabolic rate in terms of oxygen consumption [36]. Furthermore, the incidence of hyperlipidemia has been found to be higher in CLL patients, and treatment of hyperlipidemia with statins benefited them in terms of a delayed time to first treatment [52]. The same group expanded their initial study to a cohort of >2000 CLL patients in a retrospective analysis and found that both lipid-lowering drugs, as well as statin treatment prolonged overall survival by 3.7 years [53]. These findings suggest that a second mechanism mediated by LDL may be converging in STAT3 phosphorylation and generating an activated state in CLL B-cells [50].

Transcriptional profiling identified a metabolic shift into a muscle or adipose tissue-like strategy with lipid oxidation in poor prognosis Um IgHV and *LPL* expressing B CLL cells [54]. How this metabolic reprogramming ends up in a worse outcome for patients is only beginning to be understood. Long chain fatty acids, free cholesterol and vitamin E- increase STAT3 phosphorylation directed either by IL-10, IFNα or phorbol esters in CLL cells [50]. STAT3 phosphorylation in turn drives *LPL* expression directly, by binding to a GAS-like element 280 bp upstream of the *LPL* transcription start site and activating its transcription [36]. LPL expression favors FA oxidation, and this seems to result in higher cell survival as *LPL* knockdown or chemical inhibition reduced CLL cell viability [36,55], which might be explained in part by a transcriptional response [32]. Accordingly, microenvironmental induction of *LPL* expression stimulates CLL cell proliferation [35]. These findings indicate that *LPL* expression can be regulated by the microenvironment, either by autocrine or paracrine signaling and that it reflects a metabolic switch in CLL B-cells which confers an adaptive advantage. A positive

feedback loop may maintain *LPL* expression and worsen the scenario for Um patients. In CLL, STAT3 is constitutively activated which also activates *LPL* transcription [36]. LPL breaks down very low-density lipoproteins (VLDL) and chylomicrons and liberates FFAs, generating a proinflammatory state which in turn activates STAT3 [51] and further activation of *LPL* transcription. This would further increase the levels of FFAs, thus exacerbating CLL cells responsiveness to cytokine signaling. More general aspects of metabolic pathways in CLL have been nicely reviewed recently [56].

3.3. Non-Metabolic Roles of LPL in CLL

Many studies have reported an increased expression of *LPL* in poor prognosis CLL, and several metabolic pathways could be involved in cancer progression as discussed above. However, attempts to determine metabolic activity of LPL directly have failed to correlate higher expression to higher metabolic activity. A seminal study with 33 CLL patients reported lower catalytic activity in Um patients than in their Mut counterpart [30]. Another report analyzing data from 42 patients did not find differences between CLL groups and reported that LPL activity was comparable to that of healthy individuals [32].

LPL can mediate lipoprotein uptake by cells [57], chylomicron attachment to cell surface through LDL-related receptor [58], and lipoprotein margination in small blood vessels, by binding on the one hand to the extracellular surface of endothelium via GPIHBP1, and on the other to triglyceride-rich lipoproteins [59]. Besides its canonical role in lipid metabolism, an interesting—yet quite unexplored—non-metabolic function of LPL has been known for 20 years. LPL can act as a bridging molecule between cells, as in the adhesion of monocytes to endothelial cells mediated by heparan sulfate proteoglycans (HSPGs) and LPL [60], whose interaction has recently been shown to be dynamic [61]. Provided that CLL cells display HSPGs on their surface [62] and that LPL forms homodimers, it could occur that a bridging between leukemic B-cells and other cells expressing surface HSPGs or GPIHBP1 such as endothelial cells would be mediated by LPL. Although several groups have already speculated about it, a cell–cell bridging role for LPL in CLL pathogenesis still has to be demonstrated [30,35,63]. If such a bridging actually occurred, LPL would be pivoting between surface HSPGs on the B-CLL cell side, and either HSPGs or GPIHBP1 on their counterpart.

Rombout et al. have found that two SNPs commonly found in *LPL*, rs328 (premature stop codon) and rs13702 were significantly associated with CLL outcome [63]. Although both SNPs are well-known gain-of-function mutations [64,65], the authors of the aforementioned study reported not to have been able to detect significant differences in LPL mRNA, protein levels, or enzymatic activity in patients carrying the SNPs [63]. How these mutations affect clinical outcome in CLL is still unclear, but whether these SNPs might have a role—if any—in LPL non-metabolic functions has not been explored yet. Furthermore, at least nine isoelectric point isoforms of LPL have been described in human blood of healthy individuals [66], thus opening a new dimension of studies to come for LPL in CLL and other pathologies.

4. Concluding Remarks

LPL is a protein located on the luminal side of the blood vessel wall, where it is anchored to heparan sulfate proteoglycans and contains binding sites for both heparan sulfate chains and apoproteins [67]. *LPL* is overexpressed in B-cells of unmutated IgHV CLL patients, and its expression can be used to predict their clinical outcome [23–33]. Accordingly, LPL could have a bridging function in the formation of a trimolecular complex including a lipoprotein particle, LPL and heparan sulfate proteoglycans from different cells [67]. The fact that CLL B-cells display heparan sulfate proteoglycans on their surface [62], invites to speculate about whether LPL localization on the cellular membrane could affect the biological behavior of CLL cells, by favoring cell spreading, migration and intracellular signaling following activation of the tumoral clone by an activated microenvironment. If it is the case, LPL might also act as a crosstalk factor facilitating specific interactions with accessory cells in tissue microenvironments. LPL might then be added to the list of proteins implicated in the activation

of CLL proliferative pool together with integrins such as CD49d, metalloproteinases (MMP-9), antiapoptotic molecules (BCL2) as well as chemokines (CCL3, CCL4, CXCL12) [68,69]. Thus, LPL could be contributing to leukemic progression either per se through metabolic reprogramming, or through the synergistic contribution to an activating microenvironment in which the leukemic clone is continuously nourished (Figure 1).

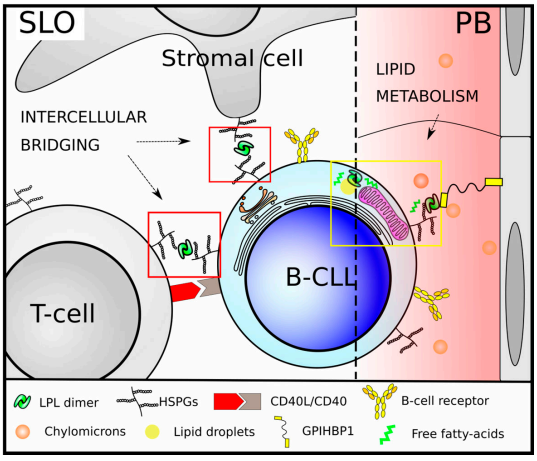

Figure 1. Hypothetical model of LPL function in CLL B-cells in secondary lymphoid organs (SLO, left) and peripheral blood (PB, right). HSPG-attached LPL molecules at the surface of B-CLL cells can bind very low-density lipoproteins and chylomicrons thus contributing to oxidative metabolism and fatty-acid signaling. LPL has been proposed to play a similar role in the intracellular compartment by releasing FFAs from cytosolic lipid droplets [36,56]. A non-canonical role for LPL in CLL B-cell surface would contribute to microenvironmental crosstalk. LPL would act as a bridging molecule between cells able to bind LPL either by heparan sulfate proteoglycans or GPIHBP1, thus facilitating modulatory interactions, exemplified here by a T-cell dependent activation through CD40/CD40L interaction.

The role that abnormal *LPL* expression could have in disease evolution, has also been addressed by previous work from Pallasch et al., demonstrating that lipase associated genes and triglyceride-specific lipase activity were significantly increased when comparing CLL B-cells to normal CD5+ B-cells [55]. The same authors reported that incubation of CLL tumoral cells with the lipase inhibitor orlistat resulted in increased apoptosis, which, could suggest that lipid metabolism and lipase activity could be functionally relevant in aggressive CLL [55]. Phenotypic analyses have shown that CLL B-cells expressing *LPL* are also enriched in FA degradation genes [54]. Recently, LPL has been shown to mediate lipolysis and subsequent FA-mediated fueling of cell proliferation in several solid tumors [49], and it has recently been shown that low-density lipoproteins may enhance proliferative responses of CLL cells to inflammatory signals [50].

A big amount of information is known nowadays about LPL some of which relates to CLL. Still, our understanding whether *LPL* overexpression in poor outcome CLL is a cause or consequence is poor. Many questions are still open and more answers will certainly come in next years.

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Discusión

La leucemia linfoide crónica (LLC) es la leucemia más frecuente en poblaciones adultas de origen caucásico. Se caracteriza por una acumulación lenta y sostenida de linfocitos B clonales en sangre periférica y órganos linfoides secundarios, que se entiende ocurre a través de un desbalance entre la proliferación celular y la muerte por apoptosis. Actualmente se considera que el enjambre de señales entre las células tumorales y su microambiente son esenciales para el mantenimiento del clon maligno, su proliferación, y la colonización de nuevos ambientes dentro del organismo, así como para lograr la evasión del sistema inmunitario. En nuestro grupo hemos descrito una población tumoral que es capaz de proliferar en respuesta señales microambientales (CD40L e interleukina 4) por medio de la regulación del microRNA-22 que activa la vía PI3K/Akt, en cuyo trabajo he participado, pero que quedan por fuera del marco de esta tesis (ver ANEXOS).

I- S100-A9 protein in exosomes from chronic lymphocytic leukemia cells promotes NF-κB activity during disease progression Actualmente, la importancia del microambiente tumoral en la progresión de la enfermedad es ampliamente reconocido. La interacción entre linfocitos B leucémicos y células accesorias en microambientes tisulares especializados dentro de la médula ósea o de los órganos linfoides secundarios, especialmente el ganglio linfático, favorecen la progresión de la enfermedad promoviendo la expansión del clon tumoral. En este contexto, la quimioterapia provee una presión de selección para su evolución clonal en los nichos proliferantes, con el desarrollo de quimioresistencia. Comprender la función de las moléculas implicadas en esa interacción célula tumoral y microambiente nos puede proporcionar nuevos caminos hacia el desarrollo terapéutico en la LLC. En los últimos años el papel de las vesículas extracelulares, y en particular de los exosomas en el juego de señales de la célula tumoral y su microambiente ha ido creciendo en importancia, a medida que va siendo mejor comprendido. Hoy se sabe que los exosomas pueden modular el nicho tumoral, suprimir respuestas inmunitarias contra el tumor, favorecer la aparición de quimio- y radioresistencia de novo, y precondicionar y sostener los nichos metastásicos. La preponderancia de la señalización mediada por exosomas como moduladores del microambiente tumoral, y la posibilidad de que la misma pudiera tener un rol en la progresión de la enfermedad nos llevo a plantearnos las siguientes preguntas:

1- ¿Cuál es el perfil proteico de los exosomas circulantes en el plasma de pacientes con LLC?
2- ¿Hay diferencias en el perfil proteómico de los exosomas de plasma de pacientes de LLC con enfermedad progresiva e indolente?
3- ¿Es posible que la carga de lo exosomas de pacientes con LLC progresiva

cambie con la evolución de la enfermedad?
4- ¿Existen cargas exosómicas exclusivas de la progresión de la LLC?
5- ¿Es posible que las proteínas que se encuentren exclusivamente en exosomas de progresión de la LLC puedan promover la proliferación del clon tumoral?

Para responder estas preguntas caracterizamos el perfil proteómico de los exosomas de plasma de pacientes con LLC progresiva e indolente al tiempo del debut y a tiempos de progresión, en el caso de pacientes con enfermedad progresiva, y a un tiempo de 4 años de sobrevida libre de progresión en pacientes con enfermedad indolente. El análisis de las redes de interacción proteica nos permitió identificar proteínas asociadas a vías de inflamación, progresión tumoral, infiltración de células leucémicas y sobrevida celular asociadas a las proteínas identificadas en pacientes con LLC progresiva al tiempo de la progresión. De esta caracterización obtuvimos sobrerepresentadas de forma consistente dos proteínas exclusivas de los exosomas de tiempos de progresión, plakoglobina (JUP), un activador de la señalización por Wnt, y S100-A9, un activador de la vía NF-κB, cuya presencia validamos mediante western blot. Debido a la importancia de la vía NF-κB, y al hecho de que S100-A9 había sido recientemente identificada en exosomas de pacientes con IgVH no mutados (Paggetti et al. 2015), decidimos investigar la función de S100-A9 en la progresión de la LLC. S100-A9 pertenece a una familia de proteínas intracelulares de unión a calcio cuyo rol en el cáncer esta ampliamente documentado, y revisado en (Bresnick, Weber y Zimmer 2015). Se encuentra fundamentalmente formando heterodímeros con S100-A8, constituyendo la calprotectina que posee sus propias funciones (Srikrishna 2011). Tanto la calprotectina (S100-A8/A9) como S100-A9 sola son activadores de NF-κB, pero lo hacen a través de vías diferentes. La calprotectina lo hace a través de estimulación del receptor TLR4, o de los receptores RAGE (receptor of advanced glycosylated end-products), que pueden ser activados por varias proteínas de la familia. S100-A9 en tanto, lo hace a través de su receptor especifico EMMPRIN (CD147, basigin-1), que cumple un papel importante en la metástasis del melanoma (Hibino et al. 2013). Por este motivo decidimos investigar si el receptor de S100-A9 EMMPRIN era expresado en las células de LLC, pudiendo entonces actuar como mediador de la señalización por exosomas durante la progresión de la enfermedad. Así, descubrimos que la isoforma 2 de EMMPRIN se expresaba y era la mayoritaria en LLC. Además, encontramos que los linfocitos B leucémicos son capaces de unir in vitro a S100-A9 recombinante, y que su localización en la membrana celular coincide con regiones en donde se localiza EMMPRIN,

sugiriendo que podría actuar como receptor de S100-A9 en LLC. Se ha descrito que S100-A9 es esencial en el establecimiento del nicho pre-metastásico, en la adquisición de quimioresistencia de novo, y subsecuente metástasis en cáncer de mama (Bresnick, Weber y Zimmer 2015) y melanoma (Peinado et al. 2012), y es necesaria para el reclutamiento de las células mieloides supresoras (MDSCs) y supresión de la respuesta inmune antitumoral (Cheng et al. 2008). En LLC se sabe que las células leucémicas son capaces de inducir MDSCs, que a su vez suprimen la activación de linfocitos T, y de promover células T regulatorias inmunosupresoras (Jitschin et al. 2014). La presencia de S100-A9 en exosomas plasmáticos de LLC durante la progresión de la enfermedad podría explicar este reclutamiento de MDSCs, y trazar su origen a la célula B leucémica. Además hemos demostrado que los exosomas cargados de S100-A9 son capaces de activar en el clon leucémico la vía canónica de NF-κB, y que la preincubación de los exosomas con anticuerpos anti S100-A9 bloquea esta activación. Estos resultados, refuerzan los hallazgos de Paggetti y cols. que mostraron que los exosomas producidos por la linea celular MEC-1 (derivada de un paciente de LLC) eran capaces de activar NF-κB en células estromales (Paggetti et al. 2015). En melanoma se ha descrito un loop de activación, en que los exosomas son capaces de inducir la expresion de calprotectina (S100-A8/A9) y S100-A9 en el nicho pre-metastásico luego de la activación de NF-κB, que a su vez se activa por calprotectina y S100-A9. Nuestros resultados sugieren que en LLC podría existir un loop análogo, en que las señales del microambiente estimulan la expresión de S100-A9 en el clon tumoral, que a su vez activa NF-κB llevando así a un incremento de las señales de proliferación y sobrevida. Esta hipótesis se sustenta en nuestro análisis de células de ganglios linfáticos de LLC, en que las células B expresan S100-A9 y marcadores de proliferación. Este loop de hipotético de activación del clon leucémico a través de S100-A9 en LLC se resume en la Figura 3. En suma, hemos descrito por primera vez distintos perfiles temporales en las proteínas de exosomas de plasma de LLC indolentes y progresivas. Asimismo, hemos descrito las variaciones individuales al tiempo del debut y de la progresión leucémica. Describimos la presencia de la proteina S100-A9 en los exosomas de LLC progresivas, y un aumento de su expresión en linfocitos leucémicos durante la progresión de la enfermedad. Por último, demostramos que los exosomas cargados de S100-A9 pueden activar NF-κB facultativamente en células de pacientes con enfermedad típicamente indolente, estableciendo un nuevo origen para la activación de NF-κB en la LLC, y destacando la importancia de los exosomas en la promoción de la progresión leucémica.

II- *Unmutated CLL cells express more LPL protein than mutated cells and it is associated with a poor clinical outcome.*

La manifestación y curso clínico de la LLC son heterogéneos (Vasconcelos et al. 2003), aunque la casuística permite identificar tres tercios con cursos diferentes. Un primer grupo debuta con una enfermedad agresiva y requiere tratamiento inmediato; un segundo grupo presenta una enfermedad indolente y no requiere tratamiento; pero el tercer grupo, que representa alrededor de un tercio de los pacientes debuta con una enfermedad indolente que puede durar años, pero que invariablemente evoluciona hacia un enfermedad agresiva que debe ser tratada (Dighiero 2003). La existencia de este último grupo ha motivado la búsqueda de buenos marcadores clínicos y biológicos con valor pronóstico para la LLC. Hoy en día, el marcador con mejor valor pronóstico es el estado mutacional de los genes de las cadenas pesadas de inmunoglobulina del clon tumoral. Este análisis requiere la utilización de técnicas de biología molecular, que impiden su universalización e inclusión en la rutina clínica de la LLC. Los análisis de perfil de expresión génica comparando grupos de pacientes de LLC demostraron que el gen que codifica la lipoprotein lipasa (LPL), es expresado de forma diferencial en pacientes mutados y no mutados (Klein et al. 2001; Rosenwald et al. 2001; Vasconcelos et al. 2005). Como resultado de estudios posteriores, se estableció que la relación existente entre la expresión de LPL y el gen de la metaloproteasa ADAM29 constituía un marcador pronóstico mejor discernimiento que los marcadores serológicos en estadios avanzados de la enfermedad (Oppezzo et al. 2005). La presencia del ARNm de LPL esta asociada a un mal pronóstico, y es el marcador molecular más robusto en la LLC después del perfil IgVH(resisado en (Prieto y Oppezzo 2017)). Se sabe que la proteína LPL se expresa en algunas células de LLC, y que posee un patrón de inmunolocalización puntiforme, lo que motivó el intento de desarrollo de un método pronóstico basado en la proteína LPL que lamentablemente fracasó, concluyendo que los niveles de proteína presentes en las células de LLC no reflejaban los niveles de su ARNm (Heintel et al. 2005). Este fenómeno no es improbable, dado que LPL posee múltiples niveles de regulación de su expresión, desde epigenético (Abreu et al. 2013), postranscripcional incluyendo microARNs (Bouvy-Liivrand et al. 2014), y postraduccional dependiente de chaperonas (Klinger et al. 2011). Debido a la relevancia de los marcadores con valor pronóstico en la LLC y su creciente consideración en la decisión terapéutica, nos propusimos trabajar en función de las siguientes preguntas:

1- ¿Qué patrón de expresión tiene la proteína LPL en la LLC?

2- ¿Existen diferencias en los niveles de proteína LPL entre pacientes IgVH mutado y no mutado?

3- ¿Por qué el grupo que lo intentó previamente no logró detectarlas?
4- ¿Se puede desarrollar un método pronóstico basado en la proteína LPL para LLC?

Para contestar estas preguntas, caracterizamos el patrón subcelular de expresión de LPL en células de LLC de pacientes que expresaban el ARNm de LPL (LPL/mRNApos) y de pacientes que no lo expresaban (LPL/mRNAneg). Utilizando inmunomarcación y microscopía de barrido láser confocal logramos repetir el patrón puntiforme previamente descrito, y precisar que todos los pacientes presentaban marcación de LPL independientemente de expresar su ARN, aunque su distribución subcelular parecía ser diferente. En una tinción doble con un marcador general de membranas observamos que en los pacientes LPL/mRNApos había dos tipos de localización de la proteína LPL: una perinuclear asociada a sistemas de membrana, y otra de naturaleza citosólica. En cambio, los pacientes LPL/mRNAneg solo encontramos la segunda población. Este inesperado resultado nos llevo a profundizar en la localización subcelular utilizando marcadores específicos de organelos, con los que pudimos determinar que el pool de LPL perinuclear asociado a membranas se localiza en la cisterna perinuclear del retículo endoplásmico, lo que coincide con un patrón de síntesis proteica de LPL en los pacientes LPL/mRNApos. Mientras tanto, la población observable como citosólica coincide parcialmente con vesículas de la vía endocítica (endosomas tardíos o lisosomas). Estos resultados fueron confirmados por inmunomicroscopía electrónica. Este segundo resultado, sumado al primero en que observamos marcación de LPL en células LPL/RNAneg nos llevó a preguntarnos si sería posible que las células de LLC estuvieran internalizando LPL del exterior, ya que LPL es una proteína que se encuentra asociada al endotelio vascular y a los quilomicrones y lipoproteínas de muy baja densidad circulantes. Esta idea se ve reforzada por el hecho de que las células de LLC poseen en su superficie proteoglicanos de heparán sulfato (Van Bockstaele et al. 2007), lo que les permitiría captar LPL desde el exterior celular. Para poner a prueba la hipótesis realizamos experimentos in vitro, en donde cultivamos a los linfocitos de LLC en un medio completamente definido libre de suero, y paralelamente con plasma autólogo o suero fetal bovino. Esta serie de experimentos nos permitió descubrir que las células de LLC son capaces de internalizar LPL desde el plasma o heterogenéamente desde el suero fetal bovino, y de enviar esa LPL internalizada a la vía endocítica. Esta información nos permitió pensar en una estrategia para potenciar las diferencias en los niveles de LPL entre las células de pacientes LPL/RNApos y LPL/RNAneg. Para reducir el aporte de señal de LPL de la superficie

celular recurrimos a la incubación con heparina en el medio de cultivo, ya que la proteína posee un sitio de unión a heparina que puede competir con la unión a proteoglicanos de heparán sulfato (Allan et al. 2017). El tratamiento con heparina para liberar LPL de sus sitios de unión se conoce desde la década de 1940, en que se descubrió que la inyección de heparina en perros era capaz de contrarrestar la lipemia alimentaria, y motivó el descubrimiento de la LPL (Hahn 1943). El tratamiento con heparina y la utilizacion de células de frescas nos permitió desenmascarar las diferencias de LPL entre los pacientes LPL/RNApos y LPL/RNAneg, y desarrollar un protocolo de marcación de LPL para citometría de flujo que resultó capaz de discriminar entre pacientes IgVH mutado y no mutado. La explicación más plausible para este fenómeno es que las células que forman parte del banco celular habitualmente son congeladas con grandes cantidades de suero fetal bovino, cargado de LPL, que las células de LLC son capaces de internalizar de forma heterogénea. Así, la utilización de células frescas más el tratamiento con heparina maximiza las diferencias entre los grupos de pacientes, constituyendo una posible estrategia para el desarrollo de un marcador pronóstico basado en la proteína LPL para citometría de flujo. Posteriormente validamos estos resultados en una cohorte mayor, y determinamos el punto de corte optimo para mayor sensibilidad del método. Con este punto de corte determinamos que el método puede predecir el tiempo al primer tratamiento diferenciando significativamente pacientes IgVH mutados y no mutados.

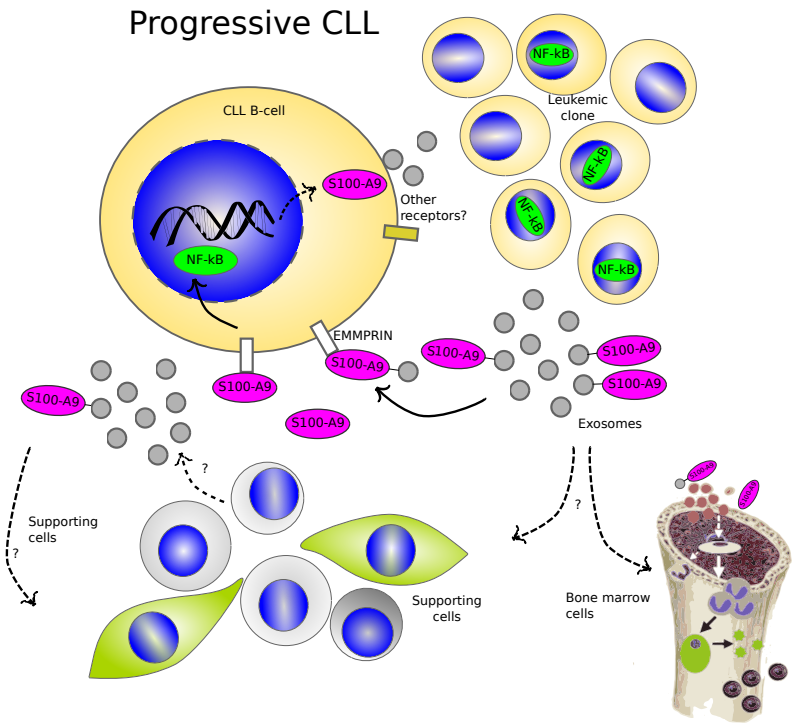

Figura 3: Modelo hipotético del bucle de activación microambiental y autoactivación del linfocito B de una LLC progresiva mediada por S100-A9. Inicialmente, las señales del microambiente tales como estimulación por antígeno, citoquinas pro-inflamatorias y otras, up-regulan los factores de transcripción NF-κB y/o AP-1 en el clon leucémico y estimulan la expresión de S100-A9. Subsecuente mente, la expresión elevada de S100-A9 en las células de LLC podría resultar en la secreción de exosomas cargados de S100-A9. Estos exosomas pueden interaccionar tanto con células accesorias del sistema inmunitario, o con los propios linfocitos tumorales estableciendo un loop de activación que aumenta la actividad de NF-κB y favorece el progreso de la enfermedad.

Perspectivas

Durante el presente trabajo de tesis hemos aportado al conocimiento básico de la LLC y hemos desarrollado una herramienta para ser validada en la clínica hematológica, que esperamos redunde en beneficio para los pacientes. Sin embargo, durante este trabajo hemos generado también un cuerpo de datos que por razones de tiempo y foco no hemos podido explorar en profundidad. Estos datos abren un abanico de perspectivas para el desarrollo de futuros trabajos de investigación en la biología de la LLC o de otras neoplasias hematológicas. La caracterización proteómica de las cargas de exosomas durante la progresión de la LLC nos permitió identificar la proteína JUP como otro posible candidato a funcionar como señal microambiental que favorezca la progresión tumoral. Investigar su papel en el escenario del nicho protumoral es una tarea inminente. Asimismo, la vía de señalización activada en el linfocito B de LLC por S100-A9 a través de EMMPRIN necesita ser caracterizada. Durante nuestro trabajo centramos la atención en EMMPRIN por ser un receptor especifico de S100-A9 y por sus amplias implicaciones en el cáncer. Sin embargo, queda por determinar cuál es el peso relativo de la señalización por EMMPRIN en el concierto de receptores que pueden unir a S100-A9, y en particular con respecto al receptor RAGE, que también puede unir calprotectina (S100-A8/A9). Sabemos que algunas señales del microambiente son capaces de activar la expresión de LPL en los linfocitos B leucémicos, pero no hemos explorado aun si las células tratadas in vitro con S100-A9 recombinante o con exosomas cargados de S100-A9 activan la expresión de LPL. Se ha descrito que la perdida parcial de función de LPL mediante interferencia de ARN en células de LLC es capaz de reducir la viabilidad de las mismas en cultivo. Sin embargo, no se ha intentado el experimento inverso, que es esencial para demostrar la importancia del mecanismo: inducir la expresión de LPL en células que normalmente no la expresan de LLC o lineas derivadas (como MEC-1) y registrar un aumento de la viabilidad en cultivo. Hemos dicho que una función atractiva y prácticamente inexplorada de LPL es la capacidad de actuar como puente intercelular, por lo que la realización de experimentos de interacción celular en cultivos organotípicos de ganglio linfático resulta tentadora y necesaria. Las recientemente descritas isoformas de punto isoeléctrico de LPL hacen inminente la caracterización de las mismas en la LLC. Finalmente, en un abordaje traslacional, la posibilidad de analizar los parámetros del ciclo celular, carga de LPL celular, y la evolución clínica de pacientes de LLC clínicamemte miniheparinizados, o tratados con heparina de bajo peso molecular constituyen desafíos científicos que seguramente serán abordados en el futuro próximo.

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Anexos

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ORIGINAL ARTICLE

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

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Chronic lymphocytic leukemia (CLL) is characterized by accumulation of clonal B cells arrested in G0/G1 stages that coexist, in different proportions, with proliferative B cells. Understanding the crosstalk between the proliferative subsets and their milieu could provide clues on CLL biology. We previously identified one of these subpopulations in the peripheral blood from unmutated patients that appears to be a hallmark of a progressive disease. Aiming to characterize the molecular mechanism underlying this proliferative behavior, we performed gene expression analysis comparing the global mRNA and microRNA expression of this leukemic subpopulation, and compared it with their quiescent counterparts. Our results suggest that proliferation of this fraction depend on microRNA-22 overexpression that induces phosphatase and tensin homolog downregulation and phosphoinositide 3-kinase (PI3K)/AKT pathway activation. Transfection experiments demonstrated that miR-22 overexpression in CLL B cells switches on PI3K/AKT, leading to downregulation of $p27^{-Kip1}$ and overexpression of Survivin and Ki-67 proteins. We also demonstrated that this pathway could be triggered by microenvironment signals like CD40 ligand/interleukin-4 and, more importantly, that this regulatory loop is also present in lymph nodes from progressive unmutated patients. Altogether, these results underline the key role of PI3K/AKT pathway in the generation of the CLL proliferative pool and provide additional rationale for the usage of PI3K inhibitors.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is characterized by accumulation of clonal mature B lymphocytes.[1] Recent evidence indicates that disease evolution could depend on the relative balance between an accumulative pool (quiescent fraction (QF)) and a smaller proliferative fraction (PF).[2] Proliferative events occur in lymph nodes (LN) and bone marrow (BM), where leukemic cells undergo favorable proliferative conditions through interaction with the microenvironment.

As several endogenous stimuli in CLL microenvironment were shown to activate different kinases, a variety of novel kinase inhibitors have been designed. In the case of CLL, these mainly include inhibitors of phosphoinositide 3-kinase (PI3K) and Bruton tyrosine kinase that have generated significant promise.[3,4] Moreover, microRNAs play a key role in the pathogenesis and progression of human cancers.[3,5] In CLL, a microRNA signature was found to be associated with prognosis and disease progression.[4]

QF and PF differ in terms of expression of several molecules, including CCL22,[5] CCL3 or CCL4 chemokines, CD38 (ref.7) and CD49d[8] progression markers, apoptosis regulators like Survivin[9] or proliferation markers such as Ki-67 and c-myc[10] as well as the $p27^{-Kip1}$ (p27), a cell cycle regulator.[10–11] Different CLL proliferative fractions have been isolated and characterized by studying different molecule markers like CD38 (ref.12) or CD5/CXCR4 molecules.[13] We first reported that leukemic cells obtained from peripheral blood (PB) of progressive unmutated (UM) cases express high levels of the mutagenic enzyme activation-induced cytidine deaminase (AID).[14] As its expression results from signals received in activated secondary lymphoid organs, we investigated whether tumoral cells expressing AID in PB correspond to proliferative cells recently egressed from proliferating centers.[14] Our results showed that AID expression is mainly restricted to a CLL subset ongoing class switch recombination process and, interestingly, that the presence of this subpopulation was associated with proliferative and anti-apoptotic expression markers and correlated with a poorer clinical outcome.[10,15]

In this work, we characterized the genomic expression profile (GEP) of the PF and QF in the same patients and compared the global mRNA and microRNA expression between both subsets. Our results suggest that the proliferative behavior of this subpopulation appears to depend on microRNA-22 overexpression that, following phosphatase and tensin homolog (PTEN) downregulation, results in activation of the PI3K/AKT pathway. Finally, we propose that a similar regulatory loop could be present in the leukemic clone in proliferating centers of progressive UM CLL patients.

MATERIALS AND METHODS

Patient samples

PB was obtained from 22 patients with a typical CLL diagnosis (Matutes score of 4 or 5).[16] Patients were segregated into the following. (1) The progressive group (12 patients) defined by UM immunoglobulin V_H gene

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(IgV_H) profile, AID and lipoprotein lipase expression at the mRNA level and presence in PB of at least 2% of CLL B cells ongoing class switch recombination process to IgG, as previously shown by us.[10] This progressive group was characterized clinically by highly progressive disease, rapid lymphocyte doubling time (1 year), lymphocytosis > to 50 000/mm^3 and FCR treatment indicated before 3 years after CLL debut. Of these 12 patients, 4 (05, 06, 07 and 11 in Table 1) were studied following fludarabine and cyclophosphamide treatment (FC), at the moment of relapse. For the other 8 patients, the sample was obtained previous to any treatment. (2) The indolent group (10 patients) included mutated VH cases, negative for lipoprotein lipase and AID molecular markers and absence of IgG subset in PB. Regarding clinical data, these cases displayed a low lymphocytosis, low lymphocyte doubling time (> 2 years) and absence of treatment requirement after 5 years. Clinical and molecular characterization of all these patients is depicted in Table 1. LN samples were obtained from 3 UM CLL patients (numbers 07, 08 and 12, 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.

MicroRNA and mRNA array

Microarray procedures. Total RNA used to perform the array for mRNA and microRNA on CLL B cells was isolated from 0.5–1 × 10^6 cells using mirVana isolation kit (Applied Biosystems, Carlsbad, CA, USA). The mRNA analysis was performed using a 4 × 44K Whole Human Genome Oligo-Microarray (G4112F, Agilent Technologies, Inc., Santa Clara, CA, USA), in a two-color design interrogating 41 000 unique human genes. Upregulated and downregulated genes (showing $P<0.01$) in PF relative to QF were used in an ontology analysis. Four biological dye swap replicates were performed. Microarray for miRNA analysis was performed using a 8 × 15K Human miRNA Microarray (G4470B, Agilent Technologies, Inc.), one-color platform containing 470 human miRNA probes according to the manufacturer's guidelines.

Microarray data analysis. Data analysis was accomplished using 'R'/Bioconductor (webmaster@bioconductor.org; Hosting provided by Fred Hutchinson Cancer Research Center).[17] Probes were flagged for filtering considering saturation, signal above background and uniformity. For microRNA and mRNA analysis, probes that had more than one replicate flagged were eliminated. Arrays were background corrected using normexp. Signal intensity was standardized across arrays via quantile normalization. Normalization was performed within and between arrays using Loess and Aquantile methods, respectively. Differential expression was assayed using the limma software and the ontology analysis conducted with GOHyperGAll function. Arrays analyses are available in GEO database (accession number GSE53270).

The mRNA and microRNA analysis expression by reverse transcription-PCR and quantitative PCR (Q-PCR). RNA isolation, reverse transcription-PCR and Q-PCR were carried out as previously described.[10] Q-PCR evaluating fold change expression of mRNAs was performed in CLL samples (01–08 of Table 1) in order to confirm the GEP. For microRNAs, PCR analyses were performed as previously described.[18] Array studies relating microRNA expression levels were assessed in four additional cases (04–08 CLLs in Table 1) to the samples used in the GEP study (01–04 in Table 1). The primers used for all reactions are provided in Supplementary Table S1.

Cell sorting studies and flow cytometry analysis

Isolation of CLL peripheral blood mononuclear cells from PB and LN, cytometry analysis and sorting experiments using the MoFlo cell sorter (Beckman Coulter, Buenos Aires, Argentina) were performed as previously described.[10] The antibodies used are reported in Supplementary Table S2. In the case of the 12 progressive CLL patients, two fractions were isolated: one expressing exclusively membrane IgM (QF) and a minority fraction expressing either IgM plus IgG or exclusively IgG (PF). Purity of isolated subpopulations assessed by flow cytometry was >98%. Isolated subsets were also evaluated for CD5 and Ki-67 expression markers, AID mRNA expression and clonal IgV_H rearrangement.

Confocal microscopy

This study was performed as previously described.[19] Briefly, after incubation for 1 h at room temperature with mouse anti-human PTEN (1:20), rabbit anti-FOXO1a (1:100), goat anti-Survivin (1:100) or mouse anti-p27 (1:1600), washings were performed and incubation was then proceeded with anti-mouse Alexa 488 (1:2000), anti-rabbit Alexa 546

Table 1. Clinical and molecular characterization of CLL patients

CLL	*Diagnostic date / Binet stage*	*Sample date/ Binet stage*	*Lymphocyte count at sampling x 10^3/μl*	*VDJ rearrangement*[a]	*FISH*[b]	*CD38%*[c]	*LPL (Q-PCR)*	*AID (Q-PCR)*	*$IgG^{(+)}$ subset (%)*[d]	*$IgG^{(+)}$/ $Ki\text{-}67^{(+)}$ subset (%)*	*Disease-related death*
01	2002/A	2003/C	128	Um - VH1–02	del 11q	38	(+)	(+)	5.2	3.9	Yes
02	2000/B	2010/B	220	Um - VH1–69*01	del 17p	16	(+/−)	(+)	3.2	3.0	Yes
03	2009/A	2009/A	54	Um - VH2–5*10	Normal	46	(+)	(+)	3.8	3.0	Yes
04	2005/A	2010/B	72	Um - VH1–69*01	Normal	19.5	(+)	(+)	4.1	2.5	Alive
05	2009/A	2011/B	140	Um - VH2–5*10	del 17p	36	(+/−)	(+)	3.3	1.5	No
06	2008/B	2010/B	170	Um - VH3–48*03	del 11q	22.5	(+)	(+)	5.0	3.2	Yes
07	2010/B	2010/B	130	Um - VH3–30*04	Tris 12	45.5	(+)	(+)	3.7	3.0	Yes
08	2011/B	2011/C	48	Um - VH3–11*01	Normal	1.9	(+/−)	(+)	2.5	1.7	Yes
09	2011/A	2011/A	105	Um - VH1–2*02	Normal	2.8	(+)	(+)	2.4	0.5	Alive
10	2012/A	2012/A	130	Um - VH1–69*01	Normal	46.5	(+)	(+/−)	3.5	0.3	Alive
11	2012/B	2012/B	160	Um - VH1–69*01	Normal	26.2	(+)	(+/−)	2.2	0.7	Alive
12	2005/A	2012/C	98	Um - VH4–34	Normal	3.9	(+)	(+)	3.2	2.7	Yes
13	2011/A	2011/A	14	Mut - VH3–48*02	N/D	N/D	(−)	(−)	Neg	Neg	Alive
14	2011/A	2011/A	8.9	Mut - VH1–03	del 13q	4.5	(−)	(−)	Neg	Neg	Alive
15	2011/A	2012/A	12	Mut - VH4–59*01	Normal	N/D	(−)	(−)	Neg	Neg	Alive
16	2010/A	2011/A	54.3	Mut - VH3–9*01	Normal	14	(−)	(−)	Neg	Neg	Alive
17	2008/A	2010/A	24.2	Mut - VH1–18*1	Normal	7.8	(−)	(−)	Neg	Neg	Alive
18	2012/A	2012/A	10.0	Mut - VH4–59*1	Normal	3.5	(−)	(−)	Neg	Neg	Alive
19	2012/A	2012/A	26.7	Mut - VH1–2*04	del 13q	N/D	(−)	(−)	Neg	Neg	Alive
20	2012/B	2012/B	21.9	Mut - VH3–23	Normal	32	(−)	(−)	Neg	Neg	Yes
21	2012/A	2012/A	17.9	Mut - VH3–48*02	Normal	8.9	(−)	(−)	Neg	Neg	Alive
22	2010/A	2012/A	6.5	Mut - VH6–1*01	Normal	N/D	(−)	(−)	Neg	Neg	Alive

Abbreviations: AID, activation-induced cytidine deaminase; CLL, chronic lymphocytic leukemia; FISH, fluorescence *in situ* hybridization; IgG immunoglobulin G; LPL, lipoprotein lipase; N/D, not determined; Neg, negative; Q-PCR, quantitative PCR. [a]Unmutated (UM) patients display <2% difference with the germline gene, whereas ⩾2% difference defines mutated (MUT) patients. [b]FISH studies. [c]Results observed by cytometry assays using CD38 cutoff of 30%. [d]Intracytoplasmic cytometry analysis of IgM and IgG subpopulations, Neg is ⩽ to 1% of $IgG^{(+)}$ CLL B cells.

(1:1000), anti-goat Alexa 633 (1:1000) and anti-mouse phycoerythrin, respectively, for 1 h at room temperature. Nuclear staining with 4′,6-diamidino-2-phenylindole or propidium iodide (0.1 μg/ml) was performed. For antibody details see Supplementary Table S2.

The microRNA transfections and CLL B-cell stimulation. We transfected peripheral blood mononuclear cells of CLL patients with miR-22, antagomiR-22 or irrelevant miR conjugated to Cy3 fluorophore using lipofectamine 2000 (Invitrogen, Grand Island, NY, USA). Peripheral blood mononuclear cells (2×10^6) were resuspended in Opti-MEM I (Invitrogen) and transfected with 50 nM of microRNA and 5 μl/ml of lipofectamine. After 6 h, the cells were washed and cultured in RPMI-1640 media (Invitrogen) supplemented with 20% fetal bovine serum and evaluated by flow cytometry and/or confocal microscopy at 24 h for PTEN and p27 protein or 48 h for Survivin and Ki-67 molecules. Transfection efficiency was evaluated by flow cytometry analysis using a Cy3 fluorophore incorporated at the C3′ position of the synthesized oligos (miR-22, antagomiR 22 and irrelevant miR, see Supplementary Table S1). Only experiments with highest transfection efficiency, ranged between 75 and 98%, were evaluated. Activation with human soluble CD40 ligand (Preprotech, Mexico City, Mexico) and interleukin-4 (IL-4; Preprotech) was performed for 4 days at 5 μg/ml and 5 ng/ml, respectively. Samples were taken for RNA extraction, flow cytometry and confocal microscopy analysis.

Statistical analyses

Comparison of different mRNA gene expression from QF and PF of the same patient was evaluated by Q-PCR using either paired Wilcoxon signed rank test or two--tailed unpaired Student's *t*-test. For protein-level evaluation of PTEN, AKT, pAKT^{Thr308}, FOXO1, Survivin, p27 and Ki-67, a paired Wilcoxon signed rank test was performed. Variables with P-values of <0.05 were considered to be significant. All analyses were done using GraphPad Prism, version 4.0 (GraphPad Software, San Diego, CA, USA). For microarray data analysis, statistical significance ($P \leqslant 0.05$) was calculated using Student's *t*-test followed by Benjamini–Hochberg false discovery rate correction on GeneSpring GX11.0.2 software (Agilent Technologies, Inc.).

RESULTS

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

Clinical and molecular characterization of CLL patients included in this study are provided in Table 1. Fluorescence in situ hybridization status in our cohort does not correspond to a classical series of CLL patients, as reported by Dohner *et al.*,[20] because our study focused in the comparison of highly progressive patients on one side and highly indolent patients on the other side. Eighty UM CLL cases were studied by flow cytometry to determine the frequency of the PF characterized by ongoing class switch recombination and AID mRNA expression. We found that 15% (12 out of 80 analyzed CLLs) exhibited this subpopulation, ranging between 1 and 3% of the total leukemic clone after surface Ig labeling. A representative case displaying the characterization of the QF and the PF is shown in Figure 1. The QF and the PF were defined as IgM$^+$/CD5$^+$/Ki-67^{low}/AID^{neg} and IgG$^+$/CD5$^+$/Ki-67^{high}/AID$^+$,

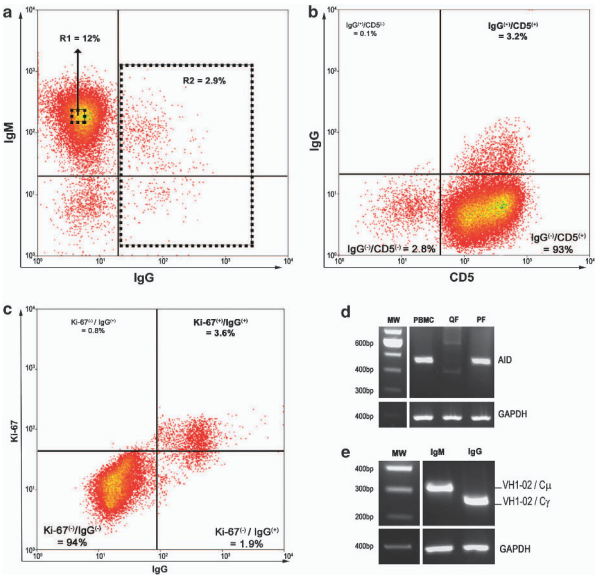

Figure 1. Proliferative and quiescent CLL fractions in a progressive UM CLL patient. (**a**–**c**) Representative flow cytometry profile from CLL patient number 03. (**a**) Surface expression of IgM and IgG CLL B cells and final sorter decision. R1is positive IgM and R2 is positive IgG surface markers. (**b**) Surface expression of IgG/CD5 CLL B cells. (**c**) Intracytoplasmic expression of Ki-67 and IgG markers. (**d**) AID mRNA expression in peripheral blood mononuclear cells (PBMCs), quiescent and proliferative fractions of patient 03. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. It is shown that AID expression predominates within the proliferative subset. (**e**) CLL patient-specific μ and γ transcripts were amplified by reverse transcription-PCR (RT-PCR). The same clonal VH rearrangement was substantiated by sequence analysis.

respectively, and both present the same clonal IgV_H rearrangement (Figures 1a–e). Cell sorter decision to isolate IgM^+ from IgG^+ cells is depicted in Figure 1a. Finally, to fully characterize both subsets we carried out GEP of the mRNA and microRNA molecules expressed by them.

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

To investigate the mechanisms involved in CLL proliferation, we evaluated the differential microRNA expression profiles of PF and QF isolated from four CLL cases (Table 1). Expression analysis of array studies followed by Student's *t*-test and Benjamini–Hochberg false discovery rate correction displayed six microRNAs as differentially expressed ($P \leqslant 0.05$). MicroRNAs, miR-22, miR-107 and miR-15b were overexpressed in the PF, whereas the microRNAs, miR-26a, miR-29a and miR-150 were downregulated (Figure 2a). The microRNAs upregulated in the PF were confirmed by Q-PCR in 8 CLL cases (CLLs 01–04 and 07–10; Figure 2b).

The mRNA GEP was also performed in the isolated PF and QF of patients 01–04 in Table 1. Our study showed a significant number of affected genes grouped in different clusters like cell activation, response to stimulus and stress and others (see Figure 2c). For a complete list of up- and downregulated genes in the PF, see Supplementary Table S3. After stringent quality control steps, we proceeded to perform gene ontology analysis to better define the different gene pathways involved in the PF. Despite the fact that these data were obtained in only four patients and they should be cautiously interpreted, the results showed that the PI3K/AKT pathway appeared to play a major role in this subset (Figure 2d and Supplementary Table S4). Among the most upregulated genes in the PF concerning PI3K/AKT pathway, we found classical cell cycle activators like Cyclin D2 (CCND2) and mitogen-activated protein kinase 1 (MAPK1), key anti-apoptotic molecules like BCL2A1, Survivin and proteins implicated in cells proliferation and migration like FYN, Talin-1 and Paxillin. In addition, PF displays low expression of tumor suppressor genes like PTEN and cell cycle regulator genes as p27 and the transcription factor FOXO1 (Figure 2e). Q-PCR of these genes was performed in order to confirm GEP analysis (Supplementary Table S5).

Interestingly, recent evidence demonstrated that miR-22 controls the signaling kinetics of PTEN/AKT/FOXO1 pathway in human cell lines and regulates this cascade.[21] As AKT pathway is a promising target for CLL treatment,[22] we investigated the molecular mechanism of miR-22 overexpression and its putative consequences on the AKT signaling in CLL proliferation.

The miRNA-22 is associated with PTEN/AKT/FOXO1 pathway activation, Survivin overexpression and p27 downregulation

In this section we explored whether the proliferative behavior of the CLL PF could be associated to miR-22 overexpression and regulation of the PTEN/AKT/FOXO1 pathway. We evaluated PTEN expression at the mRNA and protein levels and the total AKT expression in its basal and phosphorylated form ($pAKT^{Thr308}$), which is a hallmark of the PI3K/AKT pathway activation. We also evaluated nuclear or cytosolic localization of the transcription factor FOXO1 and assessed Survivin and p27 protein expression levels.

Results showed that in the PF there is a threefold overexpression of miR-22 and PTEN mRNA and a twofold underexpression of PTEN mRNA when compared with its quiescent counterpart. Significant statistical differences are shown in Figure 3a. In line with these results, significantly increased PTEN protein is visualized on the CLL B cells corresponding to the QF when compared with the PF of six CLL cases. Figure 3a depicts these results for CLL 06 patient.

As PTEN is a natural negative regulator of PI3K signaling, we investigated the expression of AKT in its basal and phosphorylated forms. Results showed a significant increase of the $pAKT^{Thr308}$ form in the PF compared with their quiescent counterpart from seven patients. In contrast, no significant differences were found in both fractions from the same CLL cases when total AKT was analyzed. A representative case (CLL 02) after flow cytometry analysis of $pAKT^{Thr308}$ is depicted in Figure 3b. As AKT activity is associated with nuclear export and cytosolic degradation of FoxO proteins,[23,24] we investigated whether high phosphorylation of AKT should result in FOXO1 inactivation. Statistical analysis of total FOXO1 protein showed that cytoplasmic expression of FOXO1 in the PF is significantly increased when compared with the QF. As shown in Figure 3c, FOXO1 displays a homogeneous nuclear localization in the QF that contrasts with the predominant cytoplasmic localization in the PF.

In CLL, p27 (ref.25) and Survivin[9] have been implicated in cell cycle arrest and survival, respectively. As both proteins are downstream of the PTEN/AKT/FOXO pathway,[26,27] we reasoned that expression analysis of these molecules could help explain the differential behavior between quiescent and proliferative CLL subsets. Our results showed that p27 expression is mainly visualized in the QF (Figure 3d). In addition, microscopy studies of the Survivin molecule showed a clear expression of this apoptotic inhibitor in the PF that contrasted with low or absent expression of this molecule in the QF (Figure 3e).

Overall, these results suggest that the proliferative behavior of this PF may result from an activated PTEN/AKT/FOXO1 pathway that in turn switches on cell survival signals like Survivin expression and downregulates cell cycle arrest proteins like p27.

The miR-22 regulates PTEN, p27 and Survivin protein expression in CLL B cells

Previous results in CLL suggest that leukemic B cells from patients with progressive disease display stronger AKT pathway activation.[28] As the results presented above suggest that miR-22 could be implicated in CLL B-cell proliferation as well as in AKT activation, we investigated whether inhibition of miR-22 might result in upregulation of PTEN and p27 molecules. To examine this possibility, we first evaluated peripheral blood mononuclear cells of the 22 CLL cases (Table 1), the phosphorylation status of AKT on Thr^{308} and the PTEN expression levels by flow cytometry in CLL B cells (Figure 4a). This experiment was used to select the five extreme cases displaying highest phosphorylated AKT signals and lower PTEN expression (CLLs 01, 03, 04, 09 and 10 in Table 1) in order to proceed with the microRNA transfection assays. Our results showed that after antagomiR-22 transfection, PTEN protein levels were increased at similar expression values of indolent CLLs (Figure 4b). PTEN expression in a representative case transfected with the antagomiR-22 and the irrelevant microRNA control is shown in Figure 4c. We then evaluated the expression of p27 protein in PB from five progressive and five indolent CLL patients (first section of Figure 4d). Interestingly, after antagomiR-22 transfection of progressive CLL cases (01, 03, 04, 09 and 10 from Table 1), a significant increase in p27 protein level was observed when compared with untransfected and with irrelevant miR controls (Figure 4d). The p27 expression in a representative CLL sample is depicted in Figure 4f. Finally, we evaluated the assumption that miR-22 could also be implicated in Survivin expression through AKT pathway. To this aim, we first evaluated Survivin basal expression in the PB of indolent and progressive CLL cases. As previously described,[9] only some responders to CD40 stimulation patients express Survivin in PB (first section Figure 4e). Then, we transfected CLL B cells from progressive cases (patients 01, 04, 08, 09 and 10 in Table 1), previously assessed as responders to CD40 ligand (CD40L; data not shown) and analyzed Survivin expression by confocal microscopy. Our results suggest that after transfection with miR-22, Survivin expression is upregulated at significant levels in the evaluated patients (Figure 4e). Upregulated Survivin displays a clear cytosolic localization, contrasting with low or no expression on irrelevant

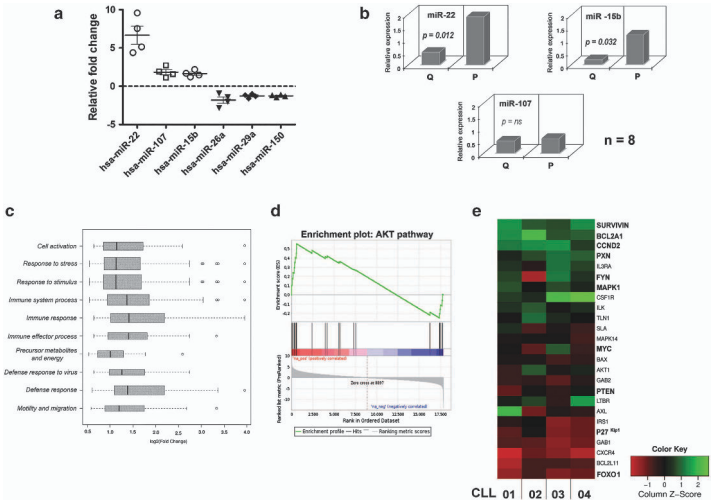

Figure 2. Comparative microRNA and mRNA gene expression analysis between proliferative and quiescent CLL fractions. (**a**) Differential microRNA expression of PF and QF. Relative fold change in the expression of miR-22, miR15b, miR-107, miR-26a, mir-29a and miR-150 in the PF. (**b**) Q-PCR of miR-22, miR15b and miR-107 expression in PF and QF of CLL B cells isolated from patients 01–04 in Table 1. The 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 were found for miR-107. The miR-22 is the most differentially overexpressed when comparing the quiescent and the proliferative CLL fractions. (**c**) Analysis of mRNA expression profile. Ontology classes with an overrepresentation in our data set are shown. (**d**) Gene-set enrichment analysis (GSEA) for AKT pathway. Microarray data were analyzed using GSEA software to verify whether AKT gene set was significantly enriched in one of the phenotypes. AKT gene set was enriched in proliferative fraction with false discovery rate (FDR) < 0.01. The X axis of the curve for enrichment scores includes 17 840 genes, with those better correlating with proliferative fraction on the left and those better correlating 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. (**e**) Heatmap representing relative proliferative/quiescent gene expression levels for genes in the AKT pathway. Relative expression levels were obtained from microarray data analysis. Red represents low gene expression in PF relative to gene expression in quiescent cells, whereas green represents high gene expression in PF 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.

miR-transfected or untransfected CLL B cells (representative case in Figure 4f).

Altogether, these results indicate that miR-22 could play an important role in the proliferative behavior of CLL B cells by preventing PTEN tumor suppressor protein expression as well as the cell cycle inhibitor p27 and increasing Survivin expression.

CD40 engagement induces miR-22 expression, AKT activation, Survivin expression and CLL proliferation

To investigate the molecular mechanism that could trigger the PI3K/AKT cascade favoring CLL proliferation, we evaluated whether CD40L/IL-4 could be at the origin of this pathway through miR-22 overexpression. Thus, we stimulated CD40L responder patients (cases 01, 04, 09 and 10 in Table 1) with CD40L/IL-4 and evaluated miR-22, $pAKT^{Thr308}$ and Survivin protein expression. Results showed that CD40L/IL-4 activation results in a miR-22-specific overexpression, whereas no or low effects were evidenced when miR1201 or miR15b expression were tested as controls (Figure 5a and Supplementary Figure S1). These studies also showed increased levels of the $pAKT^{Thr308}$ and Survivin protein compared with unstimulated samples (Figures 5b and c), suggesting that CD40 engagement switches on the PI3K/AKT pathway and Survivin expression in CLL.

To confirm the relevance of miRNA-22 upregulation on PTEN/AKT pathway and CLL proliferation, we investigated whether Ki-67 proliferation marker could be increased after miR-22 transfection on leukemic cells. Thus, purified leukemic cells from eight CLL patients, four indolent and four progressor patients responsive to CD40 stimulation (CLL 01, 04, 09, 10, 13, 16, 20 and 22 in Table 1) were stimulated with CD40L/IL4 following transfection with miR-22 and an irrelevant microRNA. Results showed a significant increase in Ki-67 expression following miR-22 transfection compared with the experimental controls (Figures 5d and e). Interestingly, transfection with miR-22 and CD40 engagement appears to act in a synergistic way resulting in increased amounts of Ki-67 expression that contrasts with the absence of effect with irrelevant microRNA transfection in CD40L/IL4-stimulated CLL B cells (Figures 5d and e). In addition, the results showed that the inhibition of miR-22 by AmiR-22 transfection during CD40L/IL4 stimulation decreased proliferation in CLL B cells. This evidence strongly supports the hypothesis that miR-22 contributes to CLL proliferation (Figure 5e). Although preliminary, these results

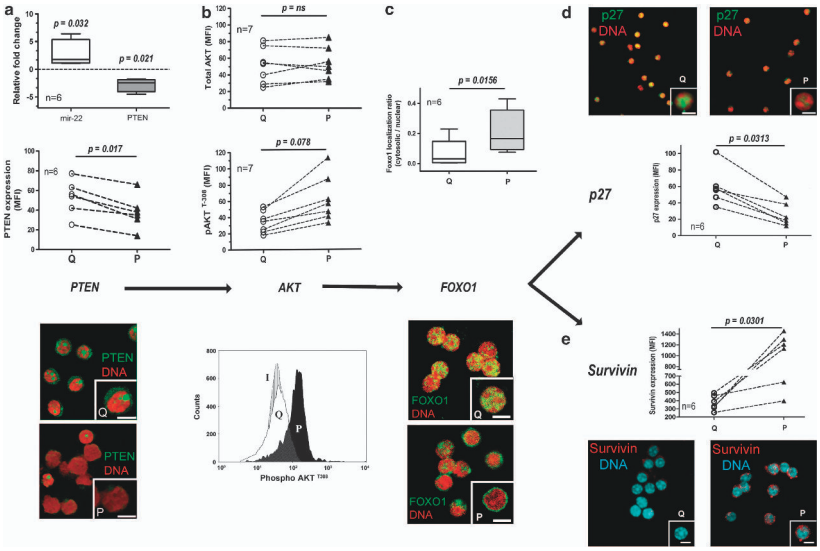

Figure 3. Characterization of miR22, PTEN, AKT and FOXO1 molecule expression levels in quiescent (Q) and proliferative (P) cells from progressive UM CLL cases. (**a**) The miR-22 and PTEN mRNA expression and PTEN protein levels. Fold change expression of miR-22 and PTEN mRNA in PF compared with QF was evaluated by Q-PCR in six CLL cases. The miR-22 is overexpressed in the PF)and PTEN mRNA is underexpressed (mean = 2.7 ± 1.2, $P = 0.032$ and mean = − 2.5 ± 0.6, $P = 0.021$, respectively). PTEN protein expression in Q (open circle) and P (black triangle) cells from six CLL cases (01–06 in Table 1) evaluated by flow cytometry. It is shown that low expression of PTEN protein levels were observed in the PF when compared with their quiescent counterpart (41.3 ± 7.0 vs 52.8 ± 7.2 respectively, $P = 0.017$, Wilcoxon matched pairs test), MFIs, mean fluorescence intensities. Representative image of confocal microscopy showing PTEN expression in QF and PF from CLL 06. Scale bar: 5 μm, green: PTEN and red: DNA. (**b**) Characterization of the phosphorylated status of AKT. Phosphorylated AKT form in Threonine 308 (pAKT^{Thr308}) and total AKT protein expression evaluated by flow cytometry in Q (open circle) and P (black triangle) from seven CLL cases (samples 01–07 in Table 1). 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). In contrast, no significant differences were found in both fractions 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). Representative pAKT^{Thr308} histogram from CLL 02 showing MFIs in the PF (black peak), QF (white peak) and isotype control (dashed peak) is shown. (**c**) Characterization of the cellular localization of FOXO1. Specific fluorescence intensity in nucleus and cytoplasm was measured in the isolated QF and PF of CLLs 01–04, 07 and 08. Between 100 and 200 cells were counted in each sample. Next, MFI for nucleus and cytoplasm was used to obtain cytosolic/nuclear ratio. The cytosolic/nuclear mean values between QF and PF were respectively, 0.070 ± 0.036 and 0.210 ± 0.056 ($P = 0.0156$, Wilcoxon matched paired test). This difference is depicted by confocal microscopy analysis from CLL 06, where it is shown that FOXO1 is mainly located in the nucleus in the QF and in the cytoplasm in the PF. Scale bar: 5 μm, green: FOXO1 and red: DNA. (**d** and **e**) Characterization of p27 and Survivin molecule expression levels. The p27 and Survivin proteins were evaluated by flow cytometry in Q (open circle) and P (black triangle) from six CLL cases (samples 01–06 in Table 1). Statistical analysis of p27 demonstrated a lower expression of this marker in the PF (mean = 33.08 ± 5.4) when compared with the QF (mean = 64.44 ± 9.64; $P = 0.0313$, Wilcoxon matched pairs test). Statistical analysis of Survivin showed high expression levels of this survival marker in the PF, whereas low or no expression was found in the QF, (mean = 1145 ± 140.7 & mean = 399 ± 35.0; $P = 0.0301$, respectively; Wilcoxon matched pairs test). A representative microscopic analysis of p27 and Survivin expression in Q and P fractions is shown. Scale bar: 5 μm. Green: p27 and red: DNA. Red: Survivin and cyan: DNA.

suggest that CD40 engagement could lead to CLL proliferation through miR-22 upregulation followed by PI3K/AKT activation and Survivin overexpression.

Proliferative behavior 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

We assumed that the proliferative behavior of this PF resulted from downregulation of the PTEN molecule and activation of the PI3K/AKT pathway that in turn induced cell proliferation underlined by downregulation of p27 and high expression of Survivin proteins. To examine this hypothesis, we studied three different LNs from UM and progressive CLLs by isolating Ki-67-positive and -negative fractions. Next, we evaluated PTEN expression, PI3K/AKT activation (pAKT^{Thr308}) and Survivin and p27 protein expression in both fractions.

The results showed 7–10% Ki-67 positive cells in the three analyzed LNs. Flow cytometry analysis confirmed that almost all of this Ki-67 subset corresponded to the leukemic CD5$^+$/CD19$^+$ B cells (data not shown). R1 and R2 depict the Ki-67-negative and the Ki-67-positive cells, respectively (Figure 6a). PTEN expression in

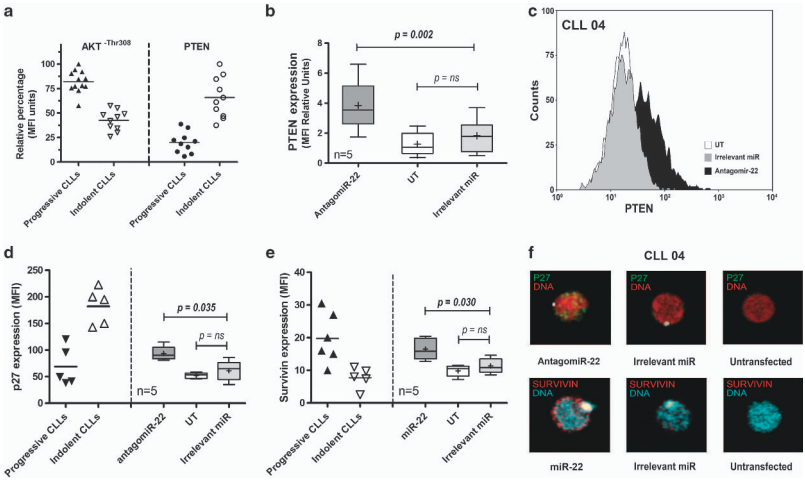

Figure 4. MiR-22 and antagomiR-22 transfection regulates PTEN, p27 and Survivin expression levels. (**a**) Higher phosphorylated AKT$^{-Thr308}$ levels are observed in 12 progressive cases (mean = 44 ± 6.6 obtained from samples 01–12 from Table 1) when compared with 10 indolent CLL cases (mean = 24 ± 4.8 obtained from samples 13–22; $P = 0.0059$) after evaluation by flow cytometry. MFIs, mean fluorescence intensities. (**b**) Lower PTEN protein expression is observed by flow cytometry in progressive CLL cases (01–10 from Table 1) when compared with indolent CLL cases (13–22 from Table 1). PTEN protein expression is also depicted after transfection with antagomiR-22, irrelevant miR and untransfected (UT) cells in five progressive CLL cases (01, 03, 04, 09 and 10 from Table 1) with low PTEN expression. Statistical analysis demonstrated a significant increase ($P = 0.002$) in PTEN expression following treatment of CLL B-cells with antagomiR-22 (mean = 3.84 ± 0.48), whereas no differences were observed when these same cells were transfected with irrelevant miR (mean = 1.67 ± 0.33) and with UT control (mean = 1.26 ± 0.23) that did not display significant differences between them ($P = 0.331$). (**c**) Representative histogram of PTEN expression in CLL B cells transfected with antagomir-22, irrelevant miR and untransfected cells. (**d**) The p27 protein expression was evaluated by confocal microscopy in five progressive CLL cases (01, 03, 04, and 09 and 10 from Table 1) and in 5 indolent CLL cases (13–15 and 17–18 from Table 1). Statistical analysis confirmed that transfection with antagomiR-22 in patients 01, 03, 04, 09 and 10 from Table 1 resulted in overexpression of p27 protein compared with irrelevant and UT cells control ($P = 0.035$, 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). A typical immunoreactive pattern of p27 protein, similar to those observed for the quiescent fraction previously described in Figures 3 and 4, was found. (**e**) Survivin protein expression evaluated by confocal microscopy in six progressive CLL cases (01, 02, 04, 07, 10 and 12 from Table 1) and in five indolent CLL cases (13–15 and 17–18 from Table 1). Survivin protein expression after transfection with miR-22, irrelevant miR and UT cells of five progressive CLL cases (CLLs 01, 02, 04, 07 and 12). Survivin expression was upregulated at significant levels ($P = 0.030$, mean = 15.3 ± 1.2) when compared with those transfected with irrelevant miR (mean = 11.3 ± 1.0). No significant differences ($P = 0.093$) were found among irrelevant miR and UT cells. (P = ns), statistically nonsignificant data. (**f**) Confocal microscopy of Survivin (red), p27 (green) and transfected cell (miR-22, antagomiR-22 and Irrelevant miR, white) are depicted in a representative sample obtained from the CLL patient number 04 after transfection with antagomiR-22, miR-22, irrelevant miR and untransfected cells.

R1 exhibited a similar profile to that of the QF obtained from UM CLL patients, whereas low or no expression was found in fraction R2 (Figure 6b). In the three analyzed LNs, we found that the Ki-67-positive fraction was enriched in cells that express pAKT$^{-Thr308}$, whereas the nonphosphorylated AKT form predominated in the Ki-67-negative fraction (Figure 6c). Interestingly and supporting our initial hypothesis, we found that the R2 fraction expresses high Survivin levels and low or absent p27 expression (Figures 6d and e, respectively).

Despite the fact that we could not study miR-22 expression in these samples because we were unable to obtain enough RNA, we could demonstrate that the PF from different LNs is also associated with low PTEN expression, PI3K/AKT activation, Survivin expression and p27 downregulation. Altogether, these results confirm the importance of the PI3K/AKT pathway in the proliferative subset of CLL and suggest that a similar regulatory loop involving PTEN/AKT/FOXO1 pathway could be relevant in an *in vivo* scenario in these UM CLL patients.

DISCUSSION

PI3K signaling pathway is a key signaling molecule in the relationship between CLL cells and the microenvironment. It mediates the transmission of signals from membrane receptors such as B-cell receptor, CXCR4 and CD40.[29] The main negative regulator of the PI3K/AKT pathway is PTEN,[30] whereas another important protein family involved in the AKT signaling are the FoxO transcription factors. When PTEN is active and AKT activity suppressed, FoxO proteins are able to enter the nucleus and upregulate genes such as p27, promoting cell cycle arrest.[21,31] Overexpression of mIR-22 has been recently proposed to regulate the PTEN/AKT/FOXO1.[21]

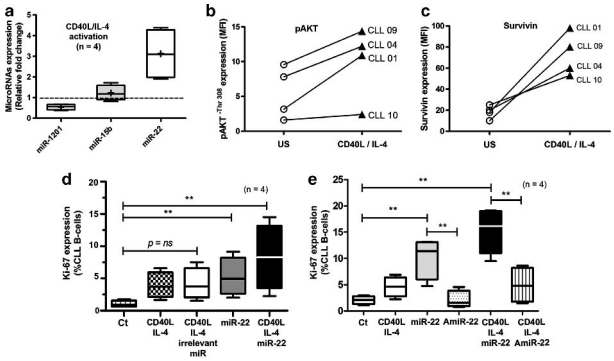

Figure 5. CD40 engagement induces miR-22 expression, activation of AKT pathway, Survivin expression and CLL proliferation. (**a**) The miR-1201, miR-15b and miR-22 fold change expression of CD40L/IL-4-stimulated cells from four CD40 responder patients (CLLs 01, 04, 09 and 10 in Table 1) compared with that of unstimulated ones. (**b**, **c**) The pAKT^{Thr308} and Survivin protein expression evaluated by flow cytometry and confocal microscopy respectively in the same unstimulated (US) and CD40L/IL-4-stimulated, CD40 responder patients. MFIs, mean fluorescence intensities. (**d**) Cell percentage expressing Ki-67 marker was evaluated by flow cytometry in CLL B cells from four indolent cases that respond to CD40 stimulation (13, 16, 20 and 22 in Table 1). Ki-67 expression was evaluated after CD40L/IL-4 stimulation, miR-22 transfection and stimulation with CD40L/IL-4 followed by miR-22 or irrelevant miR transfection. Ct, unstimulated and untransfected cells. (**e**) Ki-67 expression evaluated by flow cytometry in CLL B cells of four progressive cases. CD40 responder patients, CLLs 01, 04, 09 and 10 in Table 1, were evaluated for Ki-67 expression as proliferative marker. Percentage of positives Ki-67 CLL B cells was evaluated after CD40L/IL-4 stimulation, miR-22 transfection and miR-22 plus CD40L/IL-4 stimulation. In addition, antagomiR-22 transfection and antagomir-22 plus CD40L/IL-4 stimulation were carried out as specificity controls. AntagomiR-22 = AmiR-22 (**) = statistically significant differences ($P < 0.05$).

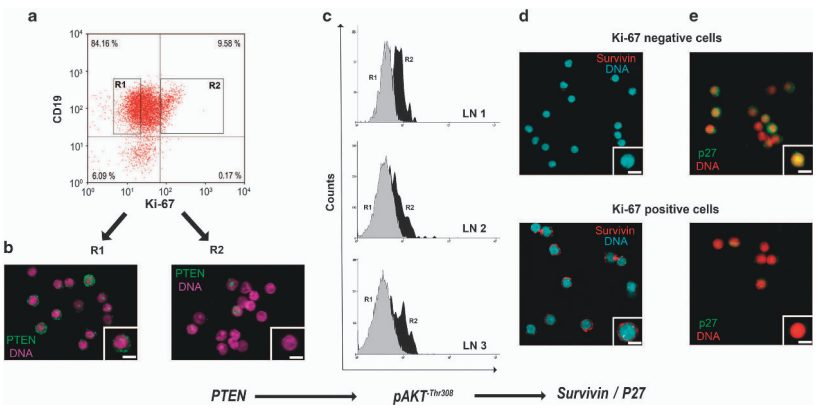

Figure 6. Isolation of Ki-67-positive and -negative fractions from B-cell CLL LNs highlight activation of PTEN/AKT cascade, Survivin expression and downregulation of p27 protein. (**a**) Representative dot plot of CD19 and Ki-67 protein expression in the LN of an UM CLL patient. R1 and R2 show the gates 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: 5 μm. (**c**) Flow cytometry histograms depicting expression of pAKT^{Thr308} expression in Ki-67-negative (R1, gray) and Ki-67-positive (R2, black) CLL B cells. (**d**, **e**) Characterization of the expression levels of Survivin (left panels) and p27 (right panels) by confocal microscopy in the Ki-67-negative and Ki-67-positive CLL B cells from a representative LN. Scale bar: 5 μm.

Understanding the crosstalk between malignant B cells and their milieu could give us new keys on the cellular and molecular biology of CLL.[6] Our group succeeded in identifying a small PF associated with a clinical poor outcome in a fraction of UM patients that is characterized by an active class switch recombination process and AID expression.[10] The present work addresses the

genomic and the molecular characterization of this subset, provides novel information about the proliferative behavior of these leukemic cells and suggests that miR-22 plays a key role in the proliferation mediated by AKT pathway in CLL. Genomic characterization at the mRNA and microRNA levels of the PF showed that AKT signaling pathway is active in this leukemic subset and that miR-22 is the most overexpressed microRNA. As previous work suggested that miR-22 regulates the signaling kinetics of PTEN/AKT/FOXO1 pathway[21] leading to tumor proliferation,[32,33] we speculated whether a similar pathway could take place in the leukemic PF of these patients. Our results showed that upregulation of miR-22 is associated with low PTEN expression at the mRNA and protein levels, and the $pAKT^{Thr308}$ form is increased in the PF compared with the QF of the same patients.

Recent work investigated PTEN/AKT signaling in CLL,[34–36] but only few evidence concerning FoxO proteins expression in this leukemia is available.[37,38] As cytoplasmic localization of FoxO proteins is a hallmark of AKT activity,[27] we proceeded to evaluate the FOXO1 protein localization in the PF and QF. As reported in other tumoral cells,[23,39] our results clearly showed that FOXO1 protein was mostly translocated to the cytoplasmic compartment in the PF of UM patients. These results are in agreement with previous evidences linking miR-22 overexpression, inhibition of PTEN, PI3K/AKT pathway activation and cancer proliferation.[40] FoxO proteins are also associated with cell cycle arrest by downregulating the p27 protein.[23,26,39,41] As mRNA array analysis displayed low and high expression of p27 and Survivin respectively (Figure 3), and as it has been described that p27 is overexpressed in the QF of CLL,[10,11] we evaluated the differential expression pattern of these proteins as well as whether this expression was related with PTEN/AKT/FOXO1 pathway activation. Results showed that in the PF, p27 protein is downregulated and Survivin molecule overexpressed (Figure 5). Assuming that microenvironment interactions trigger Survivin expression[9] and also downregulate p27 molecule,[42] our results suggest that regulation of these two key molecules might be dependent on an activated PTEN/AKT/FOXO1 pathway and play an important role in the leukemic proliferation. Results from transfection experiments with miR-22 and its specific antagomiR favor the view that miR-22 expression could play a role in PI3K/AKT activation through PTEN downregulation and suggest that this pathway is responsible for Survivin overexpression and p27 downregulation. In this line, previous results from Shehata *et al.*[34] and Martins *et al.*[43] suggest that reconstitution of PTEN activity in CLL is able to counteract the anti-apoptotic and proliferative behavior of AKT signaling.

The proliferating centers from LN and BM support leukemic cell accumulation and favor tumor-growing conditions.[2] In this sense, our results suggest that, at least in part, CD40 engagement could be responsible for miR-22 overexpression, activation of PTEN/AKT/Survivin pathway and CLL B-cell proliferation. Supporting these remarks, some reports suggest that CD40L is responsible for the AKT activation signaling,[44,45] whereas others have demonstrated that PI3K regulates Survivin through AKT activation.[46–48] Altogether, these data confirm previous results[9] showing that the proliferative behavior of some CLL B cells triggered by CD40 engagement is associated with Survivin and Ki-67 expression, but in addition propose that this proliferative potential is dependent on miR-22 overexpression and an activated PI3K/AKT pathway. Finally, the relevance of these data is supported by the *in vivo* results obtained from the LNs of three different UM and progressive CLL cases.

A recent study comparing gene expression profiling of PB to that of LN and BM tumoral cells identified LN as a key site for proliferation.[49] CLL cells in the LN showed upregulation of gene signatures (E2F, c-MYC and Ki-67 overexpression) indicating B-cell receptor and nuclear factor-κB activation. The proliferative subset (IgG^+, $Ki\text{-}67^+$, AID^+) described by our group displays very similar

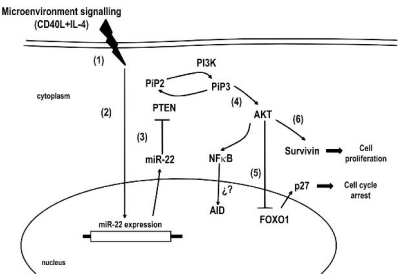

Figure 7. Hypothetical model proposed to explain the proliferative behavior of CLL subsets in UM progressive patients. (1, 2) The miR-22 expression is induced after CD40 engagement following interaction of B-cell CLL with the tumoral microenvironment of secondary lymphoid organs. (3) The miR-22 downregulates PTEN and the PI3K phosphorylates and converts PIP2 to PIP3 that recruits and activates PDK1. (4) PDK1 phosphorylates and activates the threonine protein kinase AKT ($pAKT^{Thr308}$) that inhibits the activities of the transcription factor FOXO1. (5) Inactivation of FOXO1 leads to downregulation of proteins involved in cell-cycle arrest like p27. (6) Activation of PI3K/AKT cascade triggers Survivin expression that contributes to cell progression and proliferation. AKT could also activate the NF-κB cascade that in turn might induce AID expression in the proliferative subset of these UM progressive CLL patients.

markers to those found by Herishanu *et al.*[49] in LN from CLL patients, and unpublished results from our laboratory suggest that this tumoral subset could recently egress toward the PB from a proliferating center in LN or BM. A model aiming to explain the proliferative behavior origin of this PF from progressive UM CLL patients is depicted in Figure 7. In this model, microenvironment signaling triggered by T-dependent signals as CD40L + IL4 could be at the origin of miR-22 overexpression. However, our results cannot exclude the existence of other upstream regulators of this microRNA. In this model, we propose that miR-22 overexpression downregulates PTEN, allowing that PI3K phosphorylates and converts PIP2 to PIP3 that in turn recruits and activates PDK1. Activation of PDK1 phosphorylates and activates the threonine protein kinase AKT (pAKT-Thr308) that inhibits the activities of the transcription factor FOXO1. This inactivation of FOXO1 leads to downregulation of proteins involved in cell-cycle arrest like p27. Furthermore, activation of PI3K/AKT cascade triggers Survivin expression that contributes to cell progression and proliferation. These data are in agreement with recent reports supporting the idea that Survivin acts as a proliferative marker in different hematopoietic cells.[50–52] Finally, it is also described that AKT activates the NF-κB cascade that in turn might induce AID expression in this proliferative subset.

Recently, three works by Bar *et al.*,[21] Liu *et al.*[32] and Poliseno *et al.*[33] (reviewed in Xiong[40]) have demonstrated that PTEN was a *bone fide* target of miR-22 and that overexpression of this cascade could lead to cell proliferation via PI3K/AKT pathway. The fact that this microRNA is only expressed in this small PF could explain why its overexpression was not previously reported in CLL.[4,53] Previous work suggests that optimal B-cell proliferation requires PI3K/AKT pathway activation (reviewed in Rodon *et al.*[54]). However, activation of the PTEN/AKT/FOXO1 cascade by overexpression of miR-22 triggered by CD40 engagement, which in turn regulates p27 and Survivin molecules in CLL, constitutes a novel and original contribution of this work.

Taken together, our data sustain the model of CLL B-cell 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 PI3K/AKT pathway and suggest that Survivin overexpression and p27 downregulation are two faces of the same coin that could play a key role in CLL proliferation. Finally, we propose a new proliferative mechanism involving the regulatory loop miR-22/PTEN/AKT/FOXO1 pathway that could be relevant in the maintenance of the CLL proliferative subset in progressive and UM CLL patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

FP, CA, DP, PM and SR performed experiments and collected CLL samples; TF-C, HN, GL and CR performed microarrays and statistical analysis; ANL, GD and RG performed clinical activities and data collection of CLL patients; PO and GD contributed to scientific design and revised the paper; PO and FP designed research, coordinated the study and data analysis and wrote the paper.

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Dissecting chronic lymphocytic leukemia microenvironment signals in patients with unmutated disease: microRNA-22 regulates phosphatase and tensin homolog/AKT/FOXO1 pathway in proliferative leukemic cells

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Abstract

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of clonal B cells arrested in G0/G1 stages that coexist with proliferative B cells. We identified one of these proliferative subsets in the peripheral blood from patients with unmutated disease (UM). Aiming to characterize the molecular mechanism underlying this proliferative behavior, we performed gene expression analysis of the mRNA and microRNAs in this leukemic subpopulation and compared results with those for the quiescent counterpart. Our results suggest that proliferation of this subset mainly depends on microRNA-22 overexpression, which induces phosphatase and tensin homolog (PTEN) down-regulation and phosphoinositide 3-kinase (PI3K)/AKT pathway activation. These results underline the role of the PI3K/AKT pathway at the origin of this proliferative pool in patients with UM CLL and provide additional rationale for the use of PI3K inhibitors.

Keywords: CLL microenvironment signals, unmutated patients

Introduction

Chronic lymphocytic leukemia (CLL) is an incurable disease of unknown etiology. Evidence suggests that crosstalk with accessory cells in the tissue microenvironment favors disease progression through the maintenance of a proliferating CLL B-cell subpopulation [1]. Furthermore, the tumoral microenvironment also regulates the expression of microRNAs (miRs), which play an important role in the prognosis and progression of CLL [2].

In secondary lymphoid organs, B-cells express activation-induced cytidine deaminase (AID), an enzyme that initiates somatic hypermutation and class switch recombination (CSR) processes, but might also contribute to cellular transformation and tumor progression through its mutagenic activity [3]. In CLL, we and others have demonstrated that AID is abnormally expressed in the peripheral blood (PB) of patients with unmutated CLL (UM) [4–6]. More recently, we identified that AID expression is mainly restricted to a small proliferative subset undergoing the CSR process, exhibiting high expression of proliferative molecules and correlated with a poor prognosis [7].

In this work we evaluated the gene expression profile (GEP) of the mRNA and miRs from this proliferative fraction (PF), identified as IgG+/CD5+/Ki-67high/AID+ cells, and compared it with the quiescent fraction (QF), identified as IgM+/CD5+/Ki-67low/AID−, of the same patient.

Our results suggest that overexpression of miR-22 is associated with the proliferative behavior of the PF through activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway. We found that miR-22 down-regulates the tumor suppressor phosphatase and tensin homolog 10 (PTEN), which in turn switches on the PI3K/AKT pathway. The activation of this pathway appears to be related to the cytosolic translocation of the transcription factor FOXO1, overexpression of survivin and down-regulation of $p27^{-kip-1}$ (p27). These results emphasize the importance of this PF in the PB of patients with UM CLL with a poor clinical outcome, and propose a novel miR (miR-22) as an important modulator of PI3K/AKT signaling in CLL.

Materials and methods

Patient samples

Twenty-two patients with CLL were segregated into two groups: (i) the progressive group defined by UM immunoglobulin profile, AID and lipoprotein lipase (LPL) expression at the mRNA level and presence in PB of at least 2% of CLL B-cells undergoing the CSR process to immunoglobulin G

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(IgG) [7]; (ii) the indolent group displaying a mutated immunoglobulin heavy chain variable (IgVH) profile, negative LPL and AID expression and absence of cells undergoing CSR. Patients were followed at the Hospital Maciel and provided informed consent according to the ethical regulations of Uruguay and the Declaration of Helsinki.

miR and mRNA array

RNA from CLL B-cells was isolated using a mirVana isolation kit (Applied Biosystems, Foster City, CA). mRNA microarray analysis was performed using a 4×44K Whole Human Genome Oligo-Microarray (G4112F; Agilent, Santa Clara, CA) and miRNA microarray was performed using an 8×15K Human miRNA Microarray (G4470B; Agilent) containing 470 human miRNA probes. Data analysis was accomplished using R/Bioconductor. Differential expression was assayed using limma software and ontology analysis conducted with the GOHyperGAll function. Array analysis is available in the Gene Expression Omnibus (GEO) database (accession number GSE53270).

Quantitative PCR analysis

Reverse transcription-polymerase chain reaction (RT-PCR), quantitative PCR (Q-PCR) and PCR analyses of miRs were carried out as previously described [7,8], respectively.

Cell sorting studies and flow cytometry analysis

Isolation of CLL PB mononuclear cells (PBMCs) from PB, cytometry analysis and sorting experiments were performed as previously described [7]. The QF expresses exclusively IgM surface Ig, and PF expresses IgM plus IgG, or exclusively IgG surface Ig. In all cases, the purity of isolated subpopulations was $>98\%$. Isolated subsets were also evaluated by CD5 and Ki-67 expression markers, AID mRNA expression and clonal IgVH rearrangement. We defined as the QF, IgM+ CD5 + Ki-67lowAID−, and as the PF, IgG+ CD5 + Ki-67highAID+.

Confocal microscopy and flow cytometry analysis

Analysis was performed as previously described [9]. Briefly, incubation was carried out for 1 h at room temperature (RT) with rabbit anti-FOXO1 (1:100); goat anti-survivin (1:100) or mouse anti-p27 (1:1600), washes were performed and incubation was then performed with anti-mouse Alexa 488 (1:2000), anti-rabbit Alexa 546 (1:1000), anti-goat Alexa 633 (1:1000) and anti-mouse phycoerythrin (PE) (1:1000), respectively, for 1 h at RT (Supplementary Table I to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900). Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide was performed.

miR transfections and CLL B-cell stimulation

We transfected PBMCs of patients with CLL with miR-22, antagomiR-22 or irrelevant miR conjugated to Cy3 using Lipofectamine 2000 (Invitrogen, Waltham, MA). PBMCs (2×10^6) were resuspended in Opti-MEM I (Invitrogen) and transfected with 50 nM of miR and 5 μL/mL of lipofectamine. After 6 h the cells were washed and cultured in RPMI 1640 medium (Invitrogen) supplemented with 20% fetal bovine serum (FBS) and evaluated by flow cytometry and/or confocal microscopy at 24 h or 48 h. Transfection efficiency was evaluated by flow cytometry analysis using the Cy3 fluorophore incorporated at the 3′ position of the synthesized oligos. Only experiments with highest transfection efficiency, ranging 75–98%, were evaluated. Stimulation of PBMCs from patients with CLL was carried out as previously described [9].

Statistical analyses

Comparison of different gene mRNA expressions from QF and PF of the same patient was evaluated by Q-PCR using either paired Wilcoxon signed-rank test or two-tailed unpaired Student's *t*-test. For protein level evaluation of PTEN, AKT, pAKT$^{-Thr308}$, FOXO1, survivin, p27 and Ki-67, a paired Wilcoxon signed-rank test was performed. Variables with p-values < 0.05 were considered to be significant. All analyses were done using GraphPad Prism (GraphPad Software, San Diego, CA). For microarray data analysis, statistical significance ($p \leq 0.05$) was calculated with Student's *t*-test followed by Benjamini–Hochberg false discovery rate correction (FDR) using GeneSpring software.

Results

Transcriptome and microRNAome analysis of CLL PF highlight PI3K/AKT activated pathway

Analysis of the miR GEP of PF and QF isolated from four cases of UM CLL (Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900) showed six differentially expressed miRs (p-value ≤ 0.05). miR-22, miR-107 and miR-15b were overexpressed in the PF whereas miR-26a, miR-29a and miR-150 were down-regulated [Figure 1(B)]. Up-regulated miRs in the PF were confirmed by Q-PCR in eight cases of CLL [Figure 1(C)]. Concerning mRNA GEP, a significant number of differentially expressed genes were underlined in the PF. These could be grouped into different clusters related to cell activation, motility and migration, response to stress and others, as shown in Figure 1(D). We also performed gene ontology analysis of the differential GEPs between the PF and QF. The results suggested that the PI3K/AKT signaling pathway could be activated in this PF, in which increased expression of cell cycle activators such as mitogen-activated protein kinase 1 (MAPK1) and cyclin D2 (CCND2), anti-apoptotic molecules such as as BCL2A1 and survivin, and proteins implicated in cell proliferation and migration such as FYN, talin-1 and paxillin were up-regulated [Figure 1(E)]. Also, the PF displayed low expression of the tumor suppressor PTEN and cell cycle regulator genes such as p27 and FOXO1, all of them related to the PI3K/AKT cascade [Figure 1(E)].

miR-22 is associated with PTEN/AKT/FOXO1 pathway activation, survivin overexpression and p27 down-regulation

Since recent evidence demonstrated that miR-22 down-regulates PTEN and activates the AKT/FOXO1 pathway in human cell lines [10], we explored whether the proliferative behavior of the PF could be associated with miR-22 overexpression and its regulation of the PTEN/AKT/FOXO1 pathway. For

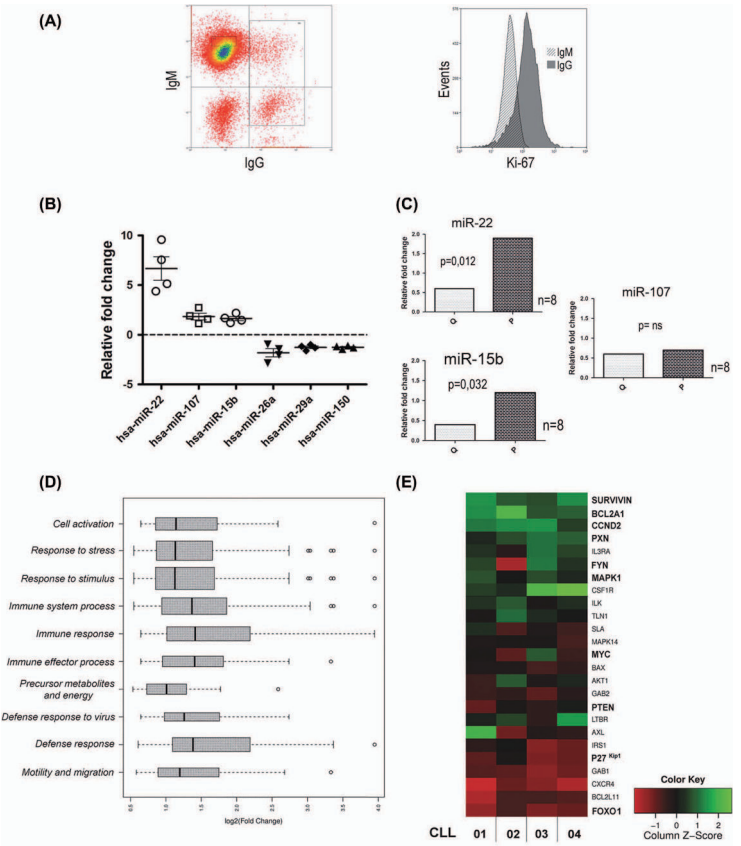

Figure 1. MicroRNA and mRNA comparative gene expression analysis between proliferative (PF) and quiescent (QF) CLL fractions. (A) Proliferative and quiescent CLL fractions in a patient with progressive UM CLL. Representative flow cytometry profile from patient with UM CLL. (A) IgM and IgG surface expression and intracytoplasmic expression of Ki-67 on both subsets (IgM and IgG) on UM CLL B-cells. Cells that express exclusively IgM surface Ig and cells that express IgM plus IgG or exclusively IgG surface Ig were isolated by cell sorter and defined as QF and PF, respectively. (B–E) Differential microRNA and mRNA expression of PF and QF. (B) Relative fold change in expression of miR-22, miR-15b, miR-107, miR-26a, mir-29a and miR-150 in the PF. (C) Q-PCR of miR-22, miR-15b and miR-107 expression in PF and QF of CLL B-cells. miR-22 and miR-15b were significantly overexpressed in the PF ($p = 0.012$ and $p = 0.032$, respectively, Wilcoxon matched pairs test), but no significant differences were found for miR-107. miR-22 is the most differentially overexpressed when comparing the QF and the PF. (D) Analysis of mRNA expression profile. (E) Heatmap representing relative proliferative/quiescent gene expression levels for genes in the AKT pathway. Relative expression levels were obtained from microarray data analysis. Red represents low gene expression in PF relative to gene expression in quiescent cells while green represents high gene expression in PF relative to gene expression in quiescent cells. Color scale is provided at the bottom of the figure. In bold are genes whose expression level was confirmed by Q-PCR.

this, we evaluated PTEN, total AKT and pAKT$^{\text{-Thr308}}$ expression, which is one of the hallmarks of the activated PI3K/AKT pathway. We also assessed subcellular localization of FOXO1 and expression levels of survivin and p27 proteins.

The results showed that in the PF, miR-22 was overexpressed by three-fold, whereas PTEN mRNA was underexpressed (two-fold) [Figure 2(A)]. A significant down-regulation of PTEN protein levels was observed in the PF when compared

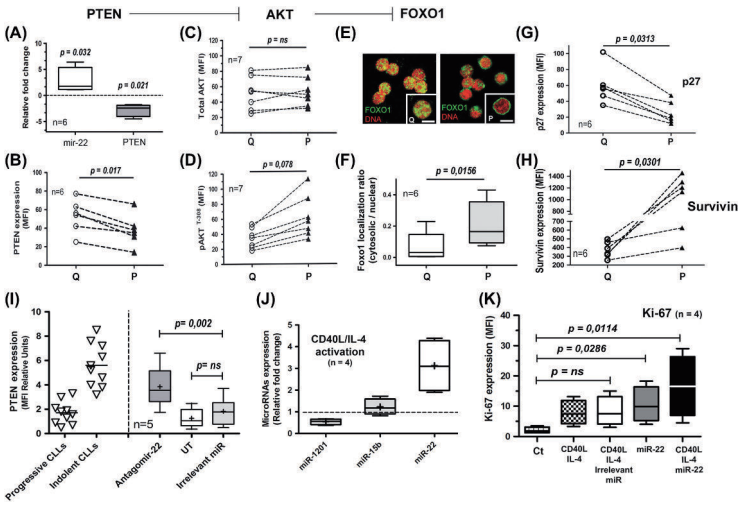

Figure 2. miR-22, PTEN, AKT and FOXO1 molecule expression levels in quiescent (Q) and proliferative (P) cells from cases of progressive UM CLL. (A, B) miR-22, PTEN mRNA and protein levels. (A) Fold change expression of miR-22 and PTEN mRNA in PF compared with QF evaluated by Q-PCR in six cases of CLL. miR-22 is overexpressed in the PF and PTEN mRNA underexpressed (mean = 2.7 ± 1.2, $p = 0.032$ and mean = − 2.5 ± 0.6, $p = 0.021$, respectively). (B) PTEN protein expression in Q (open circle) and P (black triangle) cells from six cases of CLL (01–06 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900) evaluated by flow cytometry. It is shown that low expression levels of PTEN protein were observed in the PF when compared with the quiescent counterpart (41.3 ± 7.0 vs. 52.8 ± 7.2 respectively, $p = 0.017$, Wilcoxon matched pairs test). MFI, mean fluorescence intensity. (C, D) Characterization of the phosphorylated status of AKT. Phosphorylated AKT form in threonine 308 ($pAKT^{-Thr308}$) and total AKT protein expression evaluated by flow cytometry in Q (open circle) and P (black triangle) from seven cases of CLL (01–07 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900). Results show a significant increase of $pAKT^{-Thr308}$ form in the PF (mean = 62.86 ± 10.64) when compared with the quiescent counterpart (mean = 34.29 ± 5.0; $p = 0.078$, Wilcoxon matched pairs test). In contrast, no significant differences were found in both fractions 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). (E, F) Subcellular localization of the transcription factor FOXO1. Specific fluorescence intensity in nucleus and cytoplasm was measured in the isolated QF and PF of CLLs 01–04, 07 and 08 by confocal microscopy. Between 100 and 200 cells were counted in each sample. Next, MFI for nucleus and cytoplasm was used to obtain the cytosolic/nuclear ratio. The cytosolic/nuclear mean values between QF and PF were, respectively, 0.070 ± 0.036 and 0.210 ± 0.056 ($p = 0.0156$, Wilcoxon matched pairs test). This shows that FOXO1 is mainly located in the nucleus in the QF and in the cytoplasm in the PF. Scale bar: 5 μm, green: FOXO1 and red: DNA. (G, H) p27 and survivin molecule expression levels. p27 and survivin proteins were evaluated by flow cytometry in Q (open circle) and P (black triangle) from six cases of CLL (01–06 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900). Statistical analysis of p27 demonstrated a lower expression of this marker in the PF (mean = 33.08 ± 5.4) when compared with the QF (mean = 64.44 ± 9.64; $p = 0.0313$, Wilcoxon matched pairs test). Statistical analysis of survivin showed high expression levels of this survival marker in the PF whereas low or no expression was found in the QF (mean = 1145 ± 140.7 and mean = 399 ± 35.0; $p = 0.0301$, respectively (Wilcoxon matched pairs test). (I) Role of miR-22 in PTEN regulation in CLL B-cells. Lower PTEN protein expression is observed by flow cytometry in cases of progressive CLL (01–10 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900) when compared to cases of indolent CLL (13–22 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900). PTEN protein expression is also depicted after transfection with antagomiR-22, irrelevant miR and untransfected (UT) cells in five cases of progressive CLL (01, 03, 04, 09 and 10 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900) with low PTEN expression. Statistical analysis demonstrated a significant increase ($p = 0.002$) in PTEN expression following treatment of CLL B-cells with antagomiR-22 (mean = 3.84 ± 0.48), whereas no differences were observed when these same cells were transfected with irrelevant miR (mean = 1.67 ± 0.33) and with UT control (mean = 1.26 ± 0.23), which did not display significant differences between them ($p = 0.331$). (J, K) CD40 engagement induces miR-22 expression and CLL proliferation. (J) miR-1201, miR-15b and miR-22 fold change expression of CD40L/IL-4 stimulated cells from four CD40 responder patients (CLLs 01, 04, 09 and 10 in Supplementary Table II) compared to that of unstimulated cells. (K) Ki-67 expression evaluated by flow cytometry in CLL B-cells from four indolent cases that responded to CD40 stimulation (13, 16, 20 and 22 in Supplementary Table II to be found online at http://informahealthcare.com/doi/abs/10.3109/10428194.2014.990900). Ki-67 expression was evaluated after CD40L/IL-4 stimulation, miR-22 transfection and stimulation with CD40L/IL-4 followed by miR-22 or irrelevant miR transfection. Ctr, unstimulated and untransfected cells.

with the QF of six cases of CLL [Figure 2(B)]. Since PTEN inhibits the PI3K/AKT pathway, we investigated expression levels of unphosphorylated AKT and $pAKT^{-Thr308}$, respectively, in both subsets. Results showed no significant differences in total AKT expression between the two fractions. However, significantly increased levels of $pAKT^{-Thr308}$ were evidenced in the PF compared to the QF in seven patients with CLL [Figures 2(C) and 2(D)]. Because translocation of FOXO1 from the nucleus to the cytoplasm is a consequence of an activated AKT pathway [11], we studied the subcellular localization of this

transcription factor in both subsets. Results showed that the QF displayed a homogeneous nuclear expression of FOXO1, whereas the PF predominantly exhibited a cytosolic localization of this transcription factor [Figures 2(E) and 2(F)]. Given that p27 [12] and survivin [13] have been implicated in cell cycle arrest and inhibition of apoptosis in CLL, respectively, and are associated with the PTEN/AKT/FOXO1 pathway [14], we studied their expression in the different fractions. Results showed that p27 expression was mainly found in the QF but not in the PF [Figure 2(G)], whereas high expression of survivin was mainly observed in the PF and was almost absent in the QF [Figure 2(H)].

CD40 engagement induces miR-22 expression and CLL proliferation

Since the results suggested that miR-22 could be implicated in CLL B-cell proliferation by activating the AKT pathway, we investigated whether inhibition of miR-22 might result in up-regulation of PTEN. To examine this possibility, we first evaluated pAKT^{Thr308} and PTEN expression levels in 22 patients with CLL. Then, we selected five cases displaying the highest pAKT signals and the lowest PTEN expression and proceeded with miR transfection assays. After inhibition of miR-22 by antagomiR-22 transfection, our results showed increased PTEN protein levels at similar expression values to those of indolent CLLs [Figure 2(I)]. These results suggest that miR-22 is able to down-regulate PTEN expression in CLL B-cells, enabling them to proliferate through the AKT activated pathway.

Regarding the observation that the PF expressed molecules related to the microenvironment interaction, we evaluated whether T-dependent signaling such as CD40L/interleukin-4 (IL-4) could trigger CLL B-cell proliferation through miR-22 overexpression. For that, we stimulated CD40L-responsive patients with CD40L/IL-4 and evaluated the miR-22 expression. Our results showed that CD40L/IL-4 stimulation resulted in overexpression of miR-22, whereas low effects were evidenced for miR-1201 or miR-15b expression [Figure 2(J)].

To confirm the importance of miR-22 in CLL B-cell proliferation, we evaluated Ki-67 expression in four patients with CD40L responsive indolent disease and we stimulated B-cells with CD40L/IL-4 after transfection with miR-22 or with irrelevant miR controls. Our results showed a significant increase of Ki-67 expression after CD40L/IL-4 stimulation, to the same level as the condition with CD40L/IL-4 plus irrelevant miR transfected cells, when compared to B cells without stimulus. However, B cells with CD40L/IL-4 plus miR-22 transfection showed a significant increase in Ki-67 positive cells compared to B cells with CD40L/IL-4 plus irrelevant control [Figure 2(K)]. The results suggest that miR-22 could play a role in CLL proliferation.

Discussion

Understanding the crosstalk between malignant B-cells and their milieu could give us new clues on the cellular and molecular biology of CLL. It is clear that most proliferative events occur in the tissues, where leukemic cells are able to exploit microenvironmental interactions to acquire tumoral growing conditions [1]. This PF coexists, in different proportions, with clonal B-cells which are arrested in G0/G1 stages. The balance between the two compartments could contribute to the variable clinical course of patients with CLL [15].

In the present work, we evaluated the transcriptome and microRNAome of a CLL PF and conclude that miR-22 is the most overexpressed miR in this subset, and this overexpression switches on the AKT pathway in the proliferative leukemic cells.

Since a regulatory loop involving the miR-22/PTEN/AKT/FOXO1 pathway has been demonstrated [10], we investigated the obseration that the proliferative behavior of the leukemic subset depended on miR-22 overexpression and an activated PTEN/AKT cascade. Our results suggest that up-regulation of miR-22 is associated with low PTEN expression at the mRNA and protein levels. Furthermore, in the PF, AKT is switched on probably through PDK1, which phosphorylates and activates the threonine protein kinase AKT (pAKT^{Thr308}). Also, we observed that the transcription factor FOXO1 is mainly localized in the cytoplasm of the PF cells, whereas in the QF it showed nuclear localization. These data agree with previous results suggesting that AKT activity is associated with nuclear export and cytosolic degradation of FOXO proteins [11,14]. Also, two key molecules involved in the CLL biology, p27 and survivin, which appear to be downstream to the AKT/FOXO1 pathway, were studied. The results showed that in the PF, p27 protein is down-regulated and survivin is overexpressed in the PF of UM CLL. Finally, transfection experiments with CD40L engagement and evaluation of Ki-67 expression confirmed the proliferative feature of the PF and propose that microenvironmental signals could be involved in this tumor proliferation.

Taken together, our data provide new evidence helping to understand the crosstalk between this proliferative B-cell pool and its microenvironment. These findings emphasize the importance of this proliferative subset in PB from patients with UM CLL with poor clinical outcome, and highlight the relevance of microenvironmental signals in the maintenance of the CLL tumoral clone through the regulatory miR-22/PTEN/AKT/FOXO1 cascade.

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Supplementary material available online

Supplementary Tables I–II showing further data.