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Molecular 3 e Inmunología
Universidad de Granada

Instituto de Parasitología y Biomedicina
"López-Neyra"
CSIC

Mecanismos de acción de CD38 en señalización, migración celular y patologías humanas autoinmunes

Jaime Sancho   Mercedes Zubiaur   Frances E. Lund

Memoria presentada por la licenciada Esther Cristina Zumaquero Martínez para optar el grado de doctor por la Universidad de Granada

Granada, 05 de Diciembre de 2010

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Mecanismos de acción de CD38 en señalización, migración celular y patologías autoinmunes

Esther C. Zumaquero Martínez

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F) ANEXO I: Publicaciones

- **Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signalling complexes containing CD81, Hsc-70 and Lyn**. Zumaquero E, Muñoz P, Cobo M, Lucena G, Pavón EJ, Martín A, Navarro P, García-Pérez A, Ariza-

Veguillas A, Malavasi F, Sancho J, Zubiaur M. **Exp Cell Res. 2010 Oct 1;316(16):2692-706.**

G) ANEXO II: Trabajos no publicados

- Increased CD38 expression in T cells and high blood plasma levels plasma levels of anti-CD38 IgG autoantibodies identify two different subsets of patients with systemic lupus erythematosus.

- Clinical and immunological evaluation of a patient affected with Hemophagocytic Lymphohistiocytosis associated with Leishmania infection.

- Deficient activation of peripheral blood T cells from Pemphigus patients upon stimulation with a mixture of superantigens.

H) ANEXO III: Colaboraciones

- Role of murine CD38, TRPM2 and purinergic receptors in migration of immune cells.

Resumen

Resumen

Esta tesis doctoral que presentamos a continuación ha sido el resultado de una apuesta por el trabajo en equipo, así como la colaboración e interacción con otros grupos de investigación y personal médico, que nos han permitido hacer diferentes abordajes en el estudio de CD38.

Resultados previos de nuestro laboratorio y otros colegas nos hicieron pensar que CD38 podría ser secretado al exterior celular a través de exosomas derivados de células B. Los exosomas son vesículas de origen endocítico implicadas en comunicación de células tumorales y células del sistema inmune. Además, las balsas lipídicas han sido implicadas en la selección de proteínas asociadas a exosomas. CD38 se localiza en membrana y en endosomas de reciclamiento y se dirige a la sinapsis inmunológica tras la activación de CD38. Los datos de este primer estudio demuestran que CD38 se expresa en exosomas derivados de células B. CD38 en exosomas de asocia a las moléculas de señalización CD81, Hsc70 y Lyn. De la misma manera, en balsas lipídicas, CD38 está asociado a CD81, CD19, Lyn, Gαi-2, Hsc-70 y actina. Estos resultados demuestran que existe un elevado grado de semejanza en las proteínas a las que CD38 se encuentra asociado tanto en exosomas como en balsas lipídicas. CD38 es enzimáticamente activo tanto en exosomas como en balsas lipídicas, y la activación de la vía CD38 induce activación de Akt/PKB/Erk. Este estudio indica que CD38 se localiza en balsas lipídicas y es exportado al exterior a través de exosomas pudiendo transmitir señales intercelulares.

A continuación desarrollamos un trabajo en el que demostramos que los niveles plasmáticos de autoanticuerpos anti-CD38 y la expresión de CD38 en células T era significativamente mayor en pacientes de lupus eritematoso sistémico (LES) en comparación con controles sanos. Sin embargo, la expresión de CD38 en células T de LES correlacionan con los niveles plasmáticos de ciertas citoquinas, incluyendo las citoquinas de tipo 1 y tipo 2, siendo más prevalente en

pacientes con un LES clínicamente activo, mientras que anticuerpos anti-CD38 elevados correlacionan con incrementos moderados de IL-10 e IFN-γ. Estos datos indican que la expresión incrementada de CD38 en células T de LES podría ser la consecuencia de la acción de citoquinas proinflamatorias como TNF-α e IFN-γ, e indicativo de pacientes con LES con una mayor actividad de la enfermedad mientras que la presencia de autoanticuerpos anti-CD38 en plasma podría ser indicativo de de pacientes con LES con una enfermedad relativamente controlada.

CD38 en ratón está implicado en migración de células del sistema inmune hacia un grupo de quimioquinas. Es sabido que los productos de la actividad enzimática de CD38 dan lugar a la entrada de Ca^{2+} extracelular necesaria en quimiotaxis, pero sin embargo, hasta ahora dicho canal se desconoce aunque había muchos indicios que indicaban que se trataba del canal TRPM2. Usando los ratones *Trpm2*^{-/-} hemos demostrado que éste no es el canal responsable de la entrada de Ca^{2+}.

Introducción

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I. CD38

1. Descripción

CD38 humano es una glicoproteína transmembrana de tipo II de 45-kDa (300 aa), con un dominio N-terminal citoplasmático corto (23 aa), un dominio transmembrana de 20 aa y un dominio C-terminal extracelular más largo (256 aa), donde reside su capacidad enzimática [1, 2], el sitio de unión a NAD^+ y cuatro sitios de glicosilación **(Figura 1)**. Esta molécula también puede existir en forma soluble en fluidos biológicos en condiciones normales y patológicas [3].
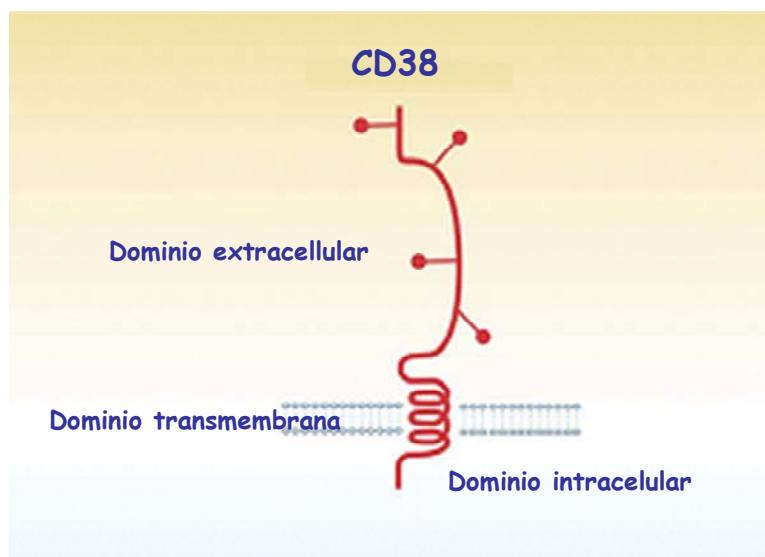
Figura 1. Esquema de los dominios que componen CD38

CD38 se expresa en diferentes tipos celulares incluyendo timocitos, linfocitos T activados, células plasmáticas, células "natural killer" (NK), monocitos, macrófagos, células dendríticas (DCs) con lo cual CD38 es una proteína ampliamente distribuida en células del sistema inmune [4, 5]. Durante la ontogénesis de los linfocitos B, CD38 está fuertemente regulado y su expresión es alta en los precursores de médula ósea, disminuye su expresión en linfocitos B naïve y de nuevo se expresa en células plasmáticas diferenciadas [6]. Este comportamiento sugiere que CD38 se expresa en

diferentes momentos durante el desarrollo de linfocitos B, cuando las interacciones célula-célula son importantes [5, 7]. CD38 también está ampliamente distribuido en tejido no linfoide como células β pancreáticas, osteoclastos, algunas células epiteliales o córnea.

Aunque CD38 está presente principalmente en la membrana plasmática, también se ha detectado en la membrana nuclear de algunos tipos celulares como en la línea osteoblástica murina MC3T3 [8], en hepatocitos de rata [9] y en células mieloides humanas HL-60 diferenciadas con ácido retinoico (ATRA) [10].

CD38 es una ectoenzima con funciones receptoras.

- CD38 es una enzima multifuncional que a partir de los sustratos NAD^+ y $NADP^+$ cataliza la formación de segundos mensajeros implicados en la regulación de los niveles de Ca^{2+} intracelular **(Figura 2)**, la ADP-ribosa cíclica (ADPRc) y el ácido nicotínico fosfato adenina dinucleótico (NAADP) movilizan el Ca^{2+} desde el interior de la célula y la ADP-ribosa está implicada en la entrada de Ca^{2+} desde el exterior celular.
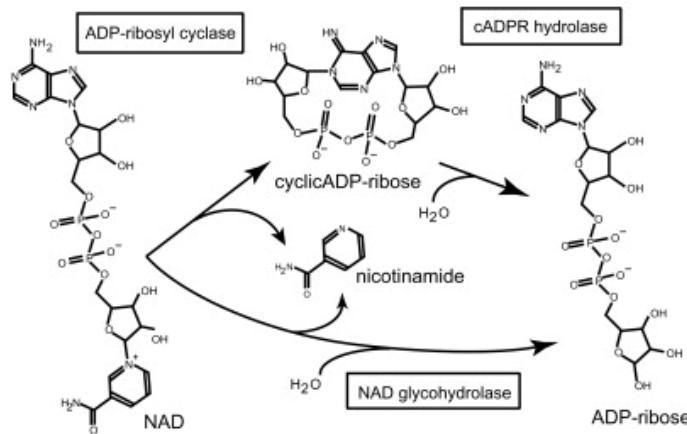
- CD38 es también un receptor, participando en procesos como movilización de Ca^{2+}, activación celular, o modulando la producción de citoquinas. Además favorece la señalización de otras moléculas como el TCR/CD3 en células B, el BCR en células B y el CD16 en células NK.

2.Gen

El gen que codifica CD38 está localizado en el cromosoma 4 [11] humano en la región p15 [12] y en el cromosoma 5 en ratón [13].

CD38 humano está codificado por un gen de copia única de más de 62 kb y que tiene ocho exones y siete intrones **(Figura 3)**, incluyendo un gran intrón que interrumpe la región 5´ codificante. El exón 1 codifica para la región N-terminal, la región de transmembrana y los primeros 33 aa de la región extracelular. El resto de los exones (2-8) codifican el dominio extracelular. La región 5´ "aguas arriba" del gen se caracteriza por la ausencia de las cajas TATA y CAAT, por la presencia de una región rica en GC inmediatamente "aguas arriba" del codón de inicio, así como la presencia de algunos sitios de inicio de la transcripción y de sitios de unión para factores de transcripción [14].
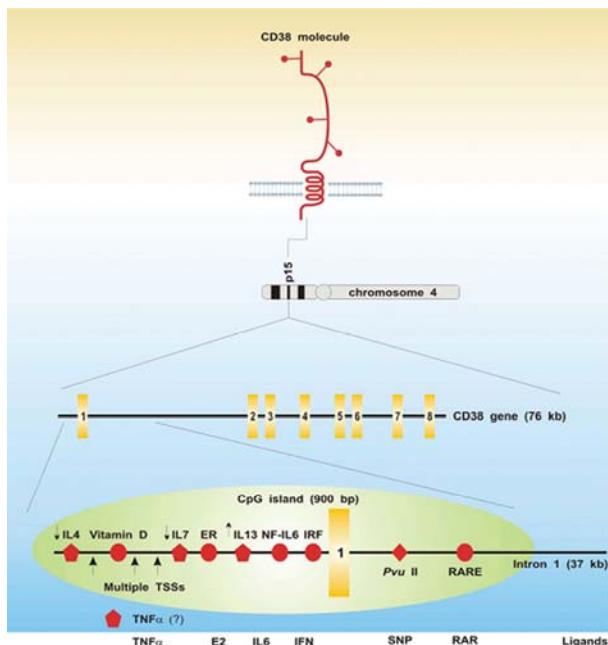
Deaglio S. y col. *Blood* 2006 (**108**); 1135-1144

Figura 3. Representación esquemática del gen *Cd38* con una ampliación de la zona "aguas arriba" más cercana al exón 1 y parte del intrón 1.

CD38 tiene un polimorfismo bien caracterizado localizado en el extremo 5´ del primer intrón (182C>G), con la aparición de un nuevo sitio de restricción *PvuII* [15]. Además este intrón contiene la isla CpG y el elemento de respuesta al ácido retinoico (RARE) responsable del aumento de expresión de CD38 inducida por ácido retinoico (ATRA) [6] **(Figura 3)**. Otra mutación puntual es C/T localizado en el exon 3, en la posición 418, dando lugar al cambio de la Arg^{418} por Trp [16]. Se ha investigado la posible asociación de polimorfismos localizados en la posición 182 del intrón 1 (C/G) y 418 (C/T) localizado en el exon 3, con la susceptibilidad y manifestaciones clínicas del lupus eritematoso sistémico (SLE). Según sus datos, el genotipo CC confiere susceptibilidad y el genotipo CG confiere protección al desarrollo de lupus discoide (cutáneo), por lo que sugieren una ligera influencia en el polimorfismo localizado en el exón 1 del gen *Cd38* al desarrollo del lupus discoide en pacientes con SLE [17].

En la familia de genes que codifican ADP-ribosil ciclasas se conocen, además de *Cd38*, otros dos genes estructuralmente relacionados, el que codifica para la proteína CD157 (anclada a membrana mediante glicosilfosfatidilinositol (GPI)) y el que codifica para la ADP-ribosil ciclasa del molusco *Aplysia californica* que es una proteína soluble [18, 19]. Los genes muestran una marcada conservación en la estructura de los exones así como en la estructura de las proteínas que codifican. Además, en las tres proteínas, la actividad ciclasa responsable de la conversión catalítica del NAD^{+} en cADPR [15] está mediada en el extremo C-terminal.

Goodrich et al. han caracterizado un nuevo miembro de la familia ciclasa que se encuentra en el parásito *Schistosoma mansoni* denominado NACE. Esta proteína está anclada a membrana mediante GPI y al igual que CD38 es una ectoenzima que cataliza la producción de NAADP a partir de NADP^{+} y la hidrólisis de NAD^{+} a ADPR. Sin embargo su actividad ciclasa (síntesis de cADPR a partir de NAD^{+}) es muy baja [20].

3. Estructura cristalina del dominio extracelular del CD38 humano (CD38hs)

Liu et al. [21] obtuvieron la estructura cristalina del dominio extracelular del CD38 humano **(Figura 4)** y compararon las secuencias de CD38hs, CD157 y la ciclasa de *Aplasia californica*.
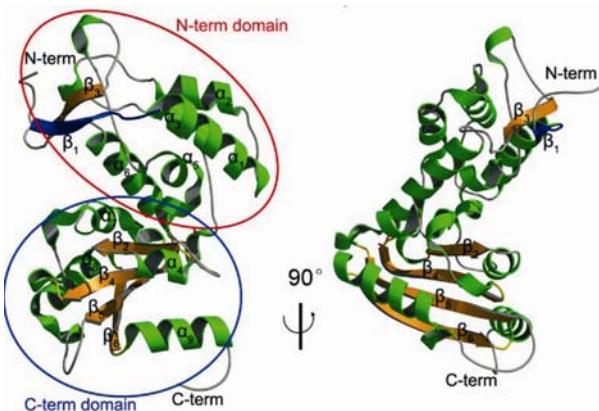
Liu, Q. y col. *Structure* (2005) Vol 13 ;1331-1339

Figura 4. Estructura del CD38 humano soluble.

Mediante mutagénesis dirigida de CD38 se han identificado varios residuos críticos en el sitio activo: Glu^{226}, Trp^{125}, Trp^{189} y Glu^{116} **(Figura 5)**.
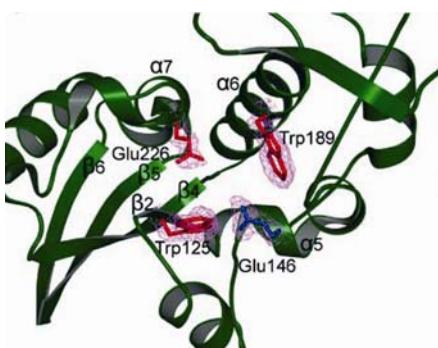
Figura 5. Estructura del centro activo de CD38

Liu, Q. et al. *Structure* (2005) Vol 13; 1331-1339

Los estudios llevados a cabo por Liu y su equipo demostraron:

a) El reemplazamiento del residuo Glu^{226} por cualquier otro residuo resulta en la pérdida completa de todas las actividades enzimáticas [22].

b) Los dos residuos de Trp parecen ser responsables del posicionamiento del sustrato.

c) La incubación de CD38 con NAD$^+$ da lugar predominantemente a la producción de ADPR gracias a su actividad NAD-glicohidrolasa, aunque también se produce una pequeña cantidad de cADPR mediante la ciclación del sustrato. El residuo Glu146 tiene un importante papel en la regulación de las actividades ciclasa y NADasa de CD38, ya que al reemplazar dicho residuo por Phe aumenta fuertemente la actividad ciclasa a un nivel similar al de la actividad NAD$^+$ hidrolasa [23].

Los residuos RWRQTW del extremo N-terminal del dominio de CD38 cristalizado tienen cadenas cargadas positivamente que están próximas a la membrana plasmática. Éstos podrían interaccionar con las cargas hidrofílicas negativas de los lípidos de las balsas lipídicas **(Figura 6)**.
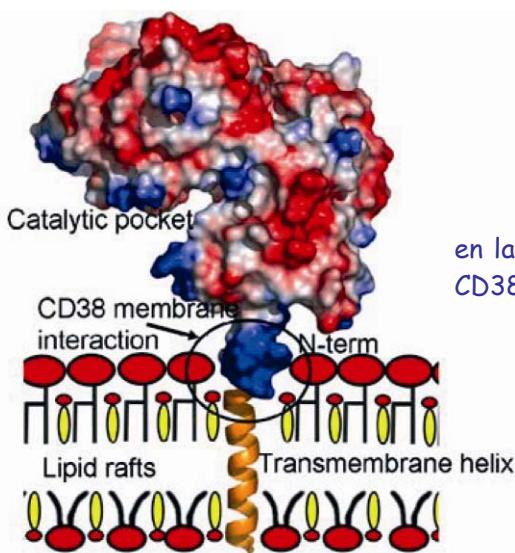
Figura 6. Posible interacción de CD38hs en la superficie de la célula e interacciones entre CD38 y las balsas lipídicas

Liu, Q. et al. *Structure* (2005) Vol 13 ;1331-1339

Esta aportación de *Liu et al.* es muy importante para nuestro grupo ya que dota de una base estructural al descubrimiento, por vez primera, en nuestro laboratorio, de que CD38 está asociado a las balsas lipídicas tanto en linfocitos T como en linfocitos B [24, 25].

4. Funciones

Todavía está en discusión si existe o no una relación entre la función enzimática y la función receptora de CD38. Los estudios sobre la evolución de la familia de las ciclasas parecen indicar que la función enzimática precede a la receptora, de manera que la adquisición de esta doble función **(Figura 7)** pudiera dar lugar a una ventaja selectiva.
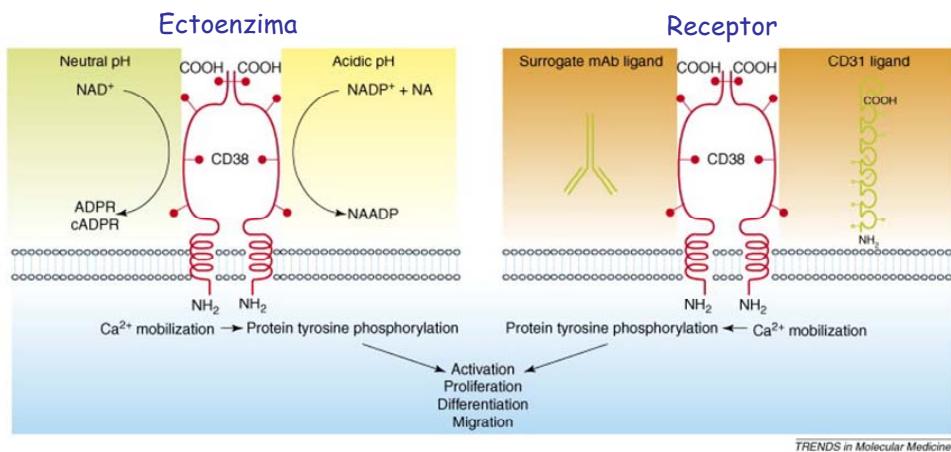
Figura 7. CD38 es una ectoenzyma que también tiene funciones de receptor

4.1 CD38 como ectoenzima

La identificación de una secuencia similar entre el antígeno humano CD38 y la ADP ribosil ciclasa de *Aplysia* [26] marcó el comienzo del estudio de las propiedades enzimáticas de CD38 así como su función en fisiología y patología. Muchos años de investigación han revelado que CD38 es una ectoenzima multifuncional que está implicada en el catabolismo del NAD^+ y $NADP^+$. La actividad enzimática de CD38 lleva a la generación de potentes agentes mobilizadores de calcio (cADPR, NAADP y ADPR); su principal producto, ADPR, puede ser covalentemente unido a proteínas y de esta manera modificar las funciones de las proteínas. La importancia de estos procesos enzimáticos han sido demostrados no sólo en el sistema inmune sino también en órganos y tejidos, como útero, bronquios, páncreas y riñón [27].

a) Actividad enzimática de CD38

Las propiedades enzimáticas de CD38 fueron formalmente demostradas al añadir NAD^+ a una forma recombinante soluble (sCD38), ésta catalizó la formación e hidrólisis

de cADPR [28]. Resultados similares se obtuvieron tras la solubilización de membranas de eritrocitos y usando un anticuerpo monoclonal anti-CD38 se obtuvieron tres actividades ectoenzimáticas, NAD glicohidrolasa (NADasa), ADP-ribosil ciclasa y cADPR hidrolasa [29].

Estos resultados se confirmaron en linfocitos T humanos, donde la actividad catalítica sobre el NAD^+ correlacionaba con la cantidad de CD38 presente en la superficie celular. Además el CD38 inmunoprecipitado de timocitos se comportaba como una auténtica NADasa, tranformando NAD^+ en nicotinamida y ADPR [30].

Experimentos llevados a cabo mediante la transfección transitoria del cDNA de CD38 en células COS1 resultó en la expresión de moléculas de CD38 capaz de convertir NAD^+ a cADPR en el medio extracelular, dando lugar a la liberación de Ca^{2+} [31]. Hay claras evidencias que indican que la cADPR es un producto de reacción más que un intermediario en la actividad glicohidrolasa [32].

Inicialmente, la mayoría de los investigadores se centraron en la producción de cADPR, dado su importante papel en fisiología celular y posteriormente se prestó atención a otras actividades enzimáticas. El dominio extracelular de CD38 media la ADP ribosilación de varias proteínas, incluyéndose a sí mismo. Este proceso tiene lugar en los residuos de cisteína, y puede ser revertido por la presencia de $HgCl_2$, que específicamente rompe puentes tioglicosídicos [33]. Se ha sugerido que durante la exposición de las células T activadas a NAD^+, CD38 es modificado por ecto-mono-ADP-ribosiltransferasas (ARTs) específicas para cisteína y residuos de arginina. La ADP ribosilación en arginina da lugar a la inactivación de la actividad ciclasa e hidrolasa de CD38, mientras que la ADP ribosilación en cisteína produce solamente la inhibición de la actividad hidrolasa. La ADP ribosilación en arginina provoca un descenso en los niveles de cADPR intracelular y una posterior disminución en los niveles de Ca^{2+}, causando la muerte de células T activadas [34]. Podemos decir que las ectoenzimas que metabolizan nucleótidos son parte de un conjunto de proteínas que funcionan de forma sincronizada y con una gran organización.

El papel de la ADP ribosilación en el control de la homeostasis de las células T fue demostrado en una serie de trabajos relacionados con la muerte celular inducida por NAD^+ (NICD). Se ha observado que la acumulación extracelular de NAD^+ induce la ADP ribosilación del receptor purinérgico $P2X_7$. Este fenómeno induce la activación ATP

independiente de este receptor iniciando un proceso de apoptosis [35]. Este modelo propone que las ARTs pueden percibir y traducir la concentración local extracelular de NAD$^+$ en los niveles correspondientes de proteínas de superficie ADP-ribosiladas, mientras que CD38 controla los niveles de ADP ribosilación de las proteínas celulares de superficie limitando la disponibilidad del sustrato por parte de las ARTs [36]. Se piensa que todo éste proceso culmina en una expansión selectiva de células T activadas a expensas de los linfocitos T naïve que son los únicos sensibles a la muerte inducida por NAD$^+$ y contribuyendo a la homeostasis de células T [37] **(Figura 8)**. En este contexto, estudios recientes muestran que el NAD$^+$ actúa como una citoquina proinflamatoria, estimulando los granulocitos humanos y reclutándolos a los sitios de inflamación [38]. Estos eventos están mediados a través del receptor purinérgico P2Y$_{11}$ sugiriendo una interacción funcional entre las ADPR ciclasas y los receptores purinérgicos [39].
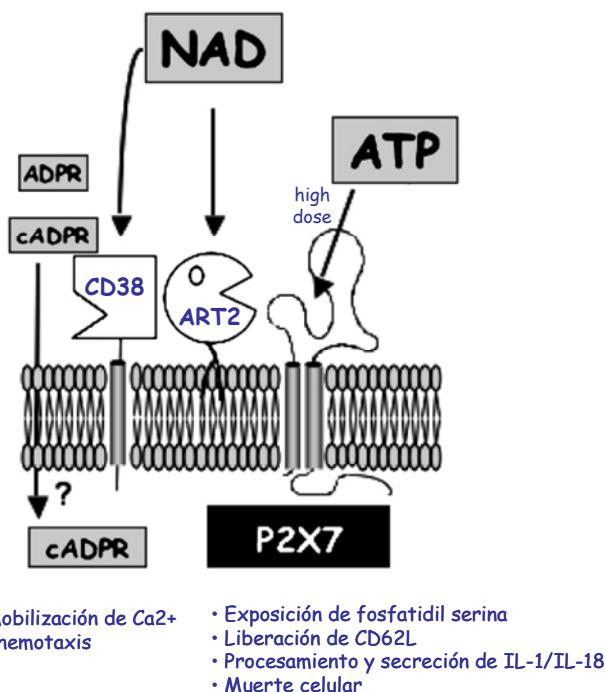
Figura 8. Acción del ATP y NAD extracellular en diferentes receptores de superficie. El ATP extracellular a altas dosis activa al receptor purinérgico P2X7. El ATP extracellular activa al receptor purinérgico. El NAD extracellular activa al receptor purinérgico P2X$_7$. El NAD extracellular sirve como sustrato para la ADP-ribosiltransferasa 2 (ART2) o bien es hidrolizado a ADPR a través de CD38. CD38 también puede sintetizar cADPR. Existe un tipo de activación ATP independiente del receptor P2X$_7$, en el que la acumulación extracellular de NAD+ induce la riboslación del receptor purinérgico por la ART2. CD38 actuaría limitando la disponibilidad del sustrato.

El papel de CD38 en el control de los niveles de NAD$^+$ ha sido examinado por el grupo de E. Chini. Estos autores, cambiando el enfoque de la superficie celular al compartimento intracelular, postularon que CD38 es la principal NADasa en células de mamífero y que regula los niveles de NAD$^+$ intracelular [40]. En un estudio en paralelo, los mismos autores propusieron que CD38 podría modular la actividad de las sirtuinas, deacetilasas dependientes de NAD$^+$ implicadas en el envejecimiento, protección celular y metabolismo energético en células de mamífero. Esta regulación tiene lugar en el núcleo y está mediada por el CD38 que se expresa en la membrana nuclear interna [41]. Recientemente se ha comprobado que la regulación CD38/sirtuina juega un papel importante en la regulación del peso corporal en ratones **(Figura 9)** [42] .
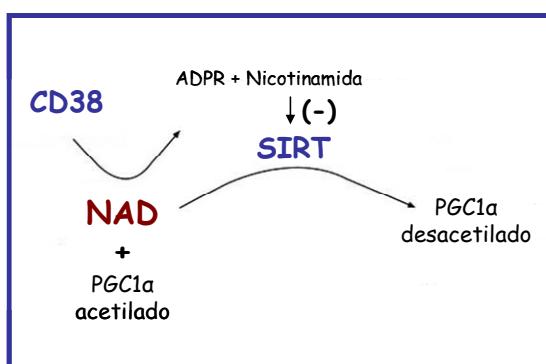
Figura 9. Los efectos de SIRT1 en obesidad y metabolismo energético son, al menos, en parte, mediados por desacetilación y activación del receptor de proliferación de peroxidasa (PGC1-a). El grupo de E. Chini ha propuesto que modulando la disponibilidad de NAD y nicotinamida , CD38 regula la actividad de SIRT1.

CD38 está también implicado en el intercambio catalítico del grupo nicotinamida de NADP$^+$ con ácido nicotínico (NA). El producto es ácido nicotínico adenín dinucleótido fosfato (NAADP), un potente movilizador de Ca^{2+} [43, 44]. Esto ocurre selectivamente a un pH ácido; la dependencia acídica del metabolismo del NAADP junto con su función biológica en la liberación de Ca^{2+} de compartimentos acídicos, sugieren que el NAADP actúa como un mensajero en orgánulos acídicos de la vía endocítica celular [45]. Hay suficientes datos que indican que el NAADP está implicado en la liberación de Ca^{2+} de lisosomas y endosomas [46]. Sin embargo, también se ha visto que el NAADP puede liberar Ca^{2+} del retículo endoplasmático [47, 48]. De manera que, además del IP3, los almacenes de Ca^{2+} pueden ser movilizados por al menos otras dos moléculas, cADPR y NAADP, que se unen a diferentes receptores y liberan Ca^{2+} de diferentes compartimentos

[49]. La importancia del NAADP como segundo mensajero ha sido confirmada en células pancreáticas, las cuales tienen propiedades que las hacen particularmente atractivas para los estudios de señalización Ca^{2+} dependiente. Varios estudios indican que hay una importante relación funcional entre el IP3, cADPR y NAADP en la transformación de picos locales de Ca^{2+} (via IP3) a incrementos globales de Ca^{2+} (via cADPR y NAADP) [50].

Recientemente se le ha prestado un especial interés a la ADPR. Aunque es el principal producto de CD38, en un principio no se conocía su papel en señalización intracelular. Más tarde se supo que la ADPR activa el canal TRPM2 al unirse al dominio Nudix [51]. Estos datos revelaron que la ADPR y el NAD^+ actúan como mensajeros intracelulares y pueden jugar un importante papel en la entrada de Ca^{2+} mediante la activación de TRPM2 en células del sistema inmune [52]. Investigaciones más recientes muestran que el cADPR y NAADP a bajas concentraciones favorecen considerablemente la activación de TRPM2 por ADPR [53-55]. Esta vía de activación ha sido estudiada en células T Jurkat activadas con altas concentraciones de concanavalina A, induciendo un incremento en la concentración de ADPR, activación de TRPM2 y eventualmente muerte celular [56].

b) Paradoja del sistema CD38-cADPR

El hecho de que el dominio catalítico de CD38 sea extracelular y los productos de su actividad enzimática (cADPR y ADPR) se generen en el exterior de la célula da lugar a una serie de reflexiones sobre

i) la accesibilidad del NAD^+, ya que está solo presente en pequeñas cantidades fuera de la célula

ii) la incorporación del cADPR/ADPR al citoplasma [57]

El NAD^+ intracelular se encuentra en concentraciones micromolares mientras que por el contrario el NAD^+ extracelular se encuentra en concentraciones nanomolares. Una posible solución podría ser que los canales de connexina 43 (Cx43) transportaran NAD^+ desde el interior al exterior de la célula [58]. CD38 podría entonces catalizar la formación de cADPR, que bien es transportado al interior de la célula mediante transportadores nucleosídicos (NT) [59], también se ha propuesto que la estructura homodímera de CD38 se comporta como un canal por el cual se transporta cADPR [60]. Hay evidencias claras de un extenso tráfico de nucleótidos y sus metabolitos a través de la membrana

plasmática y a través de la membrana de vesículas citoplasmáticas. Siguiendo con este modelo, las células Cx43+ y CD38+ permiten generar cADPR que puede funcionar de forma paracrina sobre células vecinas aumentando los niveles de Ca^{2+} intracelular [60].

La actividad ADP-ribosil ciclasa (cADPR) ha sido detectada en la mayoría de las células y tejidos siendo la cADPR un segundo mensajero implicado en muchas funciones de las células eucariotas [61].

4.2 CD38 como receptor

Las primeras evidencias sobre la función receptora de CD38 tuvo lugar al observar que un anticuerpo monoclonal anti-CD38, IB4, inducía la proliferación de células mononucleares de sangre periférica humana. Este fenómeno es dependiente de IL-2 y actúa sinérgicamente con las vías de activación de CD2 y CD3 [62]. Experimentos posteriores demostraron que CD38 también estaba implicado en la transducción de señales de activación y proliferación en células de otros linajes [63]. Resultados similares también se observaron en células B de ratón. El anticuerpo monoclonal anti-CD38, NIM-R5, provocaba un incremento de Ca^{2+} intracelular e inducía un aumento de la expresión de HLA de clase II a células B, inicialmente en reposo [64]. Todos estos resultados demuestran que CD38 se comporta como un receptor de superficie en la membrana plasmática. Esta idea cobró más fuerza tras la identificación de un ligando específico para CD38, CD31, que expresado ectópicamente inducía efectos similares a los anticuerpos anti-CD38 agonistas arriba descritos [65]. Dado que la cola citoplasmática de CD38 es muy corta y carece de secuencias o dominios señalizadores conocidos se propuso que CD38 tenía que trabajar coordinadamente con otros receptores de membrana o con moléculas señalizadoras, ya sea mediante una asociación física o funcional.

A continuación se hace un resumen de las publicaciones más interesantes relacionadas con capacidad receptora de CD38.

a) Ligandos de CD38

Las primeras evidencias de la existencia de un ligando no sustrato de CD38 humano procede de experimentos en los que el uso de anticuerpos monoclonales anti-CD38 bloqueaba la adhesión de células T CD4^{+}/CD45RA^{+} a células endoteliales. Estos experimentos de adhesión sugieren que las células T se unen débilmente al endotelio a

través de CD38 de una forma similar a las selectinas, implicadas en el rodamiento del linfocito sobre el endotelio así como en la extravasación del linfocito al tejido [66]. Con el objetivo de buscar un posible ligando para CD38 se creó un panel de anticuerpos monoclonales dirigidos a células endoteliales del cordón umbilical humano (HUVEC). Pocos anticuerpos consiguieron inhibir de forma consistente la adhesión mediada por CD38 [65], uno de esos anticuerpos, Moon-1, reconocía a CD31. Quedó demostrado que la interacción CD38-CD31 era capaz de modular no sólo adhesión sino también un incremento en los niveles de Ca^{2+} intracelular similar a los obtenidos mediante anticuerpos monoclonales anti-CD38 [67].

Esta interacción CD38-CD31 ha sido estudiada tanto en células T como en células B, NKs o células mieloides, tanto en situaciones normales como en situaciones patológicas. S. Deaglio hace una magnífica revisión de la interacción CD38-CD31 en adhesión y señalización en leucocitos humanos [68].

Otro ligando propuesto para CD38 es el ácido hialurónico, éste es un polisacárido que forma parte del tejido conectivo. CD38 tiene, tanto en el dominio extracelular como en el dominio intracelular, motivos de unión al ácido hialurónico [69].

b) Señalización a través de CD38 en diferentes tipos celulares

Un modelo muy aceptado a mediados de los años 90 era que CD38 era un receptor que por si mismo no tenía capacidad señalizadora y que necesitaba de alguna forma coordinarse o asociarse a receptores específicos de cada linaje que si tenían capacidad señalizadora ampliamente contrastada. Estas ideas propiciaron varios estudios de asociación de CD38 con el TCR/CD3 en células T, con BCR/CD19/CR2 en células B y con CD16 en células NK, que revelaron la proximidad física e interacciones funcionales de CD38 con esos receptores [70]. Mientras que el TCR/CD3, CR2 y CD16 son estructuras de unión a ligando específicas en sus respectivos linajes, CD38 está implicado en la transducción intracelular de las señales. Aunque este modelo ha sido revisado, todavía se cree que CD38 está física y funcionalmente unido a estos complejos de señalización específicos de cada linaje.

1. PBMCs y linfocitos T
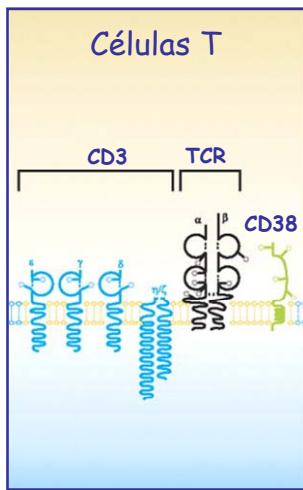
Muchos trabajos han estudiado la vía de señalización de CD38 comparándola con la del TCR/CD3 y CD2. Al igual que ocurre con estas dos moléculas, la unión de CD38 a un anticuerpo monoclonal induce la expresión de mRNA de múltiples citoquinas. Muchas de estas citoquinas también son inducidas por la activación del TCR/CD3. Sin embargo, la activación de CD38 y CD3 difieren en que los niveles de mRNA de IL-2 permanecen bajos tras la activación vía CD38, mientras que los niveles de mRNA de IL-1β e IL-6 incrementan [71]. También se hicieron estudios similares en poblaciones purificadas, la activación vía CD38 mediante el uso de anticuerpos monoclonales de células T purificadas de sangre periférica de un grupo de individuos dio lugar a la producción de ciertas citoquinas, por ejemplo, los niveles de mRNA de IL-6, GM-CSF, IFN-γ e IL-10 se incrementaron considerablemente, sin embargo se detectaron bajos niveles de mRNA de IL-2, IL-4 e IL-5 [71].

La evidencia más cercana que indica una relación funcional entre el TCR/CD3 y CD38 procede de los estudios llevados a cabo en nuestro laboratorio en células T Jurkat mutantes. Aquellos mutantes que carecen de un complejo TCR/CD3 funcional pierden la habilidad de señalizar vía CD38, la reconstitución del complejo TCR/CD3 da lugar a la recuperación de la señalización a través de CD38. Además, al igual que el complejo TCR/CD3, CD38 es capaz de inducir muerte celular por apoptosis en células Jurkat [72]. Estos resultados podrían ser debido a que la activación vía CD38 lleva a una completa fosforilación en tirosina de las cadenas CD3-ζ y CD3-ε [73], aunque la fosforilación de CD3-ζ no es totalmente necesaria para la señalización de CD38 en células T [24]. Un análisis detallado de los eventos moleculares que se producen en los linfocitos T

inmediatamente después de la unión de un anticuerpo monoclonal agonista a CD38 indica que se produce la fosforilación en tirosina de PLC-γ1, c-Cbl, ZAP-70 y Shc, un incremento en la expresión de CD69 y eventos dependientes de la activación de Ras como es la activación de Erk2. Estos eventos son muy similares a los que se producen tras la activación mediada por el TCR/CD3, aunque con cinéticas diferentes [74]. La activación mediada por CD38 y en particular la activación de Erk-2, depende fuertemente de la actividad funcional de la tirosina quinasa Lck, como se demostró en las células JCam1.6, que son mutantes de la línea de linfocitos T Jurkat en las que Lck es funcionalmente deficiente [74].

El grupo de F. Malavasi ha explorado la relación entre CD38 y el TCR/CD3 desde otro ángulo, mediante un análisis comparativo de la señalización vía CD38 en células T circulantes y linfocitos T residentes en lámina propia intestinal. A diferencia de los linfocitos T periféricos, los linfocitos T de lámina propia no movilizan Ca^{2+} tras la activación a través de CD38 y ésto es debido al fallo en la activación de PLC-γ [75].

Más recientemente, nuestro laboratorio ha contribuido enormemente al estudio de las balsas lipídicas en la iniciación de la vía de señalización de CD38. Nuestro laboratorio ha sido pionero en el descubrimiento de que CD38 se encuentra enriquecido en las balsas lipídicas. Esta presencia de CD38 en las balsas lipídicas podría mejorar la capacidad de señalización de la molécula a través de los microdominios de membrana ya que permitiría el reclutamiento de moléculas implicadas en señalización [24]. Este tema fue investigado por mis colegas de laboratorio, gran parte de CD38 se localiza en las balsas lipídicas que contienen altos niveles de Lck y la subunidad CD3-ζ, estando este último asociado con CD38. La unión de un anticuerpo monoclonal a CD38 induce la fosforilación de LAT y Lck exclusivamente en las balsas lipídicas [76]. La relevancia de Lck para la señalización de CD38 fue confirmado en experimentos que demuestran una asociación física entre la cadena citoplasmática de CD38 y el dominio homólogo de Src 2 de Lck. Estos resultados y los anteriormente descritos [74] sugirieron que la activación de los linfocitos T vía CD38 se lleva a cabo a través de la asociación de Lck [77].

2. Linfocitos B
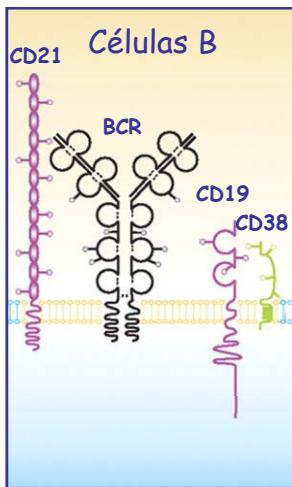
El estudio de las funciones de CD38 en linfocitos B humanos se llevó a cabo inicialmente en nódulos linfáticos, donde CD38 está fuertemente expresado. El grupo de M. Ferrarini demostró que la unión de CD38 con el anticuerpo monoclonal IB4 previene de la apoptosis de células B tonsilares humanas de los centros germinales, aunque este efecto no fue observado con otros anticuerpos monoclonales [78].

Posteriormente el grupo de D. Campana dirigió su atención al estudio de CD38 en médula ósea. La activación de CD38 en células B humanas progenitoras en cultivo en presencia de células estromales inhibe fuertemente la linfopoyesis de las células B. Este bloqueo en la diferenciación es debido tanto a la inhibición de la síntesis del DNA como a la inducción de apoptosis. Además la actividad enzimática no se afecta por la unión de CD38 a un anticuerpo monoclonal, incluso no se detectan cambios en la hidrólisis de NAD$^+$ o ADPR cíclica ni en la producción de ADPR tras la activación de CD38. Del mismo modo, la adición de NAD$^+$, ADPR o cADPR no logra compensar el efecto inhibitorio mediado por los anticuerpos monoclonales anti-CD38 [79]. Éste fue el primero de muchos estudios que indican que la unión de un anticuerpo monoclonal agonista no tiene influencia en la actividad enzimática de CD38 reforzando la idea de que la actividad enzimática y receptora de CD38 son dos funciones independientes de la misma molécula. Estos mismos estudios revelaron que CD19 es un componente principal en la cascada de señalización de CD38 en células B precursoras, que sirve de lugar de anclaje de quinasas citoplásmáticas. Además se observó que CD38 y CD19 activaban a un conjunto

de quinasas similares en células B humanas inmaduras [80]. Experimentos posteriores llevados cabo por este mismo grupo demostraron que tras la unión de un anticuerpo monoclonal agonista anti-CD38 le seguía la dimerización y la fosforilación en tirosina de la protein quinasa syk así como un incremento en la actividad quinasa de syk. La dimerización de CD38 también dio lugar a la fosforilación en tirosina de PLC-γ y de la subunidad de p85 de la fosfatidilinositol 3-kinasa (PI3K) [81], sin embargo Btk no es fosforilada en tirosina tras la activación de CD38, una peculiaridad de las células B precursoras [80]. Por otra parte este mismo grupo demostró que la actividad PI3K es esencial en la inhibición de la linfopoyesis mediada por CD38. Además la activación de cbl y PI3K suprime la proliferación celular y la apoptosis en células inmaduras linfoides [82].

Es evidente que CD38 canaliza una señal de activación/proliferación en células B maduras humanas. La activación de CD38 en células B humanas circulantes induce la expresión de CD25, HLA de clase II y mRNA de algunas citoquinas, dando lugar a proliferación. Efectos similares también han sido observados en células plasmáticas. En ningún caso se ha observado la producción de inmunoglobulinas tras la activación vía CD38 [83].

Actualmente existe un gran interés en la señalización de CD38 en células B ya que está implicado en la leucemia linfocítica crónica de células B (B-CLL), siendo un marcador de mal pronóstico de la enfermedad. Los estudios llevados a cabo por F. Malavasi, S. Deaglio y M.Zubiaur en células B tonsilares y líneas de células B indican que la señalización mediada por CD38 está regulada a diferentes niveles.

i) Organización estructural de CD38, que puede comportarse como monómero o dímero
ii) Localización de CD38 tanto en "balsas lipídicas" como en otras zonas de la membrana plasmática
iii) Asociación con complejo CD19/CD81

La localización en balsas lipídicas y la asociación con el complejo CD19/CD81 son requisitos para la señalización mediada por CD38 en células B tonsilares y en líneas celulares, la desorganización de las balsas lipídicas y/o el silenciamiento de CD19 da lugar a la pérdida de señalización mediada por CD38 [25, 84].

3. Células mieloides

La incubación de células mieloides inmaduras en presencia de ácido retinoico da lugar a un incremento en la expresión de CD38 [85], de ahí el interés de CD38 en estas células. La señalización intracelular mediada por CD38 ha sido investigada en la línea celular mielocítica humana HL-60 diferenciada con ácido retinoico. Los resultados demostraron que c-Cbl podría ser un sustrato de CD38 al ser uno de los principales sustratos fosforilados en tirosina en la vía de señalización de CD38 [86]. Además, el uso de un anticuerpo anti-c-Cbl dió lugar a la co-inmunoprecipitación de la subunidad p85 de PI3K pero solamente cuando c-Cbl estaba fosforilado [87]. Parece ser que la consecuencia última de la unión de CD38 a un anticuerpo monoclonal en líneas celulares mieloides es la de intensificar la producción de superóxido inducida por fMLP. Sin embargo, CD38 por sí solo, no es capaz de producir superóxido, con lo cual tiene que haber algún tipo de comunicación entre la fosforilación en tirosina inducida por CD38 y la via de señalización del receptor de quimioquina acoplado a proteína G [88].

CD38 y la molécula HLA de clase II están física y funcionalmente asociados en las balsas lipídicas de los monocitos humanos. Además, la integridad de estos dominios es esencial para la correcta señalización de HLA de clase II y CD38. Adicionalmente, se ha demostrado que la tetraspanina CD9 es otro componente del complejo CD38/HLA de clase II y que HLA-II, CD38 y CD9 comparten una vía de activación en monocitos humanos [89]. La expresión de CD38 en monocitos humanos está regulada por la citoquina proinflamatoria IFN-γ. El IFN-γ produce un incremento en la expresión de CD38, que es paralelo un incremento en la actividad ADP- ribosil ciclasa y actividad hidrolasa. Por el contrario el LPS, TNF-α y GM-CSF no tienen ningún efecto [89]. La señalización vía CD38 incluye la producción de IL-1β, IL-6 e IL-10 [90].
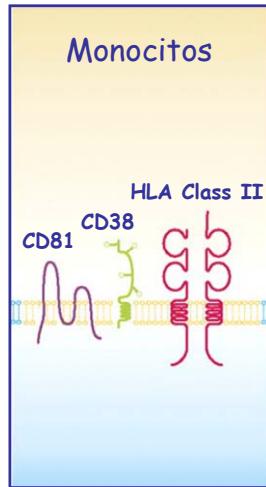
Recientemente se ha prestado cierto interés en el estudio de CD38 en células dendríticas. Hay una disminución en la expresión de CD38 durante la diferenciación a células dendríticas inmaduras derivadas de monocitos y se vuelve a expresar tras la maduración. CD38 no es un simple marcador de activación en células dendríticas, sino que también ejerce funciones receptoras. La activación de estas células con anticuerpos monoclonales anti-CD38, induce la expresión de CD83 y la secreción de IL-12 [91]. Los estudios llevados a cabo por el grupo de C.M. Ausiello en células dendríticas maduras derivadas de monocitos indican que CD38 permite una quimiotaxis así como una migración transendotelial eficiente hacia la quimioquina CCL21. Además CD38 se encuentra asociado con CCR7, CD83 y CD11b, y al igual que ocurre en monocitos, linfocitos T o linfocitos B, CD38 se localiza en balsas lipídicas [92].
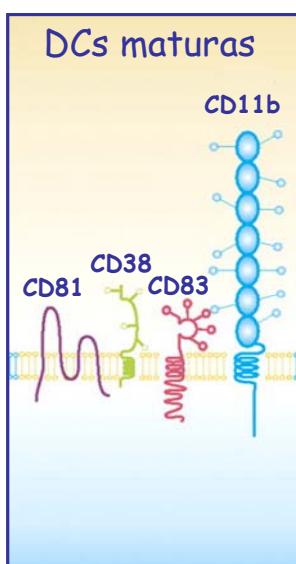
4. Células NKs

CD38 se expresa en células NK activas y en reposo. La activación de CD38 en células NK en circulación da lugar a un incremento en la concentración de Ca^{2+} intracelular, un incremento en la expresión de HLA de clase II y de CD25 así como fosforilación en tirosina de sustratos citoplasmáticos como las cadenas de CD3-ζ y FcγRI, ZAP-70 y c-Cbl. Los efectos a largo plazo de la señalización vía CD38 incluye producción de IFN-γ y GM-CSF y la inducción de funciones efectoras citolíticas [93]. CD38 forma un complejo junto con CD16, incluso se ha observado proximidad física entra CD38 y CD16. Un CD38 funcional es un requisito necesario para una correcta señalización vía CD38 [94].
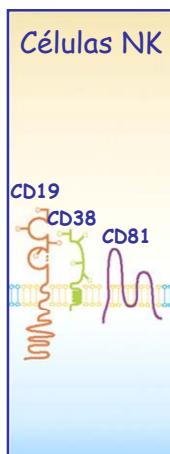
5. Ratones deficientes para *Cd38⁻/⁻*

El modelo murino *Cd38⁻/⁻* es una herramienta muy útil para el estudio de CD38 *in vivo* así como el efecto que ejercen los metabolitos producidos por CD38. No obstante, hemos de tener presente que los efectos que se pueden observar en el modelo murino, no siempre se pueden extrapolar a lo que ocurre en el humano.

Los ratones *Cd38⁻/⁻* carecen casi por completo de actividad NADasa, con una reducción significativa de los valores de cADPR aunque no total, indicando que CD157 y/u otros miembros no indentificados de esta familia podrían compensar la ausencia de CD38 [95-97]. Igualmente los niveles de NAD^+ están alterados en muchos tejidos, aunque las consecuencias fisiológicas no están aún muy claras.

Los ratones *Cd38*^{-/-} son viables y parecen reproducirse correctamente, con lo cual CD38 y su actividad enzimática no es esencial para la vida del ratón. Estudios más profundos de estos ratones confirmaron que CD38 no está implicado en el desarrollo de los diferentes linajes hematopoyéticos, aunque parece que la molécula es requerida para una correcta respuesta humoral dependiente de células T [95].

El grupo de la doctora F. Lund ha llevado a cabo diversos estudios sobre el sistema inmune en los ratones *Cd38*^{-/-} bajo ciertas condiciones, como infecciones bacterianas. La pérdida de CD38 da lugar a una mayor susceptibilidad de los ratones a infecciones bacterianas debido a la incapacidad de los neutrófilos *Cd38*^{-/-} de migrar a los sitios de infección [98]. Cuando estos ratones son infectados con *S. pneumoniae*, las bacterias diseminan rápidamente desde el pulmón a la sangre, haciendo que estos ratones sean 10 veces más susceptibles a la infección por *S. pneumoniae* y que los neutrófilos de estos ratones sean incapaces de migrar eficientemente al pulmón [98]. Este defecto se atribuye a la falta de cADPR y ADPR, que están implicados tanto en la liberación de Ca^{2+} intracelular como en la entrada de Ca^{2+} del exterior necesario en la migración de los neutrófilos tras la estimulación con fMLP, un quimioatrayente bacteriano [98]. Sin embargo, aunque los neutrofilos *Cd38*^{-/-} son incapaces de migrar en respuesta a varios quimioatrayentes y quimioquinas como fMLP o MIP-1α, son capaces de migrar correctamente hacia otras quimioquinas como IL-8 [99]. Estudios posteriores en células dendríticas demostraron que CD38 regula la migración de los precursores de las DCs de la sangre a la periferia y la migración de las DCs maduras de los sitios de inflamación a los nódulos linfáticos, a través de la producción de cADPR y ADPR. La consecuencia de ésto es que las células T no son eficazmente activadas en los ratones dando lugar a una pobre respuesta humoral [100].

Experimentos llevados a cabo en el laboratorio de F. Lund indican que la pérdida de CD38 en las células β pancreáticas incrementa la sensibilidad de estas células al daño mediante fármacos dando lugar a un desarrollo acelerado de la diabetes, sugiriendo que CD38 directamente regula la función de las células β pancreáticas. De acuerdo con ésto, experimentos llevados a cabo en el laboratorio de Jim Johnson demuestran que las células β pancreáticas aisladas de ratones *Cd38*^{-/-} tienen defectos en la respuesta de calcio a la estimulación por insulina y son más propensas a la apoptosis en respuesta a diferentes estímulos, incluyendo la falta de suero y altas concentraciones de glucosa [101]. En este contexto, *Ed Leiter y colaboradores* han descrito que la diabetes se desarrolla más

rápidamente en ratones *Cd38^{-/-}* con un fondo genético NOD/Lt (modelo murino de diabetes) [102]. En sus experimentos también demuestran que esta mayor susceptibilidad de los ratones NOD.*Cd38^{-/-}* a tener diabetes puede revertirse cuando se elimina la ART2 específica de linfocito T (mono ADP-ribosil transferasa 2) [102]. Al igual que CD38, ART2 utiliza NAD$^+$ como sustrato aunque no produce metabolitos solubles [103]. ART2 ADP-ribosila proteínas de membrana, incluyendo el receptor purinérgico P2X$_7$ [35]. La ribosilación de P2X$_7$ por la ART2 que expresan los linfocitos T permite la activación de este receptor y producir la muerte celular inducida por NAD$^+$ [35]. La pérdida de CD38 en los ratones NOD da lugar a una disminución en el número de células T reguladoras presentes en estos animales, sin embargo este fenotipo se revierte en ratones a los que les falta CD38 y ART2 [102]. Esto no es demasiado sorprendente dado que las células T reguladoras son muy sensibles a la muerte celular inducida por NAD$^+$ [104] y que la NICD dependiente de ART2 está aumentada en *Cd38^{-/-}*, principalmente debido a una mayor disponibilidad del sustrato por parte de ART2 [36]. Es razonable pensar que la eliminación de CD38 lleva a un incremento en la muerte de células reguladoras dando lugar a una respuesta autoinmune más agresiva. Aunque el desarrollo acelerado de la diabetes en este modelo es muy evidente, no está claro si en humanos la pérdida o la inactivación de CD38 podría tener el mismo impacto en el desarrollo de la diabetes, ya que en los linfocitos T humanos no expresan un homólogo al ART2 de ratón [105].

Usando ratones *Cd38^{-/-}* también se ha demostrado que CD38 regula la contractilidad del músculo liso [106, 107], tiene una función en resorción del hueso ya que en su ausencia de desorganiza la formación de osteoclastos [108] o está implicado en la regulación de la secreción de oxitocina dando lugar a alteraciones en el cuidado materno [109].

6. Enfermedades asociadas a CD38

El análisis de la expresión de CD38 se utiliza actualmente para el diagnóstico de leucemia y mieloma, siendo un marcador de pronóstico independiente de gran utilidad en los pacientes de leucemia linfocítica crónica de células B. Además, la expresión de CD38 en las células T CD8$^+$ y los valores absolutos de linfocitos CD4$^+$ son herramientas fiables que nos permiten predecir la progresión del SIDA en los pacientes infectados con VIH. La expresión de CD38 correlaciona con la carga viral en la infección temprana con HIV y está asociada con una menor supervivencia en personas con un avanzado estado de la

enfermedad (Giordi et al. Infect Dis 1999). La bajada de la expresión de CD38 es un buen indicador de la efectividad de la terapia anti-retroviral (HAART). Además, el deterioro funcional del sistema CD38/cADPR ha sido asociado con diabetes no-insulina dependiente (tipo 2).

a) Leucemia linfocítica crónica de células B

Tradicionalmente la leucemia linfocítica crónica de células B (B-CLL) se ha dividido en 2 grupos dependiendo de la presencia o no de mutaciones en los genes IgV. Los pacientes con genes IgV no mutados desarrollan una forma más agresiva de la enfermedad con un peor prognóstico.

Actualmente la presencia de CD38 de superficie y la quinasa citoplasmática ZAP-70 junto con la ausencia de mutaciones en los genes IgV define una forma agresiva de leucemia linfocítica crónica.

Entre los investigadores que trabajamos en CD38 hay un especial interés en CLL, ya que esta enfermedad podría usarse como modelo para el estudio *in vivo* de la función biológica de CD38. La hipótesis más extendida es que CD38 no es un simple marcador sino que su expresión en superficie tiene un potencial patogénico. De acuerdo con esta hipótesis, se ha observado que la unión de CD38 a anticuerpos monoclonales agonistas da lugar a proliferación y transformación a blastos de un conjunto de células procedentes de B-CLL, demostrando que CD38 puede comportarse como un receptor con función señalizadora [110]. Las propiedades de señalización mediadas por CD38 *in vitro* se incrementan por la presencia simultánea de IL-2, que actúa incrementando la expresión de CD38. Es posible que las señales mediadas por CD38 puedan ser activadas tras la interacción con CD31. Las células CD38$^+$ en presencia de fibroblastos transfectados con CD31 manifiestan un mayor crecimiento y una mayor supervivencia. Además, la interacción CD38/CD31 induce un incremento de expresión de CD100, una proteína implicada en mantener el crecimiento y supervivencia de las células B-CLL [111]. Este modelo está indirectamente confirmado por el hallazgo de que las células *nurse-like* derivadas de pacientes con B-CLL expresan altos niveles de CD31 funcional y plexin-B1, un ligando de de alta afinidad de CD100.

La estimulación vía CD38 lleva a un transitorio pero significativo incremento en la fosforilación de ZAP-70 en células CD38$^+$/ZAP-70$^+$ procedente de leucemia linfocítica

crónica. Un amplio estudio de las señales mediadas por CD38 han demostrado que la vía de señalización de CD38 es selectivamente activa en células CD38$^+$/ZAP-70$^+$. Esta observación es clínicamente relevante ya que nos proporciona un fundamento molecular para el estudio de la expresión simultánea de CD38 y ZAP-70 en pacientes con B-CLL [112].

b) Diabetes

La secreción de insulina es clave en la homeostasis de la glucosa y es una de las funciones mediada por CD38 conocida desde hace dos décadas.

Pupilli et al. demostraron la presencia de anticuerpos anti-CD38 en sueros de pacientes diabetes tipo 1 (insulina-dependientes) y tipo 2 (no insulino-dependientes) en la población caucásica y que estos anticuerpos ejercen un efecto estimulatorio de la secreción de insulina en los islotes pancreáticos humanos. La incubación de estos islotes pancreáticos con sueros CD38$^+$ daba lugar a la movilización de Ca^{2+} y potenciaba el aumento de la producción de insulina de una forma dosis-dependiente siendo independiente de la concentración de glucosa en el medio [113].

En células mononucleares de sangre periférica (PBMCs) la estimulación in vitro con anticuerpos monoclonales anti-CD38 agonistas, induce la liberación de citoquinas proinflamatorias como la IL-1, IL-6 y el factor de necrosis tumoral alfa (TNF-α), por lo tanto no es absurdo pensar que los anticuerpos anti-CD38 tengan un efecto similar contribuyendo a la a la enfermedad. (De Esther. J)

c) Lupus eritematoso sistémico

Una de las enfermedades autoinmunes de gran interés en nuestro laboratorio es el lupus eritematoso sistémico (SLE). Los estudios llevados a cabo por *E.J Pavón et al.* demuestran que la expresión de CD38 en estos pacientes está aumentada en linfocitos T CD3, CD4, CD8 y CD25. Este incremento de expresión se correlaciona con una mayor proporción de CD38 en las balsas lipídicas. Además, las células T de estos pacientes muestran una alteración en la ratio CD4:CD8, debido a una disminución en la proporción de linfocitos T CD4$^+$. Estos datos son coherentes con los obtenidos tras la estimulación *in vitro* de linfocitos T normales con un mitógeno como PHA y la consiguiente expansión con

IL-2 [114]. El incremento de expresión de CD38 en las balsas lipídicas podría modular la señalización del TCR.

En un trabajo reciente, *E.J Pavón et al* muestran que el incremento de expresión de CD38 en células T de enfermos de SLE correlaciona con los niveles de citoquinas plasmáticas de tipo 2 (IL-4, IL-10 y IL-13) y tipo 1 (IL-1β, IL-12, IFNγ y TNFα). Sin embargo la expresión de CD38 en células B correlaciona con IL-10. Es interesante destacar el hecho de al igual que ocurre en diabetes, *E.J Pavón* et al han encontrado anticuerpos anti-CD38 en plasma de un grupo de pacientes con SLE. Sorprendentemente, aquellos pacientes que presentan anticuerpos anti-CD38 presentan un perfil de citoquinas diferente al de los controles sanos y enfermos de SLE sin anticuerpos anti-CD38, con niveles más bajos de IL-6, IFN-γ e IL-10.

II. Exosomas

Los exosomas son pequeñas microvesículas secretadas por las células, que han sido objeto de un gran interés en los últimos años. Originalmente se pensó que la función principal de los exosomas era la eliminación de moléculas de superficie, sin embargo trabajos posteriores de muchos laboratorios han demostrado la importancia de los exosomas para la biología en general y el sistema inmunológico en particular.

1. Composición de los exosomas

a) Propiedades físicas y purificación de los exosomas

Los estudios bioquímicos y funcionales de exosomas presentan cierta dificultad técnica en el aislamiento y purificación. Los exosomas son fácilmente contaminados por otras vesículas membranosas como vesículas apoptóticas o vesículas procedentes de membrana plasmática, de ahí que se requiera de una rigurosa purificación. Combinando diferentes técnicas como centrifugación diferencial, filtración, concentración, centrifugación en gradiente de densidad e inmunocaptura, los exosomas pueden ser aislados de una gran variedad de líneas celulares y fluidos corporales [115]. Una vez aislados los exosomas se caracterizan mediante microscopía electrónica, citometría de flujo, espectrometría de masas y *westtern blotting* [115, 116]. El mayor problema a la hora de comparar resultados procedentes de diferentes estudios es la gran diversidad de estrategias utilizadas en la purificación de exosomas, y la posibilidad de que muchas preparaciones de exosomas estén contaminadas con otro tipo de vesículas. La **Tabla 1** nos muestra las características fisicoquímicas de los diferentes tipos de vesículas secretadas.

Tabla 1. Características fisicoquímicas de los diferentes tipos de vesículas secretadas

Característica	Exosomas	Microvesículas	Ectosomas	Partículas de Membrana	Partículas similares a exosomas	Vesículas apoptóticas
Tamaño	50-100 nm	100-1,000 nm	50-200 nm	50-80 nm	20-50 nm	50-500nm
Densidad en sacarosa	1.13-1.19 g/ml	ND	ND	1.04-1.07 g/ml	1.1 g/ml	1.16-1.28 g/ml
Apariencia en microscopio electrónico	Ligeramente alargada	Irregular	Estructura redonda bilaminar	Forma redondeada	Forma irregular	Heterogénea
Sedimentación	100,000 g	10,000 g	160,000-200,000g	100,000-200,000g	175,000 g	1,200g, 10,000g or 100,000g
Composición lipídica	Enriquecido en colesterol, esfingomielina y ceramida; contiene balsas lipídicas; exponen fosfatidilcolina	Exponen fosfatidilcolina	Enriquecido en colesterol y diacilglicerol; exponen fosfatidilserina	No determinado	No contiene balsas lipídicas	No determinado
Principales marcadores	Tetraspaninas (CD63, CD9), Alix y TSG101	Integrinas, selectinas y CD40 ligando	CR1 y enzimas proteolíticas;no CD63	CD133; no CD63	TNFRI	Histonas
Origen intracelular	Compartimentos internos (endosomas)	Membrana plasmática	Membrana plasmática	Membrana plasmática	¿Compartimentos internos?	No determinado

El prodedimiento más común para purificar exosomas procedentes de cultivos celulares consiste en una serie de centrifugaciones que permiten descartar células muertas y restos celulares, seguido de una ultracentrifugación para obtener los exosomas [117, 118]. Sin embargo este procedimiento no permite diferenciar entre exosomas y otras estructuras vesiculares pequeñas o agregados proteicos.

Los exosomas presentan un rango de densidad que oscila entre 1,13 g/ml (para exosomas derivados de células B) y 1.19 g/ml (para exosomas derivados de células intestinales) [117, 119-122]. El hecho de que, al igual que otras vesículas lipídicas, los exosomas floten en gradientes de sacarosa, nos permite separar los exosomas de material contaminante como agregados proteicos o fragmentos nucleosomales procedentes de células apoptóticas [123].

Los exosomas tienen un diámetro comprendido entre 30 y 100nm, concretamente los exosomas procedentes de células B tienen un tamaño que oscila entre 60-80nm. Mediante microscopía electrónica se ha podido observar que los exosomas tienen una morfología ligeramente alargada y están limitados por una bicapa lipídica. Dichas características coinciden con las vesículas internas que se localizan en el interior de los endosomas multivesiculares [117].

Puesto que los exosomas no son las únicas vesículas de membrana que son secretadas, es necesario filtrar el sobrenadante de cultivo a través de un filtro de 0.22µm previo a la ultracentrifugación, de esta manera reducimos la contaminación de los exosomas con vesículas de mayor tamaño que son secretadas a partir de la membrana plasmática [123].

La inmunocaptura mediante bolitas magnéticas ha sido recientemente utilizada para aislar exosomas de elevada pureza [124, 125], por ejemplo el anticuerpo Her2 (receptor 2 del factor de crecimiento humano) unido a bolita magnética ha sido usado para aislar exosomas procedentes de sobrenadante de cultivo de líneas celulares tumorales [124]. Es interesante el hecho de que este método permite la eliminación de histonas, procedente de la contaminación de vesículas apoptóticas.

b) Composición molecular de los exosomas

La identificación de las proteínas celulares que forman parte de los exosomas se realiza mediante diversas técnicas como *westtern blotting*, citometría de flujo o microscopía electrónica. Sin embargo los estudios de espectrometría de masas han permitido identificar otras proteínas que no habían sido descritas anteriormente como componentes de los exosomas. Estos estudios se han llevado a cabo en exosomas procedentes de cultivos celulares, y de exosomas procedentes de materiales biológicos tan diversos como orina, plasma, leche materna o líquido amniótico.

La composición proteica de los exosomas varía dependiendo del tipo celular, pero hay un conjunto de proteínas que se repiten en diferentes estudios proteómicos y que parecen estar presentes independientemente del origen celular de los exosomas estudiados. *Mathivanan y col.* han revisado recientemente los datos obtenidos de 19 estudios proteómicos de exosomas de diferente origen [126] **(Figura)**. Entre las proteínas encontradas podemos destacar:

- *Proteínas implicadas en la biogénesis de los cuerpos multivesiculares*
  Alix, **Tsg101** (proteína celular tumoral humana susceptible al gen 101) y **clatrina**

- *Proteínas citosólicas*:
  Proteínas Rabs: Pertenece a la familia de las pequeñas GTPasas, regula el acoplamiento del exosoma y la fusión de membranas [127]. Las proteínas Rabs activas interaccionan con proteínas implicadas en el transporte vesicular y complejos proteicos que regulan la fusión de la vesícula con la membrana aceptora [128]. **Rab4**, **Rab5** y **Rab11** se han encontrado en endosomas tempranos y endosomas de reciclamiento, sin embargo **Rab7** y **Rab9** están relacionados con endosomas tardíos [129]. Han sido identificadas hasta 40 proteínas Rab en varios estudios de exosomas.
  Anexinas: proteínas implicadas en el tráfico de membranas y fusión [119, 130].

- *Proteínas de choque térmico:* **Hsp60**, **Hsp70**, **Hsc70**, **HspA5**, **Cct2** (subunidad β del complejo T de la proteína 1) y **Hsp90** [118, 121, 123,

131-133]. Estas proteínas están implicadas en la presentación antigénica ya que pueden unirse a antígenos y participa en la incorporación del péptido al MHC (complejo principal de histocompatibilidad).

- *Tetraspaninas:* constituyen una de las familias más abundantes en los exosomas. Destacamos **CD63**, **CD81, CD82** y **CD9** [134-137].

- *Proteínas implicadas en funciones específicas:*

    MHCII: Es muy abundante en exosomas procedentes de células presentadoras de antígenos [138-140].

    MHCI: También implicada en la presentación antigénica [140].

    CD86: Se ha identificado en exosomas procedentes de DCs como una molécula coestimuladora importante para células T [117, 141].

    Proteínas transmembrana:

    Cadenas α y β de integrinas: **αM** en DCs, **β2** en DCs y células T y **α4β1** en reticulocitos [119, 142].

    Familia de la inmunoglobulinas: ICAM1 (molécula de adhesión intercelular 1) en células B o **P-selectina** en plaquetas

    Peptidasas: CD26 en enterocitos

- *Otras:*

    Enzimas metabólicas: peroxidasas , kinasas, enolasa-1

    Enzimas implicadas en señales de traducción: proteín kinasas, 14-3-3 y proteínas G [118, 119, 123, 131].

    ATPasas

    Proteínas del citoesqueleto: actina, tubulina, proteínas de unión a actina.

Al igual que las proteínas, los lípidos presentes en los exosomas son característicos de la célula de la cual proceden. Se han llevado a cabo estudios de la composición lipídica de los exosomas derivados de reticulocitos [143], mastocitos [144], líneas celulares de linfocito B [145] y células dendríticas humanas [144]. Las vesículas internas de endosomas tardíos y los exosomas de células transformadas con el virus de Eptstein Barr (EBV) están enriquecidos en colesterol, al igual que los microdominios de membrana conocidos como "balsas lipídicas" [146]. Estudios llevados a cabo por *de Gassart et al.* han demostrado la presencia de balsas lipídicas en los exosomas [147].
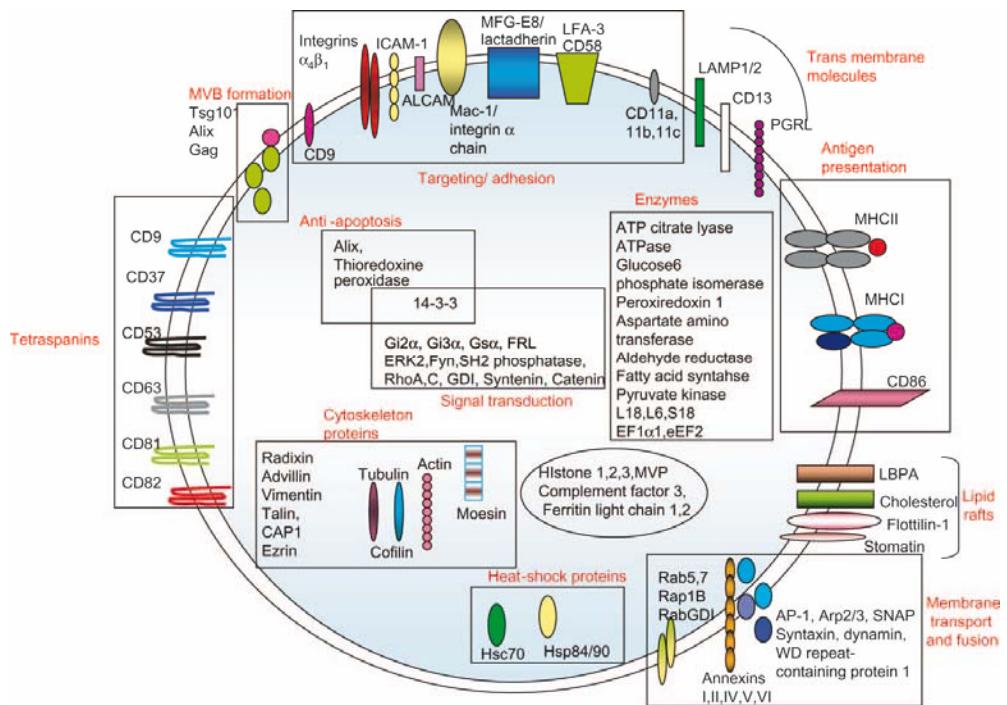
Figura 10: Composición proteica de los exosomas indicando su nombre, su localización y en algunos casos su función.

c) Los exosomas como biomarcadores

Algunas proteínas asociadas con enfermedades renales se han detectado en exosomas aislados de orina, esto podría permitir el uso de los exosomas de orina como fuente de biomarcadores [148]. Por ejemplo, *Pisitkun et al.* demostraron que los pacientes de diabetes insípida autosómica y recesiva secretaban a través de la orina exosomas que contenían la proteína acuaporina-2. Los estudios proteómicos llevados a cabo en exosomas procedentes de orina han puesto de manifiesto un conjunto de proteínas con posible valor diagnóstico [149].

Al igual que en patologías renales, la presencia de determinadas proteínas en los exosomas podría ser muy útil como biomarcadores para el diagnóstico del cáncer. Hay un especial interés en el estudio del cáncer de vejiga, ya que se han identificado un conjunto de proteínas que podrían ser usadas como marcadores en el diagnóstico de dicho cáncer [148].

Los exosomas también podrían ser usados como biomarcadores en enfermedades infecciosas y podrían ayudar a definir mejor el tratamiento de ciertas patologías, aunque ésto todavía no ha sido comprobado. Una de las mayores limitaciones en el desarrollo de un fármaco eficaz en el tratamiento de la tuberculosis es la dificultad técnica en el diagnóstico temprano de la enfermedad. Actualmente se está llevando a cabo un gran esfuerzo en identificar biomarcadores en fluidos biológicos de enfermos con tuberculosis, se cree que la presencia de determinadas proteínas en los exosomas podría utilizarse en el diagnóstico de esta enfermedad infecciosa [150].

2. Biogénesis de los exosomas

a) Origen endosómico de los exosomas

El análisis proteico de los exosomas indica que éstos no contienen proteínas del núcleo, mitocondria, retículo endoplasmático o aparato de Golgi. Todas las proteínas exosómicas que se han identificado proceden del citosol, de compartimentos endocíticos o de membrana plasmática. Si comparamos los exosomas con la membrana plasmática de las células de las cuales proceden observamos que no son simples fragmentos de membrana ya que carecen de proteínas muy abundantes en membrana plasmática, tales como receptores Fc en exosomas derivados de células dendríticas [123]; CD28, CD40L y CD45 en exosomas derivados de células T [132]; y el receptor transferrina en exosomas derivados de células B [117, 124]. Además, algunas proteínas exosómicas como CD9 o la integrina αMβ2, que a priori parecían proceder exclusivamente de la membrana plasmática, se han detectado en los compartimentos endocíticos de células dendríticas [123]. Muchas de las proteínas citosólicas encontradas en exosomas también se han encontrado recientemente en la vía endocítica, como anexina II [151], Rab5/Rab7 [151] y Tsg101. Todos estos resultados avalan la hipótesis del origen endosómico de los exosomas.

Sólo un conjunto de proteínas del sistema endosómico/lisosómico están presentes en exosomas. Por ejemplo, los exosomas no contienen proteasas lisosomales. Además los exosomas que son secretados por células B excluyen la cadena invariante (CD74), la molécula de clase II no polimórfica HLA-DM así como el marcador lisosomal Lamp2 (glicoproteína 2 asociada la membrana lisosomal) [134]. La cadena invariante está

también ausente de los exosomas derivados de DCs [140] y mastocitos [139], sin embargo Lamp2 es detectado en exosomas de DCs [140].

El mecanismo de selección de proteínas durante la formación de los exosomas en los cuerpos multivesiculares no ha sido estudiado en profundidad. Sin embargo se ha observado que la ubiquitilación del dominio citosólico de determinadas proteínas podría estar implicada [152, 153], la ubiquitín ligasa, c-Cbl, ha sido identificada en exosomas derivados de células T [132]. Sin embargo otros mecanismos independientes de ubiquitina podrían tener lugar.

b) Invaginación de la membrana endosómica

La biogénesis de los exosomas determina la orientación de la membrana. Si los exosomas se forman mediante invaginación hacia el interior de la membrana del endosoma, los exosomas deberían contener proteínas citosólicas y las proteínas transmembrana que forman parte de la membrana exosómica deberían tener la misma orientación que en membrana plasmática. Los anticuerpos que reconocen las proteínas citosólicas identificadas en los exosomas, tales como Hsc70 y anexina II, no marcan la totalidad de los exosomas mediante inmunomicroscopía electrónica, a diferencia de aquellos anticuerpos que reconocen los dominios extracelulares de la molécula MHC de clase II, CD9 o la integrina αMβ2 [119, 139]. Además, las bolitas recubiertas con anticuerpos específicos para MHC de clase II se unen a los exosomas en los sobrenadantes de cultivo [124]. Todas estas observaciones concuerdan con la orientación de la membrana propuesta y el modelo de "invaginación hacia el interior" en la biogénesis de los exosomas.

El proceso de invaginación implicado en la formación del exosoma da lugar a una orientación inversa de la membrana, a diferencia de la mayoría de los procesos de invaginación que tiene lugar en la célula. Este tipo de invaginación inversa también tiene lugar durante la apoptosis, cuando las vesículas de membrana de diferentes tamaños se desprenden de la membrana plasmática [154]. También tiene lugar en la secreción de glóbulos de leche de glándulas mamarias, o cuando las vesículas derivadas de la membrana plasmática se liberan después de la activación de plaquetas [120] o monocitos [155].

Es muy probable que todos aquellos procesos que se lleven a cabo mediante invaginación inversa requieran una maquinaria común.

c) Secreción de exosomas

Como hemos mencionado anteriormente, los exosomas son secretados al medio extracelular tras la fusión de los cuerpos multivesiculares con la membrana plasmática, dicha fusión es constitutiva en muchos tipos celulares, como células B transformadas con EBV (virus de Epstein-Barr), DCs inmaduras y células epiteliales. Sin embargo en algunas células hematopoyéticas, como células T o mastocitos, dicha fusión con la membrana plasmática depende de una estimulación acompañada de incrementos en el Ca^{2+} intracelular [156].

Actualmente se sabe muy poco sobre la maquinaria molecular que está implicada en la fusión de los cuerpos multivesiculares con la membrana plasmática. La mayoría de los eventos intracelulares de fusión de membranas se llevan a cabo a través de una maquinaria proteica específica, que incluye

- factores solubles: Nsf (proteína de fusión sensible a la N-etilmaleimida), Snap (proteína asociada al sinaptosoma) y
- complejos de membrana: Snare (receptores para Snap) [157]

Ambas membranas que están implicadas en la fusión necesitan aportar complejos de membrana específicos, denominados v-Snare (en vesícula) y t-Snare (en membrana diana).
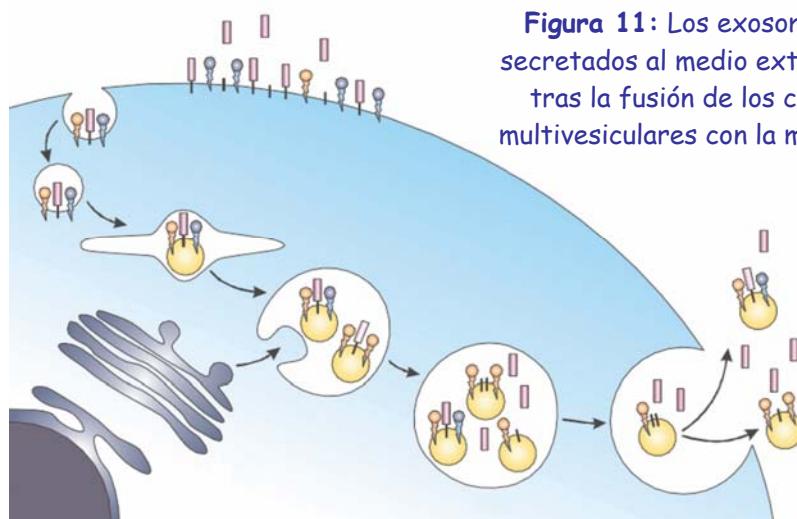
Figura 11: Los exosomas son secretados al medio extracelular tras la fusión de los cuerpos multivesiculares con la membrana

3. Función de los exosomas

En contraste con aquellas proteinas destinadas a ser degradadas a través del sistema lisosomal, los exosomas secretados son entidades biológicamente activas con importantes funciones que discutiremos a continuación.

Estudios recientes sugieren que las células pueden establecer comunicación intercelular a través de exosomas y otras vesículas de membrana [158]. Los exosomas secretados al espacio extracelular pueden permanecer en las proximidades del lugar de origen, o bien pueden recorrer largas distancias y llegar a fluidos biológicos. Esto explicaría la presencia de exosomas en plasma, orina, leche o fluido cerebroespinal. Los exosomas presentes en circulación proceden principalmente de plaquetas [159], y en menor medida de otras células sanguíneas o de células endoteliales [160]. Las células tumorales también pueden secretar exosomas, de hecho en pacientes afectados de enfermedades neoplásicas se han detectado exosomas que proceden de células tumorales [161, 162].

La función de los exosomas puede variar dependiendo del tipo celular del cual deriva y de la composición lipídica y proteica de dichos exosomas.

a) Comunicación intercelular

Los exosomas forman parte integral del espacio intercelular pudiendo ejercer una función reguladora en la comunicación intercelular. Esta idea está basada en el hecho de que los exosomas procedentes de un tipo celular concreto pueden interaccionar con otras células a través de receptores específicos [163, 164] dando lugar a una estimulación directa de la célula diana, transfiriendo receptores de membrana, proteínas funcionales o material genético, vía mRNA, microRNA (miRNA) o factores de transcripción.

a) Estimulación de la célula diana

b) Transferencia de receptores de membrana

d) Transferencia de material genético

c) Transferencia de proteína
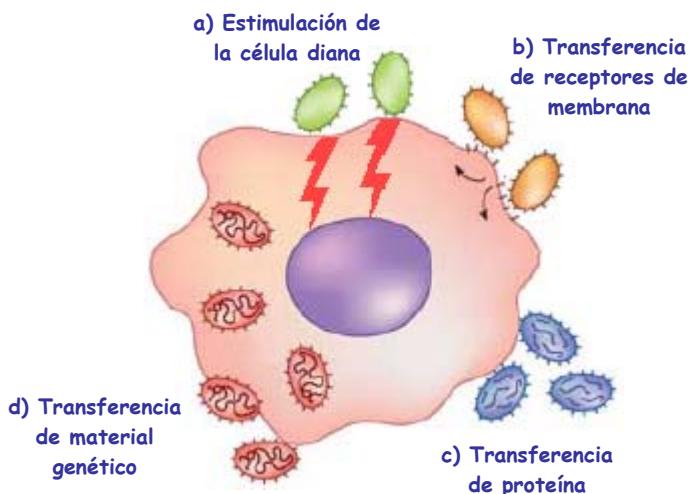
Figura 12: Representación esquemática de los mecanismos implicados en la comunicación intercelular mediada por microvesículas

i) Los exosomas pueden actuar como complejos de señalización mediante estimulación directa de la célula diana

Los estudios llevados a cabo por *Raposo et al.* demostraron por primera vez que los exosomas secretados por las células B transformadas por el virus de Epstein-Barr (EBV) eran capaces de estimular a las células T CD4+ de una manera antígeno específica [117]. Estos estudios fueron los primeros en documentar la secreción de exosomas que contienen moléculas MHC de clase II tanto de líneas celulares B humanas como murinas. Además, aquellos exosomas procedentes de DCs de ratón estimuladas con un antígeno de tumor son capaces de mediar el rechazo a tumores establecidos [140, 165]. Estos efectos antitumorales son antígenos específicos y se relacionan con las células T. También se ha descrito la estimulación directa de las células T por vesículas de membrana procedentes de células presentadoras de antígeno [166]. Por otra parte, se ha

sugerido que las células epiteliales del intestino, células T tumorales y células de melanoma son capaces de inducir tolerancia antígeno-específica y mediar apoptosis de células T mediante Fas ligando (FasL) [167, 168].

Los exosomas regulan la activación del sistema inmune mediante muerte celular inducida por activación. Un estudio reciente muestra una posible función de los exosomas en la señalización de células T durante el embarazo [169]. La placenta de mujeres embarazadas libera exosomas que contienen FasL, estos exosomas pueden suprimir importantes componentes de señalización de células T como CD3-ς y Jack-3. Esta supresión está relacionada con la presencia de Fas-L en exosomas de mujeres embarazadas [169]. Éste puede ser un mecanismo por el que la placenta contribuye a una situación de privilegio inmunológico.

En biología del desarrollo, los morfógenos juegan un papel esencial en la biología del desarrollo de tejidos. El diseño de los tejidos de Drosophila tiene lugar en respuesta a la distribución y concentración de morfógenos como Wingless y Hedgedog [170-172]. Wingless está asociado a vesículas similares a los exosomas llamados ergosomas [173, 174]. Los ergosomas se forman de una manera similar a los exosomas en los cuerpos multivesiculares.

j) **Los exosomas pueden transferrir receptores de membrana de unas células a otras**

Las células B rápidamente adquieren mediante transferencia de membranas, receptores para el antígeno (BCR) procedentes de células B activadas [175]. Esto permite la expansión selectiva de las células B que se hayan unido a un antígeno determinado favoreciendo así la presentación antigénica a las células CD4+.

Las células T, una vez activadas, capturan vesículas de membrana que son secretadas al medio de cultivo por células presentadoras de antígeno [176, 177]. Las células dendríticas adquieren proteínas transmembrana que son secretadas por otras células dendríticas [178, 179] adquiriendo una mayor eficiencia en la estimulación de células T. Aunque estos estudios no han caracterizado estas vesículas de membrana en profundidad, sí que parecen que comparten ciertas características con los exosomas. Los exosomas derivados de DCs pueden dirigirse específicamente a células T y otras DCs a través de moléculas de membrana que están presentes en su superficie [119], por ejemplo la integrina αMβ2 (Mac1) presente en los exosomas se une a Icam1 expresado

en células T y células dendríticas, y Mfge8/lactaderina presente en los exosomas se une a las integrinas αvβ3/5 expresadas en DCs.

Uno de los más claros ejemplos de la importancia de la transferencia de receptores de membrana ha sido proporcionado por *Mack et al.*, las vesículas procedentes de células de ovario de hamster y células mononucleares sanguíneas son capaces de transferir CCR5 a monocitos, células T CD4+ y células endoteliales que no expresan este receptor proporcionándole mayor susceptibilidad a infección por HIV-1 (virus de la inmunodeficiencia humana de tipo 1) [180].

k) Los exosomas pueden transferir proteínas funcionales o agentes infecciosos a las células diana.

Los exosomas juegan un papel importante en el control de crecimiento de tumores [181]. El pretratamiento de ratones con exosomas derivados de carcinomas mamarios de ratón aumenta el crecimiento de tumores debido a la inhibición de la actividad citolítica de las células NKs. Los exosomas procedentes de tumores son captados y permanecen estables en las células NKs, disminuyendo los niveles de perforina. Curiosamente, los niveles de mRNA de perforina no fueron afectados por los exosomas, lo cual indica un posible mecanismo de regulación post-transduccional, o bien que la perforina almacenada en gránulos sea degradada por alguna enzima que entre en las células NKs a través de los exosomas [181].

Se ha propuesto que existe una relación entre la evolución de los retrovirus y la biogénesis de los exosomas [182]. Los estudios con retrovirus han revelado la habilidad de los virus de utilizar la maquinaria intracelular de los cuerpos multivesiculares para ser secretados a través de la membrana plasmática [183]. En macrófagos primarios humanos, el HIV puede ensamblarse en compartimentos internos con características similares a los cuerpos multivesiculares [184] que acumulan vesículas similares a los exosomas. Tras la fusión de los cuerpos multivesiculares con la membrana plasmática de los macrófagos, las partículas infecciosas pueden ser liberadas a la circulación e infectar a otras células y contribuir a que la infección persista [184]. A pesar de que la transmisión del HIV a través de los exosomas formados a partir de macrófagos es una vía minoritaria, sin embargo esta vía de transmisión es insensible a los antivirales usados en el control de la expansión, de manera que una vía de transmisión minoritaria podría tener gran importancia biológica. Hay que destacar que en aquellas DCs [184] que capturan e

internalizan el HIV en un compartimento endosomal, los antígenos colocalizan con las tetraspaninas, marcadores característicos de los exosomas secretados de los cuerpos multivesiculares. El compartimento endosómico parece haber sido adaptado por la maquinaria vírica para favorecer su propagación [184].

Al igual que los exosomas, otras microvesículas de mayor tamaño también pueden contener un conjunto de RNAs implicados en la transcripción, angiogénesis y supervivencia celular [185, 186].

Estudios recientes han revelado un rol inesperado de los exosomas en la propagación de priones. Las enfermedades por priones producen alteraciones neurodegenerativas afectando tanto a humanos como a animales. El grupo de *Raposo et al.* han demostrado que la proteína priónica PRP en sus diferentes conformaciones utiliza los cuerpos multivesiculares para ser liberadas en exosomas [187].

I) Los exosomas pueden llevar a cabo transferencia horizontal de material genético.

Un elegante estudio llevado a cabo por *Valadi et al.* mostraba que los exosomas están enriquecidos en RNA mensajero y micro-RNA [188]. Los exosomas de líneas celulares tanto humanas como de ratón son capaces de transportar RNA a mastocitos adyacentes y ser traducido, lo cual indica que el RNA transferido es biológicamente activo. El RNA transferido a través de exosomas puede otorgar nuevas funciones a las células. Sorprendentemente, la incubación de células humanas con exosomas procedentes de células de ratón dio lugar a la producción de proteínas de ratón a partir de mRNA presente en estos exosomas de ratón. Sin embargo, el mRNA de exosomas procedentes de mastocitos fue transferido a otros mastocitos pero no a células T CD4+. Además, la transferencia horizontal de material genético es específica, ya que depende del empaquetamiento que tiene lugar en el exosoma y la célula a la que se transfiere el RNA [188].

El hecho de que los exosomas transporten RNAs con funciones específicas nos permite hacernos varias preguntas: ¿cuáles son los mecanismos para seleccionar unos mRNA específicos en los exosomas? ¿cómo seleccionan los exosomas la célula diana para transferir el RNA? ¿una vez internalizado el RNA, cómo escapa a la degradación?

m) Proteolisis del dominio extracelular de proteínas en exosomas

La mayoría de los trabajos que abordan la proteolisis de la región extracelular de las proteínas transmembrana consideran que ésta tiene lugar en la superficie de la célula. Sin embargo, estos procesos proteolíticos pueden tener lugar en cuerpos multivesiculares o en los exosomas secretados. Por ejemplo, la molécula de adhesión L1 y CD44 pueden sufrir la rotura proteolítica del dominio extracelular en los cuerpos multivesiculares o bien en el exosoma una vez secretado por acción de la enzima Adam10 [189-191]. Igualmente ocurre con las proteínas transmembrana CD46 y Tnfr1 (receptor 1 del factor de necrosis tumoral). Las líneas celulares de adenocarcinoma ovárico liberan CD46 en vesículas [192], este CD46 asociado a vesículas puede ser procesado por metaloproteasas para generar una forma soluble. Por otra parte, Tnfr1 es secretado en exosomas procedentes de células vasculares e igualmente sufre la rotura del dominio extracelular [193]. Todos estos hechos indican que los exosomas podrían tener una importante función en la proteolisis del dominio extracelular de una gran variedad de moléculas transmembrana.

Hay evidencias que indican que la secreción de los dominios extracelulares mediada por Adam y la secreción de exosomas podrían ser procesos fuertemente relacionados. Aquellos estímulos que activan la rotura proteolítica del dominio extracelular también estimulan la secreción de exosomas [189], y, de igual forma, inhibidores de Adam bloquean la formación de exosomas [193].

b) Ensayos clínicos y aplicaciones de los exosomas

Actualmente se están investigando con muchísimo interés el uso de exosomas con fines terapéuticos.

Las vacunas basadas en el uso de exosomas han sido propuestas en el tratamiento de tumores [194], ya que podrían solventar aquellas dificultades que supone la inmunoterapia basada en DCs. Esto se debe a la dificultad que supone la inmunoterapia basada en células dendríticas y la posibilidad de que los exosomas puedan solventar dichas dificultades. Los exosomas derivados de células tumorales que contienen antígenos tumorales y moléculas de MHC de clase I pueden transferir estos antígenos tumorales a las DCs e inducir una respuesta inmune antitumoral dependiente de células T CD8+ [195]. Igualmente, los exosomas secretados a partir de las DCs estimuladas con antígenos tumorales muestran una fuerte respuesta anti-tumoral. Los

datos obtenidos en ratón indican que las DCs cargadas con péptidos tumorales estimulan linfocitos T citotóxicos (CTLs) *in vivo* controlando el crecimiento de tumores establecidos en ratón de una forma T dependiente [140, 196]. Estos estudios indican que los exosomas aislados de células tumorales o de células dendríticas estimuladas con antígenos tumorales pueden llevar a cabo una respuesta inmune efectiva y podrían funcionar como otro tipo de vacuna. Algunos ensayos clínicos en fase I han sido completados y los datos obtenidos sugieren que la terapia basada en exosomas es una propuesta viable [197].

Además del uso de los exosomas en inmunoterapia del cáncer, también ha sido propuesto en el tratamiento de enfermedades infecciosas. *Aline et al.* demostraron que los exosomas derivados de DCs estimuladas con una mezcla de antígenos de taquizoíto de *T. gondii* induce una respuesta inmune protectiva contra la infección de *T. gondii.* Estos exosomas dan lugar a una respuesta celular antígeno específica y una respuesta humoral que protege frente a la toxoplasmosis tanto de tipo crónica como aguda [198]. El tratamiento de la infección neumocócica en ratón ha sido también evaluado mediante el uso de exosomas [199]. *Colino y Snapper.* demostraron que la administración de los exosomas secretados de células dendríticas derivadas de médula ósea de ratón estimuladas *in vitro* con la toxina diftérica (DT) induce la producción de inmunoglobulinas IgG2b y IgG2a específicas para la DT [200]. De forma similar, las células dendríticas derivadas de médula ósea tratadas con Cps14 liberan exosomas enriquecidos en Cps14. Estos exosomas, una vez purificados, dan lugar a la producción de inmunoglobulinas IgM e IgG3 frente a Cps14 de *S. pneumoniae* [199].

III. Enfermedades autoinmunes

1. Lupus eritematoso sistémico

a) Introducción

El lupus eritematoso sistémico sistémico (SLE), en una enfermedad autoinmune de etiología desconocida en la que hay daño celular y tisular por autoanticuerpos. Suele cursar con un amplio espectro de manifestaciones clínicas **(Tabla 2)**, y el 90% de las ocasiones afecta a mujeres en edad fértil, aunque también se puede presentar en la infancia, en décadas tardías y en hombres. En general, el SLE es una afección multisistémica, aunque al principio pueda afectarse sólo un órgano, con una aparición posterior de otras manifestaciones clínicas; la mayoría de los pacientes siguen una evolución crónica y presentan brotes o exacerbaciones de la enfermedad, intercalados con períodos de actividad.

Derrame pleural como consecuencia de tuberculosis

Exantema en alas de mariposa

Problemas de corazón

Nefritis

Artrisis

Fenómeno de Raynoud

Los síntomas del lupus eritematosos sistémico varían bastante con cada individuo

A.D.A.M
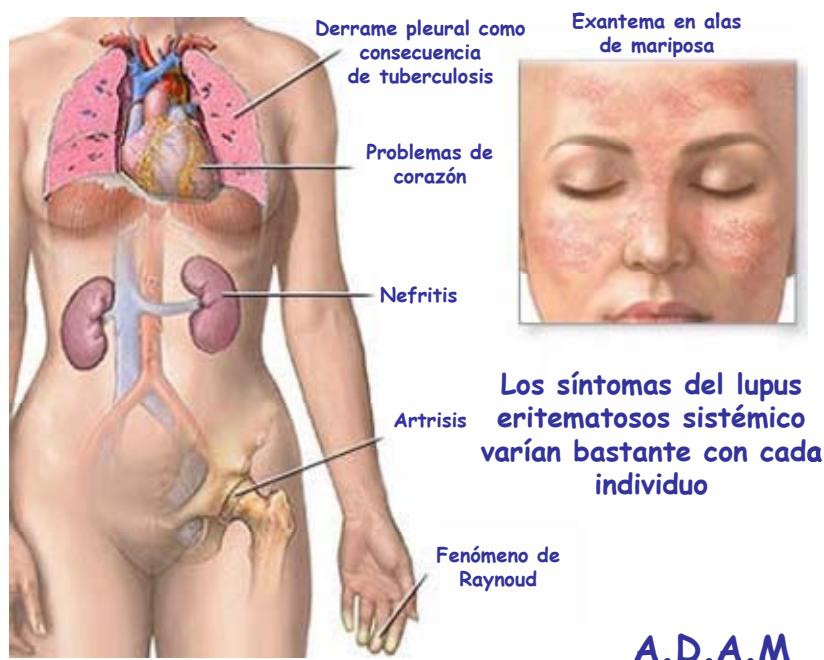
Para los clínicos, el SLE es una enfermedad potencialmente grave que se puede confundir con muchas otras enfermedades. Para nosotros, los inmunólogos, el lupus es una enfermedad muy interesante ya que prácticamente todos los componentes del sistema inmune están implicados en el desarrollo de esta enfermeda

Tabla 2. Manifestaciones Clínicas en SLE

Manifestaciones Clínica Generales	Cansancio, fiebre, anorexia y pérdida de peso
Manifestaciones musculoesqueléticas	Artralgias y/o mialgias Artritis, generalmente no erosiva Miositis/miopatía
Manifestaciones Cutáneas	*Rash* malar eritematoso *Rash* maculopapular Alopecia, generalmente parcial Úlceras orales, urticaria, vasculitis, púrpura Paniculitis, lesiones ampollosas Reacciones de hipersensibilidad o alergia Dermatitis extensa recurrente
Manifestaciones Hematológicas	Anemia, leucopenia Anticoagulante lúpico y anticuerpos anticardiolipina
Manifestaciones Cardiopulmonares	Pleuritis y derrame pleural Neumonitis lúpica Pericarditis Insuficiencias valvulares, arteriosclerosis precoz
Manifestaciones Gastrointestinales	Náuseas, diarrea y malestar inespecífico Vasculitis intestinal
Manifestaciones Renales	Nefritis
Manifestaciones del Sistema Nervioso	Ansiedad y depresión Jaqueca Convulsiones
Manifestaciones Vasculares	Trombosis
Manifestaciones Oculares	Conjuntivitis, epiescleritis, síndrome seco Vasculitis retiniana

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Tabla 2: Resumen obtenido del "Manual de enfermedades sistémicas" (Juan Jiménez-Alonso, Carmen Hidalgo-Tenorio, José Mario Sabio-Sánchez, Laura Jáimez Gámiz)

b) Etiología

La etiología de la enfermedad está pobremente estudiada o al menos se ha llevado a cabo de un modo incompleto

Se han descrito al respecto una serie de factores tanto hormonales, genéticos como ambientales:

i) **Hormonales:** El 90% de los pacientes con lupus son mujeres [201], de manera, que es obvio pensar que los estrógenos desempeñan un papel importante en la etiología de la enfermedad. Además se ha observado una disminución en la incidencia del SLE en aquellas épocas de la mujer en la que no existen unos niveles de estrógenos elevados. En los últimos años se han observado complejas interacciones entre las hormonas y las citoquinas, que podrían estar implicadas en el desarrollo de la enfermedad.

ii) **Ambientales:** Muchos fármacos pueden causar una variante del lupus. Los fármacos mejor conocidos son la procainamida, hidralazina y quinidina. Aquellos pacientes con un lupus inducido generalmente presentan manifestaciones en piel y manifestaciones, sin embargo manifestaciones renales y neurológicas con muy raras [202]. El virus del Epstein-Barr (EBV) podría ser importante ya que se ha observado una asociación temporal entre el comienzo del SLE y la infección por EBV [203]. Sin embargo, la radiacción ultravioleta es el principal factor ambiental relacionado con SLE.

iii) **Genéticos:** Muchos genes que contribuyen al SLE han sido identificados mediante el estudio del genoma completo de familias en las que muchos miembros tienen SLE [204].

c) Linfocitos T y SLE

Las células T de pacientes con SLE manifiestan ciertas anormalidades bioquímicas como alteración en la expresión de moléculas de señalización, incremento en los niveles de Ca^{2+}, y expresión alterada de factores de transcripción. Estos defectos

están implicados en un comportamiento alterado de las células T de pacientes de SLE y probablemente son importantes en la patogénesis de la enfermedad.

i. CD3ζ y FcRγ

CD3ζ es un componente del complejo CD3 y representa el principal componente de señalización del TCR. Los niveles de CD3ζ están disminuidos en células T de la mayoría de los pacientes con SLE [205], la observación en nuestro laboratorio mediante microscopía confocal de células T procedentes de pacientes de SLE no sólo indica una disminución de CD3ζ sino también un aspecto parcheado de CD3ζ en células T. Paradójicamente, esta disminución de los niveles de CD3ζ está asociada a un incremento de Ca^{2+} tras estimulación del TCR. La cadena CD3ζ es sustituida por una molécula normalmente ausente: la cadena γ del receptor Fc [206]. Esta sustitución crea una vía de señalización alternativa, en el que la cadena γ en vez de señalizar vía ZAP-70, señaliza a través Syk, una kinasa que generalmente no se encuentra en células T. Se considera que la señalización mediada por FcRγ-Syk es 100 veces más intensa que la señalización mediada por CD3ζ-ZAP70. Este reemplazamiento de CD3ζ-ZAP-70 por FcRγ-Syk explicaría en parte la hipersensibilidad de los linfocitos T de pacientes con SLE a la estimulación mediada por CD3 [207]. Una expresión forzada de FcRγ en células T normales reproduce algunos cambios descritos en células T procedentes de pacientes de SLE, como un incremento de Ca^{2+} aumentado tras la estimulación del TCR, y una disminución en la expresión de la cadena CD3ζ [208].

Figura 13: La disminución en los niveles de CD3ζ y el incremento de FcRγ da lugar a una vía de señalización alternativa FcRγ-Syk mucho más intensa que la señal de CD3ζ-ZAP-70. **J.C Crispín, G.C Tsokos / Autoinmunity Reviews 7 (2008) 256-261**

ii. CD44 y pERM

Las moléculas que median señales de activación, se localizan preferentemente en balsas lipídicas. Al ser estimulada la célula T, en aquellos lugares donde tiene lugar la estimulación del TCR se dirigen las balsas lipídicas para forman balsas lipídicas de mayor tamaño y de mayor complejidad.

Las células T de pacientes de SLE poseen mayor cantidad de balsas lipídicas, tanto en células en reposo como tras estimulación [209]. Además se ha encontrado porcentajes anormalmente altos de ciertas proteínas en las balsas lipídicas, como FcRγ, Syk quinasa activa y PLCγ1 [209, 210]. Esto tiene una importante consecuencia funcional y está directamente relacionado con el incremento en la respuesta de Ca^{2+} observado en las células T de SLE.

Las células T procedentes de enfermos de SLE tienen una mayor capacidad de adherirse y migrar en respuesta a factores quimiotácticos que aquellas células T procedentes de individuos sanos o de pacientes con RA [211]. CD44 es una molécula de superficie que participa en adhesión y migración de células T, señaliza a través de un grupo de proteínas (ezrina, radixina y moesina; ERM) que se fosforilan y dan lugar a la formación del urópodo. Las células T de pacientes de SLE tienen incrementada la expresión de CD44 así como un incremento en la fosforilación de las proteínas ERM (pERM), dando lugar a una capacidad de adhesión característico de las células T de SLE. En biopsias de riñon de pacientes con glomerulonefritis se ha observado células T que expresan CD44 y pERM [211].
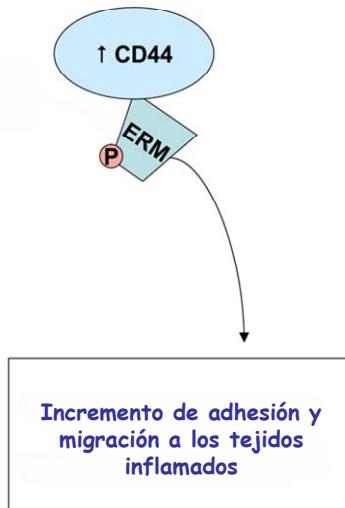
Figura 14: El incremento en los niveles de CD44 así como el incremento en la fosforilación de las las proteínas ERM da lugar a un incremento en la adhesión y migración.
J.C Crispín, G.C Tsokos / Autoinmunity Reviews 7 (2008) 256-261

iii. pCREB y CREM

Una característica fenotípica de las células T de pacientes con SLE es un fallo en la producción de IL-2 tras la activación [212]. Esta deficiencia podría explicar una serie de alteraciones presentes en el sistema inmune de pacientes de SLE: dificultad en la respuesta de la célula T, muerte celular inducida por una deficiente activación y alterada homeostasis y función de células T reguladoras.

La producción de IL-2 está principalmente controlada a nivel transcripcional. La unión de factores de transcripción al promotor del gen IL-2 está alterado en células T de pacientes SLE. La actividad nuclear de NF-kB está disminuida, la reposición de la subunidad p65 incrementa la producción de IL-2 en estas células [213]. En el promotor de la IL-2 de las células T de pacientes con SLE se ha observado un equilibrio en la ratio CREM/CREB [214], ésto se debe principalmente a que las células T de los pacientes con SLE tienen elevados niveles del factor inhibitorio CREM.
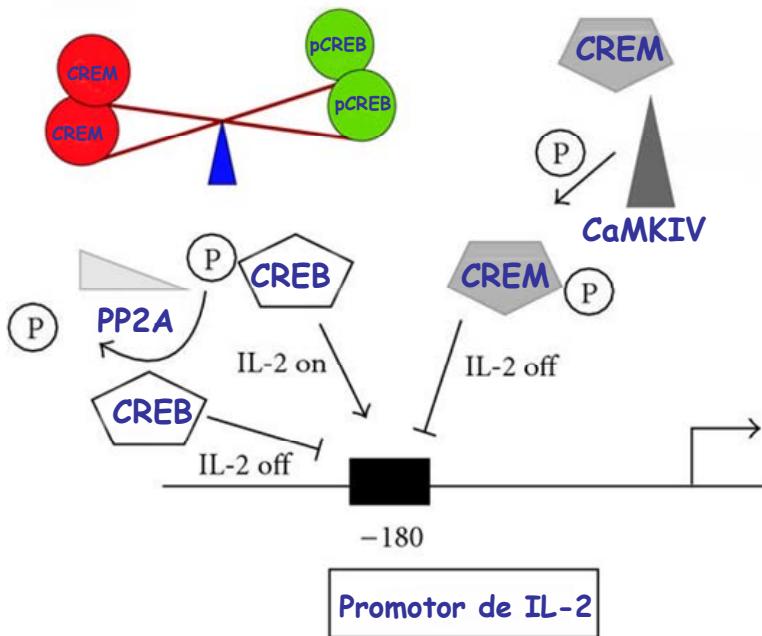
Figura 15: Regulación transcripcional de IL-2 en células T. Tras la activación de las células T, CREB es fosforilado dando lugar a la transcripción de la IL-2. CREM es también fosforilado tras la activación de células T reemplazando a la pCREB para parar la transcripción de IL-2. CaMKIV y PP2A están sobreexpresadas en SLE. **L.A Lieberman, G.C Tsokos / J Biomed Biotechnol (2010)**

Numerosos factores han sido detectados en células T procedentes de pacientes con SLE que directamente o indirectamente afectan al balance entre CREB y CREM. Un ejemplo es la proteína fosfatasa 2A (PP2A). Es la principal enzima responsable de la defosforilación de CREB en linfocitos T. Los niveles de PP2A son anormalmente elevados en células T en pacientes de SLE [215] y contribuye a una deficiente producción de IL-2 alterando la ratio pCREB/CREM.

d) Linfocitos B y SLE

La regulación de la señalización del receptor de células B es esencial para el desarrollo de la inmunidad específica y el mantenimiento de la tolerancia. El SLE se caracteriza por una pérdida de tolerancia y producción de autoanticuerpos. Junto a esta pérdida de tolerancia por parte de las células B se han encontrado alteraciones en la señalización a través del receptor de células B que se traducen en un incremento de la respuesta de Ca^{2+} y un incremento en la fosforilación de proteínas.

i. Señalización de células B en SLE

Liossis y col. fueron los primeros que observaron una señalización defectuosa a través del BCR en pacientes de lupus [216]. En este estudio se observó un incremento de Ca^{2+} intracelular y un incremento en fosforilación en tirosina tras la estimulación del BCR mediante anticuerpos anti-IgM o anti-IgD. También se ha observado *ex vivo* que los pacientes de SLE tienen un elevado porcentaje de células B con un incremento de proteínas quinasas fosforiladas basalmente [217].

ii. Lyn, CD45 y balsas lipídicas en SLE

Los ratones defientes en la proteína quinasa Lyn *(Lyn$^{-/-}$)* desarrollan una enfermedad similar al SLE con autoanticuerpos y nefritis severa [218, 219]. De acuerdo con esta enfermedad, un conjunto de pacientes con SLE tienen reducidos niveles de Lyn [220], también se ha observado que las células B de SLE además de tener una disminución de Lyn tienen incrementados los niveles de CD45 en balsas lipídicas [221]. Aunque la regulación de Lyn por CD45 no está muy clara parece ser que CD45 defosforila la fracción de Lyn que se localiza en las balsas lipídicas, con lo cual este Lyn que se encuentra próximo a importantes sustratos se inactiva y es incapaz de regular la señalización a través de BCR.

iii. Expresión y actividad alterada de FcRIIB

Una de las dianas de Lyn, el receptor de unión a inmunoglobulina FcγRIIB es un fuerte candidato que podría explicar los cambios en la señalización de células B en SLE [222]. El FcγRIIB contiene una secuencia ITIM que tras la fosforilación recluta el dominio SH2 de la fosfatasa inositol fosfato que inhibe la señalización del complejo BCR [223].

Es interesante destacar el hecho de que se han encontrado dos polimorfismos relacionados con SLE que podrían afectar a la función de FcγRIIB. Un polimorfismo en el dominio transmembrana que está asociado con el SLE en las poblaciones asiáticas y africanas [224-226] impide que el FcγRIIB se localice en las balsas lipídicas alterando la señalización a través del BCR [227, 228]. Otro polimorfismo en el promotor del FcγRIIB está asociado con el SLE en las poblaciones europeas y americanas, las células B de los individuos homocigóticos para este polimorfismo expresan menos FcγRIIB [229].

Las células B de los pacientes de SLE presentan una señalización defectuosa a través del FcγRIIB dando lugar mayores incrementos de Ca^{2+}, sugiriendo que los pacientes son menos susceptibles a la inhibición de la estimulación del BCR mediada por FcγRIIB [230]. Un posterior estudio indicó una disminución en la expresión de FcγRIIB en células B CD27^{+} de pacientes con SLE [231]. Estos resultados pueden ser reinterpretados debido a las alteraciones observadas en las diferentes subpoblaciones de linfocitos B en pacientes de SLE [232]. Las células memoria CD27^{+}IgM^{+} expresan niveles muy altos de FcγRIIB [233], parte de esa baja expresión en células B CD27^{+} en pacientes con lupus puede ser debido al bajo número de células memoria CD27^{+}IgM^{+}.

e) Autoanticuerpos y daño tisular

Las células B son hiperactivas en pacientes con SLE y producen una serie de anticuerpos espontáneamente. Los pacientes de SLE tienen anticuerpos en circulación principalmente frente a antígenos nucleares, aunque también se encuentran anticuerpos frente a antígenos citoplasmáticos o de membrana. Los anticuerpos antinucleares están presentes en el 98% de los pacientes, siendo los más frecuentes los anticuerpos que reconocen el DNA de doble hélice.
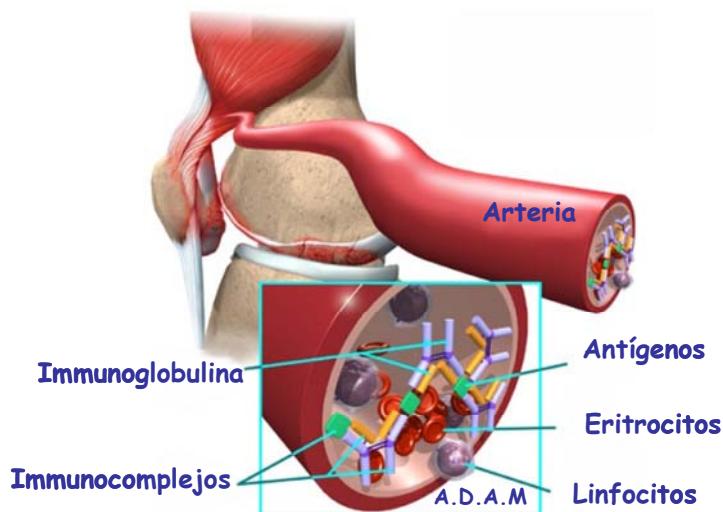
Los órganos afectados en SLE que más intensamente han sido estudiados son los riñones y la piel. En ambos casos hay inflamación y depósito de anticuerpos y componentes del sistema del complemento. En 1967, en riñones de pacientes con lupus nefrítico se encontarron anticuerpos dirigidos contra el DNA de doble cadena (ds DNA)

[234], que posteriormente fueron confirmados en la patogénesis del SLE [235]. Los anticuerpos anti-ds DNA son muy específicos para SLE; estos anticuerpos están presentes en el 70% de los pacientes con SLE y en un porcentaje menor de un 0.5% en personas sanas o pacientes con otras enfermedades autoinmunes como artritis reumatoide [236]. Los niveles de anticuerpos anti-ds DNA tienden a reflejar la actividad de la enfermedad [237], aunque no en todos los pacientes.

En un estudio de biopsias renales obtenidas de autopsias de pacientes con SLE [238], *Mannik et al.* detectaron IgG unidas a antigenos que no eran DNA, como Ro (un complejo ribonucleoproteico), La (una proteína de unión al RNA), C1q (un componente del C1 del complemento) y Sm (partículas nucleares compuestas de varios polipéptidos). La detección de anticuerpos no prueba que jueguen un papel en el desarrollo del lupus nefrítico. Más que causar la inflamación, estos anticuerpos podrían depositarse tras la apoptosis de las células del tejido.

Aunque los anticuerpos anti-ds DNA son los autoanticuerpos más estudiados en SLE, sin embargo otros anticuerpos juegan un papel en ciertas manifestaciones clínicas como anemia hemolítica, trombocitopenia, alteraciones en la piel y lupus neonatal. La presencia de anticuerpos anti-Ro y/o anti-La en el embarazo confiere cierto riesgo por bloqueo cardiaco fetal [239, 240], los anticuerpos contra el receptor N-metil-D-aspartato (NMDA) podrían ser importantes en el lupus por afectación del sistema nervioso central [241], tanto los anticuerpos anti-Ro como los anticuerpos anti-nucleosoma podrían estar implicados en el lupus cutáneo [242, 243] y aquellos anticuerpos implicados en la destrucción de eritrocitos y plaquetas son importantes en la anemia hemolítica y trombocitopenia que tiene lugar en algunos pacientes de lupus.

f) Citoquinas

Muchas citoquinas han sido implicadas en la regulación de la enfermedad así como en las diferentes manifestaciones clínicas de los enfermos de SLE.

i) Importancia de las citoquinas en SLE

La producción de citoquinas en pacientes con SLE difiere tanto de controles sanos como de otros pacientes con otras enfermedades autoinmunes como artritis reumatoide (AR). Además la producción de citoquinas es también diferente dentro de un mismo

grupo de pacientes dependiendo del tipo de SLE de cada paciente. Por ejemplo, la interleukina 6 (IL-6) está incrementada en el fluido cerebroespinal de pacientes con manifestaciones del sistema nervioso central pero sin embargo no se observa en aquellos pacientes sin síntomas neurológicos [244].

ii)   Balance de citoquinas en pacientes con SLE

Las células T cooperadoras CD4+ (Th) pueden ser divididas en varios grupos definidos por el perfil de citoquinas que producen [245, 246].

Perfil de Citoquinas

Th1	IL-2, IFNγ, TNFα
Th2	IL-4, IL-5, IL-6, IL-10 (en ratón), IL-13
Th3	TGFβ, IL-4, IL-10
Th0	todas las citoquinas anteriores

Las Th1 incrementan la activación de los macrófagos, las Th2 estimulan la respuesta humoral favoreciendo la producción de anticuerpos y las Th3 son reguladoras y pueden inducir tolerancia inmunológica.

Dependiendo del perfil de citoquinas producidas, los diferentes estadíos de la enfermedad se describen como de repuesta de tipo Th1, Th2 y Th3. Sin embargo, muchas veces es mucho más útil clasificar las citoquinas de acuerdo con su actividad anti-inflamatoria o pro-inflamatoria. En muchas enfermedades como RA y SLE los niveles de citoquinas pro-inflamatorias y citoquinas anti-inflamatorias van a determinar el grado de inflamación así como la situación clínica del paciente.

iii)   Producción de citoquinas en pacientes con SLE

IL-2: Niveles elevados de IL-2 se detectaron en el suero de un porcentaje elevado en pacientes con lupus activo, además también se ha identificado niveles altos del receptor soluble para IL-2 (sIL-2) en pacientes con lupus activo [247]. Linfocitos T y B aislados de pacientes con SLE expresan niveles incrementados de IL2R de baja afinidad.

IL-6: La IL-6 es una citoquina sintetizada principalmente por monocitos, fibroblastos y células endoteliales aunque también puede ser secretada por linfocitos T y linfocitos B.

En pacientes de SLE, los niveles de IL-6 correlacionan con la actividad de la enfermedad y los niveles de anticuerpos anti-DNA [248, 249]. Las células linfoblastoides aisladas de los pacientes de SLE producen altos niveles de IL-6 y el bloqueo de ésta da lugar a la reducción de los niveles de anti-ds DNA *in vitro* [250]. A diferencia de los individuos sanos, los linfocitos B de pacientes de SLE generan espontáneamente grandes cantidades de inmunoglobulinas. El bloqueo de la IL-6 disminuye significativamente esta síntesis espontánea de inmunoglobulinas que es restaurada tras la administración exógena de IL-6 [249]. Ha sido descrito que los linfocitos B procedentes de los pacientes de SLE secretan espontáneamente anticuerpos anti-ds DNA y curiosamente esta secreción de autoanticuerpos *ex vivo* se produce principalmente por linfocitos B de baja densidad [251], además la IL-6 puede facilitar que estos linfocitos B de baja densidad de los pacientes con SLE se diferencien directamente a células secretoras de inmunoglobulinas [251]. Se ha observado una particular relación de la IL-6 con las manifestaciones renales de SLE. Varios estudios han demostrado niveles elevados de IL-6 en orina de pacientes con lupus nefrítico y altos niveles de anticuerpos anti-ds DNA [252, 253]. Los niveles de IL-6 en pacientes con un lupus nefrítico activo son más elevados que en aquellos pacientes con la enfermedad en reposo, y además también se encuentra incrementada la expresión de IL-6 en glomérulos y túbulos en riñones de pacientes con lupus nefrítico [254]. Aquellos pacientes con manifestaciones neuropsiquiátricas tienen elevados niveles de IL-6 en líquido cerebroespinal [255]. Este nivel elevado de IL-6 en tejidos podría estar implicado en inflamación local y daño tisular.

IL-10: Es una citoquina producida principalmente por monocitos y linfocitos. Impide la activación de células presentadoras de antígenos, disminuye la expresión de moléculas co-estimuladoras, por lo tanto dificulta la activación de células T y la secreción de TNFα.

Los niveles en plasma de IL-10 son altos en pacientes con SLE [256]. Este incremento se atribuye a un incremento en la producción de IL-10 por monocitos, una subpoblación de células B y posiblemente por células memoria CD4^{+}CD45 RO^{+} [256-

258]. Los títulos de IL-10 en suero correlacionan con la actividad de la enfermedad y con los niveles de anticuerpos anti-ds DNA, y correlaciona negativamente con los niveles de C3 [259, 260]. La IL-10 estimula la proliferación de células B así como el cambio de clase de las inmunoglobulinas, dando lugar a un incremento en la producción de anticuerpos con la capacidad de entrar en compartimentos extravasculares y favorecer la inflamación en SLE [261]. Los anticuerpos anti-ds DNA y los inmunocomplejos unidos a FcγRII son potentes desencadenantes de IL-10 [262, 263]. Se ha detectado IL-10 en riñones de pacientes de SLE, aunque los datos sugieren que la ratio IFNγ/IL-10 es de mayor relevancia desde el punto de vista clínico [264]. El bloqueo de esta citoquina reduce la producción de autoanticuerpos patogénicos. *W. Emlen et al.* han observado que los linfocitos aislados de pacientes con SLE tienen una tasa de apoptosis incrementada cuando se compara con linfocitos aislados de controles sanos o pacientes RA [265]. La adición exógena de anticuerpos anti-IL-10 a los PBMCs de pacientes de SLE reduce la apoptosis [266].

IL-12: La producción de IL-2 por células mononucleares de sangre periférica es menor en pacientes de SLE que en controles sanos y esto parece ser que es debido a una disminución en la producción de IL-12 por parte de los monocitos [267]. La producción de IL-12 es también menor en aquellos pacientes con lupus activo cuando son comparados con lupus en remisión o inactivo [267].

IL-16: La concentración de IL-16 en suero está incrementada en pacientes con SLE cuando de compara con controles sanos [268].

IL-17: Es una proteína transmembrana de tipo I. Es una citoquina pro-inflamatoria producida por células T activadas, siendo las células "Th17" las principales productoras. Las células Th17 son linfocitos T CD4+ que expresan los factores de transcripción RORγt y RORα y producen las citoquinas IL-17A, IL-17F, IL-21 e IL-22 [269]. Inducen una fuerte respuesta inflamatoria secretando IL-17, un potente atrayente de neutrófilos.

En pacientes de SLE se han encontrado altos niveles de IL-17 e IL-23 y los niveles plasmáticos de IL-17 correlacionan con la actividad de la enfermedad [270]. Datos recientes sugieren que un porcentaje significativo de IL-17 en pacientes de SLE procede de células TCR-αβ$^+$CD4$^-$CD8$^-$ [271]. Además tanto las células Th17 como las células

TCR-αβ⁺CD4⁻CD8⁻ se han encontrado en biopsias renales de pacientes con lupus nefrítico, indicando que podrían tener alguna función patogénica en lupus renal [271]. También ha sido descrito que la IL-17 puede estimular la producción de anticuerpos de las células B [272].

Blys (estimulador de linfocitos B): El Blys es un miembro de la familia del ligando del factor de necrosis tumoral. Es una proteína transmembrana de tipo II, se libera en una forma soluble y biológicamente activa por la acción de proteasas [273]. Se expresa en células de la línea mieloide (monocitos, macrófagos, células dendríticas y neutrófilos activados) y su liberación está regulada por interferón-γ e IL-10 [273]. Es un importante factor en la proliferación y supervivencia de células B, y secreción de inmunoglobulinas [274].

Los niveles plasmáticos de Blys se encuentran incrementados en los pacientes de SLE y correlacionan con los niveles de anticuerpos anti-ds DNA [275].

TNFα: El factor de necrosis tumoral alfa (TNF-α) se expresa en forma de trímero en la superficie celular y en forma soluble tras la activación de macrófagos y células dendríticas. La importancia del TNFα en la patogénesis del SLE es polémico.

Hay evidencias que indican que el TNF-α puede jugar un papel pro-inflamatorio en pacientes con SLE. Los niveles séricos de TNF-α correlacionan parcialmente con la actividad de la enfermedad [276] y se ha demostrado una expresión elevada en enfermos con lupus nefrítico [277]. Sin embargo, curiosamente, se ha descrito que pacientes con otras enfermedades autoinmunes a los que se les ha sometido a una terapia anti-TNFα han desarrollado factores antinucleares y anticuerpos anticardiolipina así como un síndrome similar al lupus, además estos síntomas y estos autoanticuerpos desaparecieron al interrumpir el tratamiento [278, 279].

TGFβ (cytokine transforming growth factor β): El TGFβ está producido por células NK y tienen un fuerte poder inhibitorio en la producción de IL-6, IL-1 y TNFα por macrófagos en un sistema *in vitro* [280]. El TGFβ suprime la secreción de inmunoglobulinas de las células B [281, 282].

Los niveles de TGFβ son más bajos en pacientes con SLE y esto es probablemente debido a los altos niveles de IL-10 que suprimen la producción de TGFβ por las células NKs [282]. Puesto que el TGFβ suprime la secreción de inmunoglobulinas, éstos bajos niveles pueden favorecer los altos niveles de inmunoglobulinas obsevado en los pacientes de lupus [281].

IFN (interferón): Los interferones son proteínas con la capacidad de suprimir la replicación viral.

Los IFNs de tipo I y tipo II han sido implicados en la patogénesis de SLE. Se han identificado altos niveles de IFN-α e IFN-γ en suero de pacientes de SLE [283-287]. Los pacientes con SLE activo a menudo presentan síntomas similares a la gripe, como fiebre y fatiga, que reflejan elevada concentración de IFN-α en suero [288-290]. Los mayores productores de IFN-α son las células dendríticas plasmacitoides (pDCs) [291]. Un par de trabajos han identificado un gran número de pDCs en las lesiones de lupus cutáneo [292, 293]; por el contrario se ha observado una disminución en el número de pDCs en sangre periférica, sugiriendo la capacidad de estas pDCs de migrar hacia los tejidos periféricos [294-297]. El grupo de N. Nishimoto ha identificado la sobreexpresión de varios genes inducibles por IFN en células de sangre periférica de enfermos de SLE, estos datos están de acuerdo con otros trabajos que demuestran un patrón de expresión de genes en los que están sobreexpresados genes inducibles por IFN [298-301].
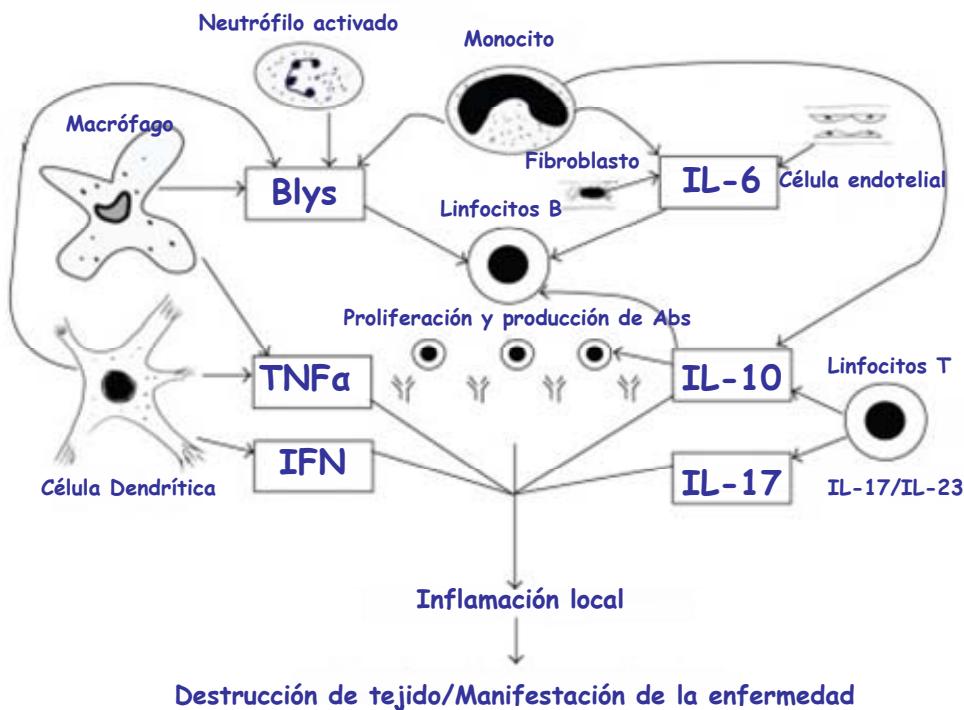
Figura 16: Diagrama esquemático que muestra la interacción entre diferentes células del sistema inmune y citoquinas que dan lugar a la patogénesis del SLE.

2. Pénfigo

a) Introducción

El pénfigo (del Griego "pemphix" o "akantha") es una enfermedad autoinmune ampollosa que afecta a la piel y a las mucosas. La característica histológica principal, responsable de la formación de ampollas, es la acantólisis, pérdida de adhesión entre los queratinocitos, en la zona suprabasal de la epidermis.

Está mediada por la producción de autoanticuerpos patógenos que van dirigidos contra las desmogleínas (Dsg), glucoproteínas transmembrana de adhesión que forman parte de los desmosomas, uniones de adhesión intercelular responsables de la unión de queratinocitos en piel y mucosas [302-304].

Se distinguen principalmente dos subtipos de pénfigo, el pénfigo vulgar (PV) y el pénfigo foliáceo (PF), en los que los autoanticuerpos van dirigidos hacia la Dsg3 y Dsg1,

respectivamente. En la forma mucocutánea de PV ambos autoanticuerpos están presentes, anti-Dsg1 y anti-Dsg3. Los anticuerpos anti-Dsg1, presentes en el pénfigo foliáceo no pueden producir ampollas en mucosas o en las porciones profundas de la piel ya que en esas localizaciones existe una representación significativa de otra proteína del desmosoma, la Dsg3, que es suficiente para mantener la cohesión intercelular. Los anticuerpos anti-Dsg1 son capaces de producir ampollas cutáneas superficiales ya que a ese nivel la expresión de la desmogleína 3 es suficiente para mantener la adhesión entre los queratinocitos. Por otra parte, los anticuerpos anti-Dsg3, presentes en el pénfigo vulgar, pueden producir por sí solos ampollas a nivel de mucosas, ya que a ese nivel la expresión de Dsg1 es muy escasa y no puede mantener la adhesión intercelular. A nivel de piel los anticuerpos anti-Dsg3 sólo producen lesiones limitadas a la capa basal debido a la presencia de Dsg1 en la piel. Si se producen anticuerpos anti-Dsg1 y Dsg3 se producen lesiones tanto en piel como y en mucosas [305, 306].

b) Etiología

La interacción entre factores genéticos y ambientales podría desencadenar la enfermedad.

El PV es una enfermedad autoinmune asociada al complejo principal de histocompatibilidad (HLA). En la población judía hay asociación entre el PV y los alelos HLA-DRB1*0402 [307], sin embargo en la población no judía la asociación es con los alelos HLA-DQB1*0503 [308], principalmente. Estos alelos codifican para un complejo principal de histocompatibilidad de clase II (HLA-II) implicado en la presentación de epítopos de Dsg3 a linfocitos T autoreactivos ya que tienen una carga negativa en un sitio crítico que es ocupado por una carga positiva de algunos residuos de la Dsg3 [309]. De ahí que el tener alguno de estos haplotipos incremente el riesgo de padecer la enfermedad.

Las personas genéticamente susceptibles tienen mayor riesgo de desarrollar la enfermedad al estar expuestas a ciertos fármacos como captopril [310] o penicilina [311], factores ambientales como pesticidas [312] o virus como el virus herpes humano (HHV-8) [313].

c) Linfocitos T y pénfigo

El pénfigo es una enfermedad mediada por anticuerpos, sin embargo no podemos olvidar que la producción de éstos no es posible de no haber interacción entre linfocitos T y linfocitos B, con lo cual la inmunidad celular es una parte importante de esta enfermedad.

Estudios inmunohistoquímicos han permitido conocer la distribución de algunas células en la piel de los pacientes con PV, observándose un incremento en el número de células mononucleares en la dermis de la zona lesionada en comparación con la dermis perilesional. Un elevado porcentaje de estas células mononucleares son linfocitos T [314].

La presencia de linfocitos T activados se manifiesta por la presencia del receptor soluble de interleuquina 2 (IL-2) en plasma y líquido de las ampollas, cuyos niveles está relacionado con la actividad de la enfermedad [314].

Actualmente, han sido identificados linfocitos T cooperadores (Th) autoreactivos frente a Dsg3 tanto en pacientes con PV como en personas sanas con los alelos HLA DRB1*0402 y DQB1*0503 [315, 316]. Se ha hecho un gran esfuerzo en los últimos años en la búsqueda de aquellos factores que hacen que se desarrolle la enfermedad. Hasta el momento se han encontrado principalmente dos diferencias:

a) **La relación entre linfocitos con respuesta tipo Th1 y Th2 es distinta.** [315]

Los linfocitos Th1 favorecen predominantemente una respuesta inmune celular y sus citoquinas estimulan la producción de IgG1 e IgG3, mientras que los linfocitos Th2 promueven una respuesta inmune humoral y sus citoquinas inducen principalmente la síntesis de IgG4 e IgE. En personas sanas con los alelos asociados a PV se han aislado linfocitos Th1 autoreactivos (productores IL-2 e IFN-γ), pero no linfocitos Th2. por el contrario en pacientes con PV se aislaron linfocitos Th2 (productores de IL-4, IL-5 e IL-13) en cantidades similares en los diferentes estadíos de la enfermedad, aunque también se aislaron linfocitos Th1 en cantidades variables. Los enfermos crónicos tuvieron el mayor número de linfocito Th1, seguidos por los enfermos en remisión y finalmente por enfermos activos. Además, la relación Th1/Th2 se correlacionó directamente con la cantidad de anticuerpos anti-Dsg3 de tipo IgG1 e IgG4, ésto explicaría el hecho de que los pacientes con pénfigo activo presenten autoanticuerpos de tipo IgG4 [317] mientras

que los pacientes con enfermedad crónica tienen tanto anticuerpos de tipo IgG4 como IgG1 [318].

b) **La cantidad de linfocitos T reguladores es distinta.**

La mayoría de los linfocitos T reactivos contra Dsg3, en personas sanas, son T reguladores de tipo 1 (Tr1), éstos tienen una acción inhibitoria sobre la respuesta proliferativa de los clones de los linfocitos Th auto-reactivos mediada por IL-10 y TGF-β. Por el contrario, las personas con pénfigo vulgar, tienen principalmente linfocitos Th autorreactivos y una minoría de Tr1, favoreciendo la pérdida de tolerancia [316].

d) Linfocitos B, anticuerpos y pénfigo

Los linfocitos B son las células productoras de anticuerpos, y por lo tanto son necesarias para que se produzcan autoanticuerpos contra la Dsg3. En sangre periférica de pacientes con PV se han detectado linfocitos B autorreactivos contra Dsg3 [319, 320]. La interacción entre linfocitos B y Th autorreactivos es necesaria para que tenga lugar la producción de anticuerpos anti-Dsg3. Experimentos llevados a cabo estimulando *in vitro* con Dsg3 una mezcla de linfocitos de sangre periférica de pacientes con PV se detectó la producción de anticuerpos, sin embargo, al eliminar los linfocitos CD4 de la mezcla, los linfocitos B autoreactivos no produjeron anticuerpos, sugiriendo el importante papel de la células T en la producción de autoanticuerpos [320].

Los autoanticuerpos en PV son de tipo IgG y policlonales, sin embargo el subtipo IgG4 es el más frecuente en pacientes con enfermedad activa y los subtipos IgG4 [317] e IgG1 en pacientes con enfermedad crónica [318].

Hay una clara evidencia de la patogenicidad de los autoanticuerpos en pénfigo:
1) Los bebés de madres con pénfigo pueden presentar la enfermedad durante las primeras semanas de vida [321].
2) Al inyectar suero de pacientes con PV a ratones neonatos BALB/c se produce acantólisis con histología típica de estos pacientes y formación de ampollas (modelo murino de transferencia pasiva) [322].
3) El papel patogénico de la Dsg3 también ha sido demostrado en un modelo murino. En este modelo murino de PV, los ratones deficientes para la Dsg3 (Dsg3-/-) son inmunizados con Dsg3 de ratón. Una vez inmunizados los esplenocitos de los ratones Dsg3-/- son transferidos a los ratones Rag2-/-, dando lugar a la

producción de anticuerpos anti-Dsg3 en circulación y a un fenotipo similar al de PV.

4) La IgG de pacientes con pénfigo, sin complemento y sin células inflamatorias, puede inducir pérdida de adhesión celular en cultivos de queratinocitos [323].

5) Aquellos anticuerpos dirigidos contra la porción amino terminal de la región extracelular de la Dsg3 son los que causan más daño [324].

e) Células NKs y pénfigo

No hay muchas publicaciones que hayan evaluado la función de las células NKs en la patogénesis del pénfigo. Se ha observado que las células NKs procedentes de pacientes de pénfigo presentan una elevada expresión de MHCII *ex vivo*, sin la adición de citoquinas exógenas. Las células T CD4$^+$ de pacientes de PV cultivadas junto a células NKs CD56$^+$CD3$^-$ que expresan moléculas MHCII y la molécula coestimuladora B7-H3 proliferan y producen niveles significativos de IL-6, IL-8 e IFN-γ [325]. Un estudio del fenotipo de los NKs en pacientes de PV muestra un incremento en la expresión del marcador de activación CD69$^+$, disminuación en la expresión de los genes que codifican para IL-12Rβ, perforina y granzima, alteración en la fosforilación de Stat4 inducida por IL-12, incremento en la expresión de IL-10, disminución de la producción de IL-10 tras estimulación *in vitro* vía IL-2/IL-12 e incremento de expresión de IL-5 en función de la actividad de la enfermedad [326]. Todos esto datos sugieren que las células NKs podrían estar implicadas en la patogénesis de la respuesta inmune en pacientes de PV favoreciendo una respuesta Th2.

Podemos concluir diciendo que el pénfigo es un claro ejemplo de enfermedad autoinmune órgano-específica debido a:

i)   su asociación a moléculas HLA

ii)   presencia de linfocitos T y B autorreactivos y

iii)   pérdida de tolerancia periférica por un desequilibrio en las poblaciones de linfocitos autorreactivos efectores y reguladores.

3. Linfohistiocitosis hemofagocítica

a) Introducción

El síndrome hemofagocítico (linfohistiocitosis hemofagocítica, HLH) representa una situación de hiperinflamación con síntomas prolongados de fiebre, citopenia, hepatoesplenomegalia y hemofagocitosis debido a una proliferación incontrolada, no maligna, de histiocitos, generalmente acompañada de linfocitos T con activación macrofágica y estado de hipercitoquinemia. Entre los marcadores bioquímicos se destacan niveles elevados de ferritina y triglicéridos, y bajos niveles de fibrinógeno. Mientras que en niños varias inmunodeficiencias pueden llevar a este síndrome, en la mayoría de los adultos no se conoce un defecto genético responsable. No obstante, una función alterada de las células NKs y células T citotóxicas (CTL) es característico de la forma genética o adquirida de la HLH. Los agentes desencadenantes son agentes infecciosos, principalmente virus del herpes. Algunos linfomas malignos, especialmente en adultos pueden estar asociados con HLH. Una forma especial de HLH en enfermedades reumaticas de denomina síndrome de activación macrofágica (MAS).

b) Clasificación

La HLH tiene lugar en todas las edades. No es una enfermedad concreta sino un síndrome que se puede encontrar asociado a diferentes condiciones pero que dan lugar a un mismo fenotipo de inflamación.

i) **HLH primaria o genética:** Es aquella en la que existe una alteracción genética, es heredada de una forma autosómica recesiva o asociada al cromosoma X y puede dividirse en dos subgrupos:

- HLH familiar: el 25% de todos los casos de HLH son familiares.
- HLH en pacientes con inmunodeficiencias: [327, 328]

  Síndrome de Chediak-Higashi (CHS1)

  Síndrome de Griscelli (GS2)

  Síndrome linfoproliferativo asociado al cromosama X (XLP)

ii) **HLH secundaria o adquirida:**

- Síndrome hemofagocítico asociado a infección: [329-334]

  - *Virus:* Epstein-Barr virus, cytomegalovirus, parvovirus, HIV
  - *Bacterias:* tuberculosis

- *Parásistos:* leishmaniasis
- *Hongos*
- Síndrome hemofagocítico asociado a linfoma [335-340]
- Síndrome de activación macrofágico: tiene lugar en enfermedades autoinmunes [341-343]

Se puede poner aquí la figura2 del paper hemofagocitic síndrome

c) Manifestaciones clínicas y datos clínicos

Los síntomas característicos del HLH son: fiebre prolongada, hepatoesplenomegalia y citopenia. Otras manifestaciones menos frecuentes son: linfoadenopatía, erupción, ictericia o síntomas neurológicos. Estos pacientes tienen altos niveles de triglicéridos, ferrritina, transaminasas, bilirrubina y LDH, sin embargo tienen bajos niveles de fibrinógeno [344-347]. La cadena alpha del receptor soluble de la interleukina-2 (sCD25) es un marcador importante debido a los altos niveles durante el HLH activo [348]. Una característica de la HLH, tanto de la forma genética como la adquirida es la deficiencia o ausencia de función de las células NKs y células T citotóxicas [349]. En los pacientes con HLH familiar, el número de células NKs son normales pero no funcionan correctamente. Los pacientes con la forma adquirida de HLH tienen bajos niveles de células NKs y normalmente su función está disminuida en situaciones de actividad clínica, sin embargo tras el tratamiento se recupera la actividad de las NKs.

Todos los síntomas de HLH pueden ser explicados por altas concentraciones de citoquinas inflamatorias [349, 350] y la infiltración de órganos por linfocitos activados e histiocitos. La fiebre está inducida por IL-1 e IL-6, y la pancitopenia es además la consecuencia de altos niveles del factor de necrosis tumoral α e interferón γ. El TNFα inhibe la proteín lipasa dando lugar a un incremento en los niveles de triglicéridos. Los macrófagos activados secretan ferritina y activador de plasminógeno que resulta en niveles altos de plasmina e hiperfibrinolisis. Los linfocitos activados son el origen de las altas concentraciones de la cadena α del receptor de IL-2. Finalmente, la hepatoesplenomegalia, enzimas hepáticas incrementadas y bilirrubina así como síntomas neurológicos son la consecuencia de la infiltración de órganos por linfocitos e histiocitos

activados. El papel del IFNγ junto con las células T CD8+ para el desarrollo de HLH se ha demostrado en un modelo de ratón deficiente para la perforina *(perforina$^{-/-}$)* [351].

d) Genética y patofisiología

Tras la activación del sistema inmune con un agente infeccioso, los histiocitos (macrófagos y células dendríticas), las células NKs y las células CTLs son activadas y además se estimulan mutuamente unas a otras. Esto lleva a matar a la célula infectada, eliminar el antígeno y posterior finalización de la respuesta inmune. Esta actividad citotóxica alterada no sólo afecta a la eliminación de antígenos sino también a la regulación de la respuesta immune. La activación persistente del sistema inmune junto con niveles altos de citoquinas lleva a las diferentes manifestaciones clínicas de HLH.

Las células NKs y los linfocitos T citotóxicos eliminan a sus dianas a través de vesículas citolíticas (gránulos) que contienen perforina y granzima. Tras el contacto de la célula efectora y la célula diana se forma la sinapsis inmunológica y tiene lugar el tráfico de vesículas al sitio de contacto, el anclaje y la fusión con la membrana plasmática y la liberación de su contenido [352].

Todos los defectos que se conocen en HLH parecen que están envueltos en este proceso.

No está muy claro como la función de las células NKs y células T citotóxicas se ve comprometida en pacientes inmunocompetentes con HLH adquirida. Los virus pueden interferir con la función de las células T citotóxicas, al igual elevadas concentraciones de citoquinas. En algunos pacientes cierta susceptibilidad genética puede estar presente al observarse leves episodios de HLH. En pacientes con linfomas, la secreción de citoquinas por las células malignas podrían explicar la HLH.

IV. Referencias

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Justificación y Objetivos

Justificación y Objetivos

Los **<u>OBJETIVOS</u>** que nos planteamos en esta tesis son los siguientes:

1. Estudio de la secreción de CD38 a través de exosomas derivados de células B así como su distribución en microdominios de membrana del linfocito B.

2. Identificar las proteínas asociadas a CD38 en exosomas y en microdominios de membrana en los que localiza CD38 y la naturaleza de dicha asociación.

3. Identificar proteínas implicadas en la vía de señalización de la célula B a través de CD38.

4. Estudiar la expresión de CD38 en linfocitos T, así como en sus distintas subpoblaciones (CD25, CD4 y CD8), en células B, monocitos, células NK, células NKT y granulocitos de baja densidad en pacientes de lupus eritematoso sistémico, pénfigo y un caso de un síndrome hemofagocítico asociado a una infección por *Leishmania*.

5. Hacer un análisis de expresión diferencial de proteínas en células mononucleares de sangre periférica mediante citometría de flujo en pacientes de lupus eritematoso sistémico (LES), pacientes de pénfigo y controles sanos.

6. Determinar la presencia de autoanticuerpos anti-CD38 en pacientes de (LES).

7. Determinar si existe relación entre la expresión de CD38, citoquinas plasmáticas, presencia de autoanticuerpos y actividad de la enfermedad en pacientes con LES.

8. Demostrar si el canal TRPM2 está implicado en la entrada de Ca^{2+} extracelular que tiene lugar en migración leucocitaria dependiente de CD38.

Discusión

Discusión

Uno de los aspectos más destacables de esta tesis doctoral ha sido la capacidad de abordar temas muy diversos, en los que se han utilizado diferentes tecnologías e incluso diferentes modelos biológicos, aunque siempre manteniendo nuestro interés en el estudio de CD38. Fundamentales han sido las colaboraciones que hemos llevado a cabo, tanto con destacados expertos en el ámbito de las ectoenzimas, y especialmente de CD38, como el *Prof. Fabio Malavasi* (CD38 humano) y la *Dra. Frances E. Lund* (CD38 murino), como con el *Dr. Norberto Ortego* (Jefe de la Unidad de Enfermedades Autoinmunes Sistémicas, Hospital Clínico, Granada) y el *Dr. Salvador Arias* (Servicio de Dermatología, Hospital Clínico, Granada), que nos han proporcionado no sólo las muestras de los pacientes sino un mayor conocimiento de las enfermedades estudiadas.

Un trabajo previo llevado a cabo por nuestra compañera *P. Muñoz et al.* mostraba que los endosomas de reciclaje que contenían CD38 se localizan en la sinapsis inmunológica tras la activación del TCR, donde se fusionan con la membrana plasmática [1]. Además, nuestro colaborador el *Prof F. Malavasi* ya había observado hace algunos años mediante microscopía electrónica la presencia de CD38 en cuerpos multivesiculares que fusionaban con la membrana plasmática [2]. Estos datos llevaron a la *Dra. Mercedes Zubiaur* a liderar un trabajo en el que se planteaba la posibilidad de que los exosomas procedentes de células B estuviesen enriquecidos en CD38 y de la existencia de posibles interacciones específicas entre CD38 y otras proteínas de señalización asociadas a exosomas. Este CD38 exosómico, bien a través de su actividad enzimática o bien a través de estas interacciones podría ejercer una función señalizadora. Además, las balsas lipídicas han sido relacionadas en el proceso de selección de proteínas asociadas a exosomas, con lo cual nos planteamos la posibilidad de que proteínas asociadas a CD38 en exosomas también lo estarían en balsas lipídicas. Por lo tanto, este posible complejo de señalización asociado a CD38 se incorporaría a un conjunto de balsas lipídicas que posteriormente mantendrían su asociación en la vía exocítica.

Nuestros indicios fueron confirmados y observamos por primera vez que CD38 se localiza en la superficie de exosomas derivados de células B humanas, además, este CD38 en exosomas conserva la actividad enzimática, lo cual es muy interesante ya que aquellos exosomas que contienen CD38 podrían, a través de la actividad enzimática de

CD38, ejercer un efecto paracrino sobre células adyacentes. Es interesante destacar el hecho de que el CD38 encontrado en exosomas se presenta tanto en forma monomérica como en forma de dímero, dicha organización estructural de CD38 ya ha sido previamente descrita [3-5]. Hemos de incidir en el hecho de que el CD38 presente en exosomas es diferente al CD38 soluble encontrado en varios fluidos biológicos, y en sobrenadantes de cultivo, el cual es una molécula de 39 KDa [6]. No obstante, hay ejemplos claros en la literatura que indican que los exosomas podrían tener una importante función en la proteolisis del dominio extracelular de una gran variedad de moléculas transmembrana [7-10].

Debido a la implicación de las balsas lipídicas en la selección de proteínas asociadas a los exosomas [11], estudiamos la presencia de CD38 en las balsas lipídicas. Las balsas lipídicas fueron obtenidas a partir de lisados celulares en Brij 98 a 37ºC, preservando de esta manera los complejos tetraspanina-tetraspanina y tetraspanina-proteína así como su capacidad de localizar en fracciones de baja densidad en gradientes de sacarosa [12]. Las balsas lipídicas procedentes de lisados en Brij 98 presentan características físicas y funcionales diferentes de aquellas obtenidas de lisados en Tritón X-100 [13, 14], siendo más parecidas a los microdominios enriquecidos en tetraspanina [15]. Los estudios llevados a cabo en las balsas lipídicas indicaron que CD38 es muy abundante y además es enzimáticamente activo. El tratamiento de las células con metil-β-ciclodextrina alteró la distribución de CD38 observándose una disminución de CD38 en las balsas lipídicas, así como una disminución en la actividad enzimática. Además, el uso del anticuerpo monoclonal anti-CD38 HB137 dio lugar a la activación de Akt/PKB y Erk, la oligomerización de CD38 y un incremento en la resistencia de CD38 a la solubilización en Brij 98.

A continuación, identificamos una serie de moléculas asociadas a CD38. Está ampliamente documentado el hecho de que la interacción de CD38 con otras moléculas de señalización de superficie es crucial para la señalización mediada por CD38 [6, 14, 16]. En los exosomas, CD38 interacciona con CD81, Hsc-70 y Lyn. Interacciones similares se observaron en las balsas lipídicas, donde se encuentra gran parte de CD38 en las líneas celulares estudiadas. La interacción CD38-CD81 tiene lugar en los exosomas y en balsas lipídicas en presencia de Brij 98 y ODG a 15mM, que favorece la solubilidad. La interacción CD38-CD81 también fue detectada en las fracciones solubles aisladas de las células B lisadas en Brij 98 a 37ºC. Las fracciones solubles corresponden

a proteínas de membrana solubilizadas y componentes citosólicos. Sin embargo, esta interacción no se mantiene cuando los exosomas son lisados en NP40 al 1% a 4ºC, una condición que rompe los complejos tetraspanina-proteína [12]. Estos resultados indican que los complejos CD38-CD81 no son artefactos debido a una solubilización incompleta, sino que representan unidades discretas de tamaño moderado que pueden ser totalmente solubilizados. De la misma manera, en monocitos humanos, la tetraspanina CD9 se encuentra asociada con CD38 tanto en balsas lipídicas como en otras fracciones [17].

Nuestros resultados también demuestran una asociación recíproca entre CD38 y CD19 exclusivamente en las balsas lipídicas. CD19 se ha encontrado asociado a CD38 tanto en linfocitos B normales como en células B procedentes de leucemia linfocítica crónica [3, 18]. Por otra parte, CD19 y CD81 están probablemente fuertemente unidos ya que la interacción entre ellos se mantiene en estrictas condiciones de detergente [15]. Además CD38 sirve de enlace entre CD38 y CD19.

La chaperona intracelular Hsc-70 [19] es otro componente del complejo CD38/CD81 tanto en exosomas como en balsas lipídicas. La Hsc-70 está constitutivamente expresada en exosomas y está implicada en la selección de proteínas en los cuerpos multivesiculares [20]. Los complejos asociados a CD38 en exosomas aislados están enriquecidos en Hsc-70 y Lyn. La asociación de Hsc-70 con Lyn parece ser independiente de CD38; Lyn inmunoprecipitado de los exosomas procedentes de las células B Namalwa CD38^{+} lleva asociado a la Hsc-70, pero no a CD38. Los complejos Lyn-Hsc70 también son detectados en los exosomas procedentes de las células B HOM2 CD38^{-}. La ausencia de CD38 en los inmunoprecipitados de Lyn de los exosomas procedentes de Namalwa es sorprendente ya que Lyn manifiesta una asociación bidireccional con CD38 en las balsas lipídicas. Las balsas lipídicas contienen altas concentraciones de tirosín-quinasas como Lyn [21]. Asumiendo que la interacción CD38-Lyn-Hsc70 es de baja estequiometría, diferencias en la concentración relativa de estas proteínas en exosomas en comparación con las balsas lipídicas podrían explicar estos resultados. Imágenes obtenidas mediante microscopía confocal revelan que la expresión de CD38 en la superficie de las células Namalwa tiene una apariencia parcheada. La co-precipitación de Lyn con CD38 en las balsas lipídicas, incluso sin la estimulación de la célula mediante un anticuerpo monoclonal anti-CD38, sugiere la presencia de un conjunto de CD38 que interacciona con Lyn en las balsas lipídicas. Por lo tanto, Lyn es parte de un

complejo de señalización que incluye CD38 y BCR/CD19/CD21/CD81 [21, 22]. Otro miembro del complejo CD38/Lyn es la subunidad Gαi-2. El mecanismo de asociación entre Gαi-2 y CD38 en las balsas lipídicas es actualmente desconocido. En células epiteliales, CD59, un receptor anclado a glicosilfosfatidilinositol, recluta Lyn y Gαi-2 vía interacciones de tipo proteína-proteína e interacciones lípido-lípido. Posiblemente Lyn sea activado por la unión de Gαi-2, que induce la unión de CD59 a la F-actina, crucial en el reclutamiento y activación de PLC-γ2 [23]. Por lo tanto, en células B, la interacción entre CD38, Lyn y Gαi-2 en balsas lipídicas puede ser funcionalmente relevante y representar un enlace con actina u otras proteínas citoesqueléticas. Este modelo queda reforzado al encontrar actina asociada a CD38 en las balsas lipídicas.

La hipótesis original de la formación de las balsas lipídicas atribuía un papel central a los lípidos que las componen en la estabilización y formación de plataformas señalizadoras más grandes. Es decir, las proteínas que se asociaban a las balsas lipídicas no tenían un papel reconocido. Sin embargo, esta teoría ha sido modificada recientemente a la luz de nuevos experimentos realizados mediante técnicas microscópicas de alta resolución [21, 24-27]. Se han identificado una serie de proteínas que son claves para la captura y estabilización de dominios lipídicos, que por si solos son extremadamente inestables y de tamaño muy pequeño. Estas proteínas facilitarían la formación de complejos o estructuras señalizadoras diferenciadas del resto de la membrana plasmática, denominados en inglés *membrane rafts* y que sustituiría a la antigua denominación de *lípid rafts.* En este estudio las imágenes obtenidas mediante microscopía confocal revelan que CD38 colocaliza con el gangliósido GM1. Trabajos anteriores indican que gangliosidos complejos inhiben la actividad enzimática de CD38 [28], posiblemente mediante interacciones físicas todavía por determinar. También, la estructura del dominio extracelular de CD38 indica la presencia de una zona rica en aminoácidos básicos en la proximidad del dominio de transmembrana, que originalmente se pensó podría facilitar la interacción de CD38 con las balsas lipídicas [5]. Según la nueva hipótesis, esta región de CD38 podría tener un papel activo en la captación y estabilización de los microdominios lipídicos mediante la interacción con los grupos cargados negativamente de los esfingolípidos y gangliósidos, componentes habituales de estos microdominios. Las tetraspaninas también se asocian fuertemente con el colesterol y los gangliósidos [29], posiblemente como consecuencia de la palmitoilación que sufren [15]. En efecto, los exosomas contienen grandes cantidades de colesterol, esfingomielina, el gangliósido GM3 [30] y de otros lípidos que también están normalmente presentes en

las balsas lipídicas y en los microdominios enriquecidos en tetraspaninas. La presencia de CD38 en estas estructuras en las células B podría ser importante para que las vías de señalización de Ca2+ que dependen de cADPR, ADPR y NAADP se lleven a término eficazmente como se observa en otros sistemas celulares [6, 31].

Durante una estancia en el laboratorio de la **Dra. Lund** en Saranac Lake (N.Y) observamos que tras el tratamiento de células B de bazo de ratón con αIgM e IL-4 se producía una clara disminución en la expresión de CD38. Un reciente trabajo demuestra que las células B de bazo de ratón producen una gran cantidad de exosomas tras la estimulación vía CD40 e IL-4, estos exosomas expresan niveles elevados de MHC-I, MHC-II, B220, Ig de superficie y las tetraspaninas CD81 y CD9 [32]. Quizás esa regulación de expresión de CD38 en la célula se deba a su secreción vía exosomas. Quizás en el futuro podamos comprobar si efectivamente CD38 es secretado en exosomas de células B de ratón, si esto es así, y puesto que disponemos del ratón *Cd38⁻/⁻* se podrían plantear de una manera más sencilla una serie de experimentos que nos permitan dilucidar una posible función de CD38 en exosomas.

En conclusión, lo novedoso de este estudio es la inesperada presencia de CD38 en la membrana externa de los exosomas liberados por células B. Esta presencia podría estar regulada por la incorporación previa de CD38 a las balsas lipídicas o a los microdominios enriquecidos en tetraspaninas y su conexión con las correspondientes redes señalizadoras asociadas a estos microdominios. La presencia de CD38 en los exosomas podría tener un papel específico en la regulación de las interacciones intercelulares homo y heterotípicas (por ejemplo: interacción con células CD31⁺). Por último, los exosomas CD38⁺ podrían contener otros elementos singulares (por ejemplo metabolitos como cADPR y ADPR, o proteínas asociadas con CD38) capaces de modular respuestas antígeno-específicas (respuesta a tumores o patógenos).

Como ya hemos indicado en la introducción, un incremento en la expresión de CD38 en diferentes tipos celulares ha sido asociado a ciertas enfermedades humanas [33, 34], e incluso representa un marcador de mal pronóstico en la leucemia linfocítica crónica de células B [35]. Además, parece ser que CD38 juega cierto papel en la patogénesis de la diabetes mellitus, ya que se han encontrado anticuerpos anti-CD38 en diabetes de tipo 1 y tipo 2 en pacientes Caucásicos [36]. También se han detectado autoanticuerpos anti-CD38 en pacientes con tiroiditis autoinmune crónica y enfermedad

de Graves [37]. Sin embargo, no se han llevado a cabo estudios similares en otras enfermedades autoinmunes, como el lupus eritematoso sistémico (SLE). Con todos estos antecedentes nos propusimos desarrollar un segundo trabajo bajo la supervisión del *Dr. Jaime Sancho* y en colaboración con el *Dr. Norberto Ortego* con el objetivo de estudiar la presencia en plasma de autoanticuerpos anti-CD38 en un amplio grupo de pacientes de SLE mediante el uso de un ensayo inmunoenzimático, y la posible relación de estos autoanticuerpos con la expresión aumentada de CD38 en la superficie de las células T, la presencia de citoquinas en el plasma sanguíneo, o con alguna de las características clínicas específicas de los enfermos con lupus.

Nuestro trabajo demuestra niveles séricos incrementados de autoanticuerpos anti-CD38 de tipo IgG en pacientes de SLE y mayores porcentajes de células linfoides CD38$^+$ circulantes (células T, células B y células NK) que en controles sanos. Por el contrario no se detectaron niveles incrementados de autoanticuerpos anti-CD38 de tipo IgM.

Es muy probable que los autoanticuerpos anti-CD38 presentes en el plasma de pacientes con LES sean más diversos que los que se han detectado en este estudio, sobre todo en relación a los epítopos reconocidos de forma natural (formas anormalmente glicosiladas, productos proteolíticos, etc). En este sentido, la proteína recombinante GST-CD38 que hemos utilizado en los experimentos de ELISA no está glicosilada y no es reconocida por los anticuerpos monoclonales HB136 u OKT10, que son específicos contra epítopos conformacionales de CD38, mientras que, por el contrario, esta proteína es reconocida de forma eficiente por al anticuerpo policlonal H-170, que es específico contra epítopos no conformacionales de CD38. Por lo tanto, es bastante probable que los anticuerpos anti-CD38 detectados en pacientes con LES sean contra formas no glicosiladas de CD38, y/o epítopos no conformacionales. En este sentido, la mayoría de los trabajos publicados sobre autoanticuerpos anti-CD38 en otras enfermedades han utilizado como antígeno una proteína CD38 recombinante no glicosilada, tanto en ensayos de *Western blot* [38, 39] como de ELISA [40], por lo que también seguramente están minusvalorando el repertorio potencial de autoanticuerpos anti-CD38.

Se desconoce qué papel pueden jugar una tasa elevada de autoanticuerpos anti-CD38 en el contexto de una enfermedad autoinmune como LES. Podrían tener capacidad agonista e inducir liberación de calcio intracelular y la secreción de citoquinas

proinflamatorias o antiinflamatorias en células CD38^{+}. Así, en experimentos realizados anteriormente en el laboratorio se demostró que un anticuerpo monoclonal anti-CD38 podía bloquear la respuesta antígeno específica de los linfocitos T CD38+ e inhibir la secreción de IL-2 e IFN-γ (referencia Muñoz et al., Blood). Otro anticuerpo monoclonal anti-CD38 diferente bloqueaba en células dendríticas humanas la señalización mediada por CD38 inhibiendo la secreción de IL-12 inducida por LPS, mientras que la secreción de la IL-10 no se alterada [41]. Además, este tratamiento alteraba la polarización de las células T naïve hacia Th1 [42]. En este estudio, observamos una correlación entre los niveles plasmáticos de autoanticuerpos anti-CD38 de tipo IgG con niveles plasmáticos de IL-10 e IFNγ moderadamente incrementados, sugiriendo que estos pacientes tienen una enfermedad relativamente bien controlada, corroborado por el bajo SLEDAI de estos pacientes. Aunque en individuos normales la presencia de IL-10 se asocia con fenómenos de autorregulación del sistema inmune, en el caso de los pacientes con LES se asocia al mantenimiento de niveles de autoanticuerpos e inmunocomplejos elevados [43, 44]. Así, las células B reguladoras normales, que principalmente producen IL-10, ejercen funciones en el control de la respuesta autoinmune. Recientemente se ha descrito en controles sanos humanos una subpoblación de células B CD19^{+}CD24^{+++}CD38^{+++} productoras de IL-10 que inhiben la producción y secreción de las citoquinas proinflamatorias IFNγ y TNFα por las células T CD4^{+} [45]. Esta subpoblación de células B no es funcional en los pacientes con SLE, al menos tras la activación vía CD40 [45]. Sería interesante saber qué efecto tienen los autoanticuerpos anti-CD38 sobre la función de esta subpoblación de células B reguladores. Es interesante resaltar que en nuestro estudio hay una correlación positiva entre el incremento de expresión de CD38 en los linfocitos B y los niveles plasmáticos de IL-10., que quizá podría estar relacionado con el mantenimiento de altos niveles de anticuerpos anti-CD38.

En muchos tipos celulares la expresión de CD38 está regulada por una variedad de citoquinas como IL-1β, TNFα, IL-13, IFNγ e IFNα [46-49]. Es interesante destacar el hecho de que el TNF-α y los IFNs (β o γ) regulan sinérgicamente la expresión de CD38 en las células del músculo liso de las vías respiratorias, haciendolas refractarias a la acción anti-inflamatoria de los esteroides [50]. En este estudio, la expresión incrementada de CD38 en células T correlaciona fuertemente con niveles plasmáticos incrementados de varias citoquinas como TNFα, IFNγ e IL-13, que a su vez pueden contribuir a la inducción y estabilización de la expresión de CD38, en particular en aquellos pacientes de SLE en tratamiento con glucocorticoides. La sobreexpresión de CD38 en células T de

pacientes de SLE podría contribuir a perpetuar el perfil de citoquinas observado en los plasmas de los pacientes de SLE. Además, el incremento de expresión de CD38 en células T normales incrementa la movilización de Ca^{2+} mediada por TCR, y la producción de IL-2 de una forma antígeno dependiente [1], por el contrario, resultados opuestos se han obtenido disminuyendo la expresión de CD38 en superficie mediante el uso de RNA de interferencia [1]. El aumento de la expresión de CD38 en los linfocitos T de pacientes con LES, así como la presencia incrementada de CD38 en las balsas lipídicas de estos pacientes [51], puede contribuir, junto con otras moléculas de señalización que también se encuentran alteradas en la superficie del linfocito T, al defecto general en la señalización temprana de las células T de pacientes con SLE [52].

En este estudio se ha observado que los pacientes de SLE que presentan los niveles más altos de anticuerpos anti-CD38 de tipo IgG son aquellos que tienen los niveles más bajos de CD38 en células T. Por el contrario, aquellos pacientes con mayor expresión de CD38 en células T, son aquellos con los niveles más bajos de anticuerpos anti-CD38 de tipo IgG. Una posible explicación es que los anticuerpos anti-CD38 en plasma de los pacientes de SLE se puedan unir a CD38 de superficie de las células linfoides en circulación y sature los sitios de unión de CD38, impidiendo la unión del anticuerpo monoclonal anti-CD38 usado en citometría de flujo. Alternativamente, los autoanticuerpos anti-CD38 podrían modular la expresión de CD38 en la superficie celular por un mecanismo activo de señalización, como se ha observado in vitro en células T Jurkat CD38$^+$ estimuladas con un anticuerpo monoclonal anti-CD38 agonista [53]. Otra posibilidad es que los autoanticuerpos anti-CD38 induzcan la secreción activa de CD38 en forma de exosomas (Zumaquero et al., 2010), o en forma de CD38 soluble por activación indirecta de una ectopeptidasa (Funaro et al., 1996; Mallone et al., 2001).

Los niveles plasmáticos de citoquinas en pacientes de SLE claramente muestran un desequilibrio en la ratio de citoquinas Th2/Th1 al comparar los niveles de IL-6, IL-5, IL-10, o IL-13 con los niveles de IFNγ en cada individuo. Resultados similares se han obtenido usando la ratio IL-10/IL-12. Todos estos datos apoyan la idea de que en pacientes con SLE activo hay un cambio en el perfil de citoquinas, con un predominio de linfocitos T productores de citoquinas de tipo Th2 [54, 55]. Sin embargo, en pacientes con LES no se observado una ratio Th1/Th2 elevada cuando se compara la proporción de linfocitos T CD4+ productores de la citoquina de tipo 1 IFNγ versus los que producen citoquina de tipo 2 IL-4 [61]. De forma similar, en nuestro trabajo no hemos encontrado un

aumento en la ratio IL-4/IFNγ en el plasma de pacientes de SLE. Por el contrario la ratio IL-13/IFNγ está claramente incrementada, datos que están de acuerdo con otros trabajos que muestran un incremento en la IL-13 pero no de IL-4 en suero de pacientes de SLE [62]. Además, en PBMCs no estimulados de pacientes de SLE se ha observado un incremento en los niveles de mRNA de IL-13 y una disminución de mRNA de IL-4 [63, 64]. La IL-13 tiene actividades biológicas similares a la IL-4 aunque la producción de estas citoquinas por las células T se regula de manera diferente [65].

En las células T de los pacientes de SLE hay un incremento en la señalización mediada por Ca^{2+} que lleva a la activación del factor de activación nuclear en células T (NFAT), y a una activación alterada de la proteína activadora 1 (AP-1). Esto podría explicar una producción de citoquinas de tipo 2 como IL-10, e IL-13, pero no de IL-4 en pacientes de SLE [66]. Puesto que algunas son claves en el crecimiento y función de linfocitos, una alteración en el equilibrio de los niveles de citoquinas podría alterar la regulación de la autoreactividad, e iniciar autoinmunidad en el lupus.

No obstante, estas citoquinas no son exclusivamente sintetizadas por células T. Así, los monocitos secretan IL-6, que, junto con el TGFβ, favorece la diferenciación de células Th17 [56]. Los pacientes con lupus tienen niveles séricos de IL-6 elevados (obsevado en este trabajo y en otros,[57]), que podrían inducir crecimiento y diferenciación de células T aumentando la producción de IL-2 [58]. Es sabido que la IL-6 juega un importante papel en la hiperactividad de las células B y en la inmunopatología del SLE humano, y podría estar implicada en el daño tisular [59]. En este sentido, la mayoría de los pacientes con SLE tienen niveles elevados de IL-2, y porcentajes incrementados de células T $CD38^{+}CD8^{+}$ [60], tal y como hemos podido confirmar en este estudio.

Sería interesante evaluar en un futuro si los cambios en la expresión de CD38 en células linfoides o en los niveles plasmáticos de autoanticuerpos anti-CD38 pudiese ser útil para predecir estados activos de la enfermedad. Nuestro estudio indica que la elevada expresión de CD38 en las células T procedentes de pacientes de SLE podría ser consecuencia de la acción de citoquinas proinflamatorias como IFNγ o TNFα, siendo indicativo de pacientes con un estado más activo de la enfermedad y con un patrón de citoquinas Th1 y Th2 alterado. Por el contrario, la presencia de autoanticuerpos anti-CD38 podría ser indicativo de pacientes de SLE con una enfermedad relativamente controlada, con un bajo SLEDAI y niveles de citoquinas más bajas.

También junto con el **Dr. Norberto Ortego** hemos llevado a cabo un estudio inmunológico de una paciente afectada por un síndrome hemofagocítico (HLH) que posteriormente fue asociado a una infección por *Leishmania*, para ello, al igual que en los pacientes de SLE, hemos analizado los niveles de 10 citoquinas en plasma y mediante citometría de flujo hemos estudiado diferentes subpoblaciones celulares en 6 extracciones de sangre durante un periodo de tiempo de 20 meses. Adicionalmente hemos llevado a cabo la extracción de mRNA de parte de los PBMCs de la paciente y en la actualidad estamos analizando los niveles de transcritos de un conjunto de citoquinas y factores de transcripción.

Previo al diagnóstico de infección por *Leishmania*, los altos niveles de IL-10 en plasma, junto con la presencia de neutrófilos activados (CD10$^+$CD64$^+$), así como un incremento en la expresión de CD64 en monocitos nos hizo pensar desde un primer momento en una infección crónica.

La IL-10 ha sido relacionada con una serie de infecciones parasitarias, bacterianas o víricas [67-69]. De entre todos los mecanismos propuestos [70-73], parece ser que la producción de IL-10 es una de las estrategias más importantes adoptadas por *Leishmania* para evadir la respuesta inmune. Aunque la IL-10 se produce para disminuir la respuesta Th1 y T CD8$^+$ y así prevenir ciertas inmunopatologías, una excesiva producción de IL-10 inhibe la respuesta proinflamatoria dando lugar a una mayor susceptibilidad a infección por *Malaria* [74, 75], *Leishmania* [76, 77], virus coriomeningitis linfocítico (LCMV) [78, 79] y *Mycobacteria* spp [80]. Varios estudios demuestran que uno de los mayores factores que contribuyen a la progresión de la enfermedad en la leishmaniasis es la IL-10. El bloqueo del receptor de la IL-10 en ratones infectados con *L. donovani* prácticamente elimina la infección [81]; y los ratones *Il-10$^{-/-}$* son muy resistentes a la lesismaniasis visceral (VL) [82]. Ha sido descrito que varias poblaciones celulares expresan IL-10 durante la infección por *Leishmania*, células T reguladoras naturales [70], células Th1 [71, 83], células NK [84], macrófagos [85], células B [84] y DCs [86]. En humanos, los niveles plasmáticos de IL-10 están fuertemente ligados a susceptibilidad a una serie de enfermedades infecciosas como malaria [87], lepra [88] o sida [89]. Una infección persistente por *L. donovani* está asociada con niveles incrementados de IL-10 en el suero de pacientes con VL [90], tal y como ocurre en nuestra paciente, especialmente en las primeras extracciones. Aún no se conocen las células productoras

de esta IL-10, aunque hay un estudio muy reciente que demuestra que los individuos con VL acumulan transcritos de IL-10 en células T CD25^{+} Foxp3^{+} en el bazo [91].

Otro mecanismo de evasión adoptado por *L. donovani* es la capacidad de escapar a la respuesta celular T CD8^{+} [72], esencial en el control de infecciones. Estudios llevados a cabo en un interesante modelo de ratón indican que *L. donovani* evade la respuesta celular T CD8^{+} limitando la expansión clonal e induciendo un agotamiento funcional de estas células [72]. Esta limitada capacidad de expansión puede ser debida a múltiples factores, bien a una disminución en la capacidad de las DCs de procesar y presentar el antígeno [92, 93] o bien debido a un ambiente supresivo, causado principalmente por altos niveles de IL-10, atenuando la función efectora y alterando la formación de células T memoria. Hasta el momento no hemos analizado en profundidad las células T CD8^{+} de la paciente, aunque sí nos ha llamado la atención la elevada expresión de CD38 en las células T, especialmente en las células T CD8^{+} debido a una subpoblación fuertemente positiva para CD38. Además, estudios llevados a cabo mediante RT-PCR en PBMCs de la paciente indican niveles muy bajos de granzima, característica de células T CD8+ agotadas.

La reciente observación de que las infecciones víricas persistentes pueden ser resueltas *in vivo* neutralizando los efectos de la IL-10, sugiere que otras enfermedades infecciosas crónicas también asociadas con IL-10 pueden, igualmente, ser tratadas de la misma manera. En humanos, los niveles plasmáticos de IL-10 están relacionados con la situación patológica de aquellos enfermos infectados por *Leishmania* [90], y la pérdida de función de las células T se recuperan *in vitro* tras el bloqueo de la IL-10 mediante el uso de un anticuerpo monoclonal [94].

El estudio de las diferentes poblaciones celulares mediante citometría de flujo nos ha revelado datos muy interesantes. En primer lugar hemos encontrado un elevado porcentaje de granulocitos de baja densidad (LDGs) en PBMCs en la paciente con HLH, estos granulocitos se caracterizan por dos subpoblaciones, una con baja y otra con alta expresión de CD15. Los antígenos de superficie de los neutrófilos sufren varios cambios durante su maduración, mientras que algunos antígenos se mantienen otros aparecen o desparecen. Con el objetivo de estudiar el fenotipo de estos LDGs hemos marcado con CD10 (marcador de neutrófilos maduros) y CD64 (marcador de neutrófilos inmaduros).

Sin embargo, CD64 es también un marcador de neutrófilos maduros activados. A diferencia del control sano, en el que la mayoría de los LDGs son CD10$^+$CD64$^-$, y por lo tanto neutrófilos maduros, los LDGs de la paciente muestra dos fenotipos diferentes, una subpoblación es CD10$^-$CD64$^+$ y podría corresponder con neutrófilos inmaduros, y la otra es CD10$^+$CD64$^+$, neutrófilos activados maduros. Además estos LDGs son muy positivos para CD38.

CD64 se expresa constitutivamente en fagocitos mononucleares y está ausente en neutrófilos maduros en reposo [95, 96]; sin embargo CD69 se regula fuertemente por acción de citoquinas pro-inflamatorias como IFNγ, el cual está ligeramente elevado en la paciente, o G-CSF que se producen durante infecciones o tras exposición a endotoxina [97]. Actualmente existen muchos artículos que investigan el uso de CD64 como un marcador de infección viral, bacteriana, sepsis e inflamación [98-100]. Además, ha sido descrito que la expresión simultánea de CD64 y CD35 podría distinguir entre inflamación y enfermedad infecciosa [101, 102]. Se ha sugerido que los cambios sufridos por los neutrófilos como consecuencia de algunos factores en circulación pueden facilitar su unión al endotelio y producir daño tisular. Además, este incremento en la expresión de CD64 podría dar lugar a altas concentraciones de citoquinas en circulación y además podría usarse como indicador de neutrófilos activados.

Puesto que la activación de monocitos/macrófagos es clave en la patogénesis del síndrome hemofagocítico, analizamos la expresión de los marcadores de activación CD64 y CD38, al igual que HLA-DR. Los monocitos expresan CD64 e incrementan la expresión de este receptor durante la activación. Los monocitos procedentes de la paciente presentan una mayor expresión de CD64 que aquellos procedentes del control. La expresión de CD64 en neutrófilos activados correlaciona con un incremento, aunque más débil, en la expresión de CD64 en monocitos [103], tal y como ocurre en la paciente. Algunos investigadores han demostrado que en monocitos humanos y macrófagos murinos, la expresión de CD64 puede ser regulada por IL-10, tanto *in vivo* como *in vitro* [104-106], sorprendentemente la IL-10 falla a la hora de estimular la expresión de CD64 en neutrófilos humanos [107].

Además, al igual que CD64, la paciente muestra una mayor expresión de CD38 en monocitos. El IFN-γ regula la expresión de CD38 en monocitos de sangre periférica y líneas celulares de monocitos, al igual que la IL-2, aunque en menor medida [47]. El

efecto del IFN-γ en la regulación de la interacción CD38-CD31 [47] podría jugar un papel fundamental en la extravasación de monocitos en infecciones o enfermedades autoinmunes en las que existe una prevalencia de la respuesta Th1. Sabemos que en monocitos humanos, CD38 está asociado a la molécula HLA de clase II, actuando como un co-receptor en la activación inducida por superantígenos [108]. La mayor expresión de HLA-DR y CD38 observado en los monocitos de la paciente podría tener consecuencias en la presentación antigénica de células T.

El estudio de las células NKs en la paciente indica que el porcentaje de estas células es muy bajo. Las células NKs se dividen en dos subpoblaciones, una minoritaria denominada CD56^{bright} caracterizada por una elevada expresión de CD56, que producen altos niveles de IFN-γ y son poco citotóxicas; por otra parte están la llamadas CD56^{dim}, que producen pocas citoquinas pero tienen una elevada capacidad citotóxica y expresan altos niveles de perforina [109]. Un estudio en detalle de estas células NKs nos indica que la paciente tiene una población CD56^{bright} poco definida y difícil de distinguir, mientras que la población CD56^{dim} está dividida en dos subpoblaciones diferenciadas por una diferente expresión de CD56 así como diferente tamaño/complejidad. A diferencia de las células NKs, el porcentaje de células NKTs en la paciente es bastante similar al del control, pero sorprendentemente son muy positivas para CD38. La expresión de CD38 en células NKTs ha sido relacionado con un fenotipo activado en pacientes infectados con HIV -1 o *Mycobacterium tuberculosis* [110].

Otro de los aspectos que nos ha llamado la atención ha sido el bajo porcentaje de células B, sin embargo el porcentaje de células B plasmáticas de la paciente es mucho mayor que el del control.

Tanto el estudio como el tratamiento de esta paciente se presentan como una tarea difícil de afrontar. No es fácil dar una explicación convincente de cómo un parásito como la *Leishmania* es capaz de generar un cuadro hemofagocítico de tal magnitud. Hemos encontrado marcadores de activación celular incrementados, como CD64 y CD38, bajos porcentajes de células NKs y células B, así como altos niveles de citoquinas como IL-10 e IL-6, y niveles algo elevados de IFN-γ e IL-2. El análisis en profundidad de estos resultados nos permitirá diseñar nuevos experimentos que nos permitan un estudio inmunológico en mayor detalle. Probablemente, el análisis de la actividad citotóxica de las células así como el estudio de los niveles de mRNA de citoquinas, factores de

transcripción, marcadores de actividad o marcadores implicados en citotoxicidad será clave para entender el cuadro patológico de esta paciente.

El *Dr. Salvador Arias* (Servicio de Dermatología, Hospital Clínico, Granada) nos propuso el estudio de un grupo de pacientes diagnosticados de pénfigo. El pénfigo se caracteriza por la producción de autoanticuerpos frente a la desmogleína (Dsg), moléculas de adhesión responsables de la unión de los queratinocitos en piel y mucosas.

Uno de los hechos más interesantes observados en este grupo de pacientes es una disminución en la formación de conjugados de tipo T:B en PBMCs estimulados con una mezcla de superantígenos en comparación con un grupo control. Una interacción estable entre la célula B y célula T es clave para que se transfieran señales estimuladoras entre ambas células, se sabe que la interacción LFA1-ICAM es esencial [111]. Otra molécula que podría mediar el contacto T:B es CD28. Además, CD28 puede funcionar como una molécula de adhesión, ya que los anticuerpos monoclonales anti-CD28 afectan a la formación de conjugados T:B, y la disminución en la formación de conjugados T:B correlaciona con una menor expresión de CD28 [112]. Además, las células T de ratón *Cd28*^{-/-} forman un menor número de conjugados estables [113]. Ha sido publicado que el porcentaje de células T CD8^{+}CD28^{-} está aumentado en pacientes de pénfigo [114], esta observación podría explicar la disminución en la formación de conjugados en nuestro grupo de pénfigos. Muchos individuos que tienen un elevado número de células T CD28^{-} tienen una enfermedad autoinmune. Otra posibilidad a tener en cuenta es que los tratamientos inmunosupresivos afecten a las integrinas y den lugar a una disminución en la interacción célula T – célula presentadora de antígeno (APC) [115]. La activación de CD28 en presencia de anti-CD3 o antígeno da lugar a la proliferación de células T y producción de IL-2 [116, 117]. Los datos de este estudio muestran una baja producción de IL-2 por los PBMCs de los pacientes de pénfigo después de 18h de incubación con una mezcla de superantígenos. Presumiblemente, este menor número de conjugados en pacientes de pénfigo está relacionado con una producción disminuida de IL-2 y otras citoquinas como IFN-γ, IL-5, IL-10 o IL-12 [118]. La co-estimulación vía CD28 también media una fuerte regulación del receptor de la IL-2 (CD25) [119, 120], un menor incremento en la expresión de CD25 tras la estimulación con la mezcla de superantígenos se observa en el grupo de pacientes de pénfigo de este estudio.

Los niveles de citoquinas también son analizados en este grupo de pacientes, observándose niveles incrementados de IL-6 al compararlo con el grupo de controles sanos. Estudios llevados a cabo por otros autores indican niveles incrementados en suero de IL-1, TNFα e IL-6 en un estado activo de la enfermedad, pero sólo la IL-6 se encuentra elevada tanto en estados activos como en remisión en pacientes de pénfigo [121]. Uno de nuestros pacientes, en un estado muy activo de la enfermedad, presenta una muy elevada concentración de IL-6, así como otras citoquinas como IL-1β, IL-10, IL-12 o TNFα. También hemos podido acceder a biopsias de pacientes de pénfigo y penfigoide, otra enfermedad autoinmune de la piel, que actualmente están siendo analizadas mediante RT-PCR con el objetivo de evaluar los niveles de mRNA de un conjunto de citoquinas así como factores de transcripción. La sobreexpresión de IL-1α y TNFα en lesiones ha sido confirmada por otros autores mediante RT-PCR en pacientes de pénfigo vulgar [122].

Los análisis de citometría de flujo muestran una población de LDGs en PBMCs procedentes de los pacientes de pénfigo. LDGs han sido previamente detectados en pacientes con SLE y se caracterizan por tener un fenotipo activado [123], al igual que los LDGs procedentes de la paciente con síndrome hemofagocítico asociado a una leishmaniasis que hemos comentado anteriormente. La baja densidad de estos granulocitos es probablemente debido a la degranulación y liberación de citoquinas proinflamatorias. Además, estos LDGs procedentes de pacientes de SLE tienen la capacidad de sintetizar elevados niveles de TNFα e IFNγ, y son capaces de inducir citotoxicidad endotelial pero, sin embargo, tienen disminuido su potencial fagocítico [123]. Futuros estudios en estos granulocitos nos permitirán conocer en mayor profundidad las características fenotípicas de los LDGs de los pacientes de pénfigo. Los análisis de FACs de los PBMCs de los pacientes de pénfigo muestran un mayor porcentaje de células B con elevada expresión de CD38, probablemente corresponden con células plasmáticas. La patogénesis del pénfigo está asociada con autoanticuerpos frente a Dsg3 y Dsg1 y los anticuerpos en plasma anti-Dsg3 y anti-Dsg1 correlacionan con la actividad de la enfermedad. Es clara la correlación entre la mejoría clínica de los pacientes y la disminución de los autoanticuerpos [124, 125]. Un incremento en el porcentaje de células plasmáticas podría estar relacionado con la producción de autoanticuerpos. Otro resultado interesante es el menor porcentaje de CD56^{bright} observado en estos pacientes en comparación con el grupo de controles sanos. Esta disminución de NK

inmunoreguladoras en circulación periférica podría reflejar el reclutamiento de estas células a los sitios de inflamación [126].

En un futuro próximo nos planteamos hacer un estudio fenotípico de estos LDGs en los pacientes de pénfigo, quizás compartan similitudes a aquellos procedentes de la paciente diagnosticada de síndrome hemofagocítico causado por infección de *Leishmania.* Un estudio en detalle de las proteínas de adhesión implicadas en la formación de conjugados T:APC será clave para demostrar si CD28 u otra proteína es la responsable del menor número de conjugados. Es llamativo el hecho de que las citoquinas plasmáticas se encuentren en unos niveles tan bajos, quizá el estudio de estas citoquinas en las lesiones nos permita obtener resultados más satisfactorios.

Debido a mi interés por los mecanismos que controlan la respuesta inflamatoria a infecciones, procesos alérgicos y enfermedades autoinmunes decidí llevar a cabo una estancia en el laboratorio de la **Dra. Frances E. Lund** en Rochester (N.Y). La **Dra. Lund** está interesada en el papel de los nucleótidos extracelulares en los procesos inflamatorios. La ectoenzima CD38 es clave, ya que como hemos indicado en la introducción usa como sustrato NAD^+ y genera metabolitos implicados en la señalización del Ca^{2+}. Sabemos que CD38, a través de su actividad enzimática, regula la respuesta inmune innata y adaptativa modulando la señalización a través de receptores de quimioquinas y controlando el tráfico de neutrófilos, monocitos y células dendríticas a los sitios de infección. Nuestros datos y los de otros laboratorios van en la misma dirección, los niveles extracelulares de NAD^+ al igual que otros nucleótidos extracelulares como ATP, UTP o UDP juegan un importante papel en la regulación del sistema inmune y la respuesta inflamatoria.

El equipo de la **Dra. Lund** ha demostrado en múltiples trabajos que los productos de CD38, cADPR y ADPR, inducen la movilización del Ca^{2+} intracelular y extracelular regulando la quimiotaxis de fagocitos y la migración de células del sistema inmune [127-130]. Los mecanismos moleculares de cómo estos metabolitos llevan a cabo su actividad no se entiende completamente, aunque está claro que la entrada de Ca^{2+} extracelular al interior de la célula a través de un canal de Ca^{2+} es clave en la respuesta quimiotáctica de los neutrófilos y DCs a muchas quimioquinas. Aunque todavía no conocemos la identidad de este canal, sabemos que es sensible a la presencia de antagonistas de cADPR, es dependiente de la liberación de Ca^{2+} intracelular, puede ser bloqueado con un análogo de

ADPR y no es un canal clásico de tipo SOC. Durante mucho tiempo se ha especulado con la hipótesis de que el principal metabolito de CD38, la ADPR, directamente activa el flujo de Ca^{2+} a través del canal Trpm2 [130, 131]. Esta idea está fuertemente avalada por el hecho de que tanto pequeñas concentraciones de cADPR como el Ca^{2+} intracelular potencian el efecto de la ADPR sobre este canal [132]. La teoría más extendida es que la cADPR sinergiza con la ADPR o bien induce liberación de Ca^{2+} vía receptores de rianodina y este Ca^{2+} intracelular actúa junto a la ADPR para inducir la entrada de Ca^{2+} desde el exterior. En conclusión, la ADPR sola o en combinación con la cADPR, podría jugar un importante papel en la entrada de Ca^{2+} dependiente de CD38 y en migración celular.

Es difícil de entender, y actualmente no está bien argumentado, como una ectoenzima, como CD38, es capaz de regular una serie de respuestas a través de sus productos, cADPR y ADPR, que aparentemente funcionan intracelularmente pero que son generados en el exterior de la célula. La cADPR tiene que unirse a los receptores de rianodina para inducir la liberación de Ca^{2+} intracelular, y la ADPR tiene que unirse a un dominio citoplasmático del canal Trpm2 para inducir la entrada de Ca^{2+} [133, 134]. Sin embargo, ha sido descrito que los productos de CD38 podrían ser transferidos por transportadores de nucleósidos al interior de la célula [135], pudiendo de esta manera acceder a sus sitios de unión en el citoplasma.

El primer objetivo que nos propusimos fue demostrar si el canal Trpm2 está implicado en la vía de señalización de CD38. Hasta muy recientemente no hemos podido llevar a cabo este estudio debido a que no existen inhibidores selectivos para el canal Trpm2 y los ratones deficientes para este canal *(Trpm2-/-)* no habían sido generados. Una vez que hemos tenido acceso a los ratones *Trpm2-/-* hemos llevado a cabo experimentos de quimiotaxis usando diferentes tipos celulares hacia quimioquinas que señalizan de una forma tanto CD38 dependiente como independiente. Durante mi estancia en el laboratorio de la **Dra. Lund** hemos examinado la respuesta quimiotáctica de los neutrófilos de medula ósea de los ratones *Trpm2-/-* hacia fMLP e IL-8. Hemos generado *in vitro* DCs maduras e inmaduras derivadas de ratones *Trpm2-/-* y llevado a cabo experimentos de quimiotaxis a SDF-1 y ELC, respectivamente. El poder quimiotáctico de SDF-1, ELC y SLC sobre las células T CD4+ y SDF-1 y BLC sobre las células B de ratones *Trpm2-/-* también ha sido analizado. Finalmente hemos examinado la migración de monocitos a MCP-1 de medula ósea prodedentes de los ratones *Cd38-/-* y *Trpm2-/-* hacia MCP-1.

Ninguno de los tipos celulares *Trpm2^{-/-}* mostró una migración alterada a las quimioquinas estudiadas. Estos resultados preliminares indican que probablemente el Trpm2 no es el canal de membrana plasmática implicado en la vía de señalización de CD38.

Los productos obtenidos por CD38 juegan un papel crítico en la regulación de la migración de los leucocitos, sin embargo, uno de los datos más interesantes es que CD38 controla la quimiotaxis a algunas pero no a todas las quimioquinas, ya que el sistema CD38/cADPR/ADPR no controla la señalización a través de todos los receptores de quimioquinas o quimioatrayentes. Por ejemplo, una eficiente migración de los neutrófilos de ratón hacia IL-8 y MIP2 no requiere de la activación del flujo de Ca^{2+} por el sistema cADPR/ADPR. Además, *Partida-Sánchez et al.* [128] han demostrado que CD38 regula la migración de las DCs maduras desde la sangre hasta los sitios de inflamación así como la migración de las DCs maduras desde los sitios de inflamación hasta los nódulos linfáticos. CD38 y sus metabolitos modulan la movilización de Ca^{2+} en las DCs estimuladas con quimioquinas y son esenciales para la quimiotaxis de las DCs inmaduras a MCP-1 y SDF-1 y DCs maduras a SLC e ELC.

Se sabe que los receptores de quimioquinas se acoplan a proteínas G triméricas en las que la subunidad α es generalmente Gαi. Para que se produzca una quimiotaxis adecuada se requiere la activación de la subunidad αi, con la subsecuente liberación de las subunidades βγ [136]. Sin embargo, los receptores de quimioquinas pueden acoplarse a otras proteínas G, como por ejemplo a la proteína Gαq [137]. En el caso de CD38, se pensaba inicialmente que para ejercer su acción debería estar acoplado a una proteína Gαi-2. Sin embargo, *Shi et al.* [138] han dado un paso clave en el entendimiento de la vía de señalización de CD38 al demostrar que la Gαi-2, aunque necesaria, no es suficiente para inducir una quimiotaxis adecuada de leucocitos primarios a un gran grupo de quimioatrayentes. Se requiere de una vía de señalización alternativa acoplada a Gαq. Esta vía de señalización alternativa acoplada a la proteína Gαq es muy importante en migración celular tanto *in vivo* como *in vitro*, al menos en repuesta a estímulos inflamatorios. La proteína Gαq, al igual que CD38, regula la entrada de Ca^{2+} extracelular en células estimuladas con ciertas quimioquinas. Curiosamente los autores sugieren que CD38 y la Gαq son coreguladores del mismo canal de Ca^{2+}. Resumiendo, para que haya una correcta quimiotaxis de los neutrófilos de médula ósea de ratón al péptido formilado fMLP, de las DC inmaduras a las quimioquinas MCP-1 y SDF-1 y de las DC maduras a

SLC y ELC, es necesaria la expresión de CD38, una vía de señalización acoplada a Gαq y flujo de Ca^{2+} extracelular.

Una vez demostrado que el canal Trpm2 no estaba implicado en la vía de señalización de CD38, nos propusimos investigar otras posibilidades. Una de las opciones que *a priori* nos parecieron de las más interesantes fueron los receptores purinérgicos. Varios estudios indican que los nucleótidos y derivados nucleotídicos pueden jugar un importante papel en quimiotaxis a través de su acción sobre receptores purinérgicos, además algunos de estos receptores del subtipo P2Y están acoplados a la proteína Gαq. Curiosamente, el sustrato de CD38, NAD^+, ha sido recientemente asociado al receptor $P2Y_{11}$ en granulocitos y receptores $P2Y_1$ en músculo liso visceral [139, 140]. Además, el NAD^+ ha sido también relacionado con los receptores ionotrópicos P2X, *Granher et al.* [141] han demostrado que la adición extracelular de NAD^+ da lugar a un incremento en el flujo de Ca^{2+} extracelular en monocitos humanos, esta entrada de Ca^{2+} extracelular se inhibe mediante el uso de antagonistas de receptores purinérgicos. Más interesante aún es el hecho de que productos del CD38, el NAADP y la ADPR puedan actuar como agonistas de los receptores P2Y. El NAADP ha sido identificado como un agonista del receptor purinérgico humano $P2Y_{11}$ [142] dando lugar a un incremento sostenido de Ca^{2+} debido a la entrada de Ca^{2+} extracelular. La ADPR extracelular también actúa como agonista de un receptor P2Y y un receptor de adenosina en células β pancreáticas [143]. El derivado nucleotídico UDP-glucosa actúa como un agonista de $P2Y_{14}$, de manera que es razonable pensar que otros derivados nucleotídicos, como la ADPR puedan actuar como agonistas de los receptores purinérgicos.

Con el objetivo de investigar la posibilidad de que los receptores purinérgicos estén implicados en la vía de señalización de CD38, primero analizamos mediante RT-PCR la expresión de un grupo de receptores purinérgicos en neutrófilos aislados de médula ósea de ratones *salvajes*, *Cd38⁻/⁻* y *Trpm2⁻/⁻*. Nuestro estudio muestra elevados niveles de mRNA de $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_6$, $P2Y_{13}$ y A_3. A_{2B} y $P2Y_{12}$ también se expresan pero en menor medida. A_{2A}, $P2Y_4$, $P2Y_{11}$ y $P2X_6$ no se incluyeron en este estudio. En segundo lugar probamos el efecto de los antagonistas no selectivos, suramina y CGS-15943, y el agonista natural ATP sobre la quimiotaxis de los neutrófilos de ratón. La suramina es un antagonista no selectivo de los receptores P2, a altas concentraciones afecta a todos los subtipos a excepción del $P2Y_4$, sin embargo a bajas concentraciones boquea a los receptores $P2X_1$, $P2X_2$, $P2X_3$, $P2X_5$, $P2X_{2/3}$, $P2X_{1/5}$, $P2Y_1$,

P2Y$_2$, P2Y$_{11}$ y receptores P2Y$_{13}$. La quimiotaxis de los neutrófilos de ratón a fMLP está fuertemente inhibida usando 10µM de suramina, sin embargo no afecta a la quimiotaxis hacia IL-8, esto significa que la suramina está afectando a la vía de señalización de fMLP, o en otras palabras que la suramina interfiere en la vía de señalización de CD38. Por otra parte, el CSG-15943 es un potente antagonista no selectivo de los receptores P1. La quimiotaxis de los neutrófilos de ratón a fMLP e IL-8 está fuertemente inhibida por 10µM de CGS-15943, con lo cual el receptor de adenosina o receptores que estamos bloqueando con este antagonista está implicado en las dos vías de señalización. El efecto del ATP en la quimiotaxis de los neutrófilos a fMLP, no es fácil de explicar, observamos que los neutrófilos de ratón incubados con ATP producen una débil disminución de la quimiotaxis a fMLP. No podemos excluir la posibilidad de que la señalización llevada a cabo por un teórico receptor purinérgico sea interferida por ATP. La situación se complica aún más teniendo en cuenta que el ATP es rápidamente hidrolizado, y sus metabolitos, principalmente ADP y adenosina, pueden por sí mismo señalizar a través de otros receptores purinérgicos. Nosotros no hemos probado el efecto del ATP sobre la quimiotaxis de los neutrófilos hacia IL-8, pero sería interesante saber si el ATP también produce esta débil disminución en la quimiotaxis hacia IL-8. Todos estos datos demuestran que los receptores de adenosina probablemente estén implicados en la respuesta quimiotáctica de los neutrófilos regulando tanto las vías de señalización dependiente e independiente de CD38 y que la suramina parece bloquear un receptor que está implicado exclusivamente en la vía de señalización de CD38. Sin embargo, es importante destacar que el uso de la suramina presenta ciertas limitaciones ya que inhibe algunas proteasas, ecto-nucleotidasas, proteínas quinasas y subunidades de la proteína G. Sin embargo, es evidente que la suramina inhibe la migración de los neutrófilos a fMLP pero no a IL-8.

Nuestro estudio de expresión de receptores purinérgicos muestra elevados niveles de mRNA de los receptores P2X$_1$, P2X$_4$ y P2X$_7$. Estos receptores son co-expresados en la mayoría de las células del sistema inmune, como mastocitos, linfocitos B y T, macrófagos o microglia [144]. Sin embargo, los transcritos de P2X$_2$, P2X$_3$ y P2X$_5$ son muy bajos. No tenemos los datos de expresión de P2X$_6$, aunque sabemos que la suramina es un débil antagonista.

De este subgrupo el P2X$_1$ requiere una mención especial. El P2X$_1$ ha sido implicado en la respuesta quimiotáctica. Recientemente, *Lecut et al.* [145] mostraron por

primera vez que los canales $P2X_1$ estaban implicados en el control de la quimiotaxis de los neutrófilos, tanto *in vivo* como *in vitro*. $P2X_1$ parece ser un buen candidato entre los receptores de tipo P2X ya que está fuertemente expresado en neutrófilos, es muy sensible a la suramina y su activación da lugar a la entrada de Ca^{2+}. Sin embargo, es difícil de entender como los productos de CD38 pudieran actuar activando un receptor cuyo principal agonista es el ATP. El uso del antagonista selectivo para $P2X_1$ NF449 o el uso de los ratones *P2x1$^{-/-}$* en futuros experimentos será clave para saber la posible implicación de este canal en la vía de señalización de CD38.

Entre los receptores de tipo P2Y hay varias posibilidades interesantes, por una parte tenemos aquellos que están acoplados a Gq como $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, o $P2Y_{11}$, por otra parte tenemos aquellos que están activados por dinucleótidos derivados de adenina, como $P2Y_1$, $P2Y_{12}$ o $P2Y_{13}$.

En un principio el receptor $P2Y_{11}$ nos pareció un muy buen candidato ya que es sensible a la suramina, está acoplado a Gαq y lo más interesante es que uno de los productos de CD38, el NAADP, ha sido identificado como agonista de los receptores $P2Y_{11}$ humanos [142] y además parece tener un rol en maduración y migración de DCs [146]. Sin embargo no se han encontrados transcritos de este receptor en ratas o ratones con lo cual no fue incorporado en nuestro estudio de expresión.

A pesar de que los niveles de los transcritos de $P2Y_1$ sean bajos, éste parece ser un buen candidato, es el único que además de ser activado por ADP está acoplado a proteína Gq. Estos receptores están implicados en el incremento de los niveles de Ca^{2+} en respuesta a ADP en macrófagos del peritoneo en ratones [147]. Los ratones *P2y1$^{-/-}$* presentan un fenotipo con una disminución en la agregación plaquetarias y hemorragias [148, 149]. Además, el "knockdown" del receptor $P2Y_1$ presenta una migración defectuosa de los progenitores neuronales a la zona subventricular [150].

Los otros receptores activados por ADP, el $P2Y_{12}$ y $P2Y_{13}$ son también interesantes. El receptor $P2Y_{12}$ está implicado en quimiotaxis de microglía [151, 152], y en algunos sistemas experimentales, los receptores $P2Y_{12}$/$P2Y_{13}$ se han encontrado acoplados a incrementos de Ca^{2+}.

Probablemente la situación a la que nos enfrentamos sea mucho más compleja de lo que pensamos en un principio, y quizás, varios receptores estén implicados. En la literatura hay varios ejemplos de "cross-talk" entre receptores purinérgicos. Hay un trabajo muy interesante llevado a cabo por *Tolhurts et al*. [153] que muestra que tanto $P2Y_1$ como $P2Y_{12}$ son necesarios para una completa activación de un canal que permite la entrada de Ca^{2+} y Na^+ en megacariocitos derivados médula ósea y además, favorecen la secreción de ATP y ADP, activando a los receptores $P2X_1$, $P2Y_1$ y $P2Y_{12}$ y de esta manera acelerando la entrada de Ca^{2+}. Esta compleja inter-relación entre estos tres receptores purinérgicos permite la entrada de Ca^{2+} durante la activación de plaquetas.

En el futuro, el uso de ratones deficientes en receptores purinérgicos junto con el uso de inhibidores específicos nos permitirá clarificar los mecanismos dependientes de CD38 implicados en quimiotaxis.

Para concluir hemos de resaltar el hecho de que tanto CD38 como algunos receptores purinérgicos se localizan en balsas lipídicas. Durante mi estancia en el laboratorio de la **Dra. Zubiaur** aislamos las balsas lipídicas tanto de esplenocitos como de células B purificadas de bazo de ratón y un elevado porcentaje de CD38 se encontró en las balsas lipídicas, así que, presumiblemente, una parte de CD38 se localice en las balsas lipídicas de los neutrófilos de ratones. Las concentraciones locales de nucleótidos alrededor de las balsas lipídicas están fuertemente controladas y podría dar lugar a un determinado patrón de señalización vía receptores purinérgicos en diferentes microdominios. Es fácil predecir que la composición de las balsas lipídicas que contienen CD38 en neutrófilos estimulados con fMLP de aquellos estimulados con IL-8.

Probablemente CD38, junto con otros receptores, estén implicados en una señalización integral en forma de cascada coordinada en diferentes pasos. Sin duda estamos en un momento fascinante en el estudio de los mecanismos implicados en migración celular.

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Conclusiones

Conclusiones

1. **CD38 está expresado en la superficie de exosomas derivados de células B. CD38 en exosomas está asociado a CD81, CD19, Lyn, Gαi-2, Hsc-70 y Lyn. Igualmente, CD38 en balsas lipídicas está asociado a CD81, CD19, Lyn, Gαi-2, Hsc-70 y actina.**

2. **CD38 es enzimáticamente activo en balsas lipídicas y exosomas.**

3. **La activación de la célula B vía CD38 induce activación de Akt/PKB y Erk.**

4. **Los pacientes de LES tienen mayores niveles de anticuerpos anti-CD38 IgG y mayor expresión de CD38.**

5. **La expresión de CD38 en linfocitos T de LES correlacionan con los niveles plasmáticos de ciertas citoquinas, siendo predominante en pacientes en un estado clínicamente activo.**

6. **Los anticuerpos anti-CD38 correlacionan con niveles plasmáticos moderadamente incrementados de IL-10 e IFN-γ.**

7. **Los pacientes de pénfigo presentan una formación de conjugados T:B disminuida, una menor activación de linfocitos T y menor producción de citoquinas tras la activación con superantígenos.**

8. **El canal TRPM2 no es el canal implicado en la entada de Ca^{2+} en quimiotaxis dependiente de CD38 en células de ratón.**

Anexo I: *Publicaciones*

Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signalling complexes containing CD81, Hsc-70 and Lyn
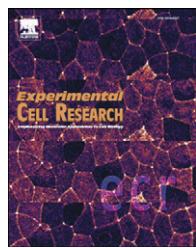
Research Article

Exosomes from human lymphoblastoid B cells express enzymatically active CD38 that is associated with signaling complexes containing CD81, Hsc-70 and Lyn[☆]

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ARTICLE INFORMATION

ABSTRACT

Exosome vesicles of endocytic origin are involved in communication between tumor and immune cells. In addition, membrane rafts (MR) may support the sorting of proteins associated with exosomes. CD38 is found at the plasma membrane and in recycling endosomes, which are both redistributed toward the immunological synapse (IS) upon T cell antigen receptor (TCR) engagement. The data of this study provide evidence that CD38 is expressed on the surface of secreted exosomes derived from lymphoblastoid B cells. Exosomic CD38 is associated with the signaling molecules CD81, Hsc-70 and Lyn. Likewise, in MR, CD38 is associated with CD81, CD19, Lyn, Gαi-2, Hsc-70 and actin. Therefore, a high degree of overlap in the pattern of signaling proteins associated with CD38 in exosomes and MR exists. Exosomic and MR CD38, by virtue of these interactions, have signaling potential. Indeed, CD38 is enzymatically active in both exosomes and MR, and CD38 ligation induces Akt/PKB and Erk activation, which is accompanied by increased translocation of CD38 into MR. In conclusion, the present study indicates that CD38 localizes to MR, where it promotes cell signaling, and it is exported out of the cells through the exosome-mediated exocytic pathway, where it may act as an intercellular messenger.

Introduction

Ectoenzymes are a family of pleiotropic molecules that are thought to play increasingly important roles in cell fate and in the regulation of intercellular interactions [1]. One member of this molecular family is human CD38, a type II transmembrane glycoprotein that is ~45 kDa in its monomeric form. CD38 is an ADP-ribosyl cyclase that converts NAD^+ to cyclic ADP ribose (cADPR) and NADP to nicotinic acid-

adenine dinucleotide phosphate (NAADP). Both of these end products are endogenous activators of intracellular Ca^{2+} release [1–3]. Similar to other ectoenzymes, CD38 acts as a receptor and interacts in a selectin-type fashion with CD31, a non-substrate ligand expressed by endothelial cells and other cell lineages. The signals mediated by CD31/CD38 interactions appear to be partially dependent on lateral membrane associations with surface signaling receptors and membrane adaptors [1,4]. Formal confirmation of this finding was recently obtained in T lymphocytes [5]. Indeed, membrane CD38 was shown to localize to specific areas of the IS [5]. Also, unexpectedly, a second pool of CD38 that is derived from endosomes was found. Surface and cytoplasmic pools are redistributed at the IS upon TCR engagement, and the redistribution of both pools are dependent on tyrosine kinase Lck-mediated signals [5].

Exosomes are nanometer-sized vesicles (40–100 nm) of endocytic origin secreted upon fusion of multivesicular endosomes to the surface of cells of hematopoietic and non-hematopoietic origins. A growing number of functions have been attributed to exosomes such as scavenging archaic proteins, transmitting pathogens and receptor activity. Exosomes from cytotoxic T cells and B lymphocytes may be involved in targeting molecules for cell death or antigen presentation [6,7]. Moreover, it has been reported that MR may support the sorting of proteins such as MHC class II molecules that are found partially associated with MR domains isolated from B cell exosomes [6].

MR [8,9] are small (10–200 nm), heterogeneous, and highly dynamic sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Protein–lipid and protein–protein interactions contribute to their formation. The presence of receptor and effector proteins in MR has led to the concept of a signalosome domain: MR are thought to act as molecular sorting machines that are capable of coordinating the organization of signal transduction pathways within limited regions of the plasma membrane and organelles [8]. MR are also involved in membrane trafficking [8,10]. Furthermore, MR can be successfully isolated at a physiological temperature (37 °C) using Brij 98, a member of the polyoxyethylene ether series of non-ionic detergents [11]. This novel extraction procedure has allowed the isolation and characterization of small membrane vesicles (~67 nm) [12–15], which are more typical of the physiological MR described above than the MR isolated using Triton X-100 at low temperatures (0–4 °C).

The goal of the present study was to provide a topographical analysis of CD38 and to characterize CD38 associated molecules purified from different cell districts in B cells. CD38 has shown to be physiologically relevant in normal and neoplastic human B lymphocytes [16]. CD38 play a key role in the delivery of growth signals to chronic lymphocytic leukemia (CLL) cells and in regulating cellular migration to growth-permissive niches [17]. First, we tested whether exosomes released from B cells are enriched in CD38 and whether specific interactions exist between CD38 and other well-characterized exosome-associated signaling proteins. Based on these interactions, exosomic CD38 is a potential signaling molecule and may provide an alternative route for context-dependent signal delivery or for the transmission of materials relevant for immune responses. Second, MR may support the sorting of some proteins associated with exosomes; therefore, the signaling proteins retrieved with MR-associated CD38 were analyzed in parallel. There is a high degree of overlap in the pattern of signaling proteins associated with CD38 in both compartments, as opposed to the proteins in soluble fractions. This phenomenon most likely occurs because these molecules are part of

pre-formed signaling complexes that are incorporated into the same MR subset [13] and may maintain their association along the exocytic pathway.

Materials and methods

Cell lines

Human lymphoblastoid B cell lines Namalwa and Raji ($CD38^+$) were from ATCC and HOM-2 cells ($CD38^-$) were generously provided by Dr. D. Jaraquemada. The cells were cultured in RPMI-Hepes, with 5% to 10% heat-inactivated fetal bovine serum (FBS), supplemented with antibiotics, as described [18] and kept at 37 °C in a 5% CO_2 atmosphere.

Antibodies and reagents

Anti-human CD38 monoclonal antibodies (mAbs) used: HB136 (IgG1), OKT10 (IgG1), IB4 (IgG2a) (generously provided by Dr. A. Horenstein) and clone GR7A4-PE (phycoerythrin) (IgG1, Immunostep, Salamanca, Spain). Other antibodies used (from BD Biosciences, USA): anti-human CD81-PE (clone JS-81, IgG1) and anti-human HLA-DR, DP, DQ- FITC (fluorescein isothiocyanate-conjugated) (clone Tü39, IgG2a) that reacts with all major histocompatibility Class II HLA. The isotype-matched controls used: IgG1-PE, IgG2a-FTIC and other isotype-matched controls PE and FITC conjugated (from BD Biosciences and Immunostep). A rabbit IgG fraction was purified by affinity chromatography from pre-immune serum, for immunofluorescence experiments [18]. For stimulation experiments it was used an affinity purified (AP) F(ab′) 2 fraction of a goat antibody to mouse IgG (whole molecule) (GαMIg) (Cappel, Organon Teknika, USA). AP rabbit and goat polyclonal antibodies and mouse mAbs used (from Santa Cruz Biotechnology, USA): anti-Erk-2 (C-14), anti-CD38 (H-170), anti-Fyn (FYN3, sc-16), anti-Lyn (44, sc-15 and H-6, sc-7274), anti Gαi-2 (T-19, sc-7276 and L5, sc-13534); anti-CD19 (C-20, sc-8498), anti-Gβ (T-20, sc-378); anti-Hsc-70 (B-6, sc-7298), anti-CD81 (5A6, sc-23962), anti-c-Cbl (15, sc-170), and anti-Rab-5 (FL-215, sc-28570). Anti-phospho-p44/42 MAPK-Erk (Thr202/Tyr204) E10 mouse mAb (9101), rabbit mAb antibody anti-phospho-Akt/PKB (Ser473) (9271) and rabbit polyclonal anti-total Akt (9272) were obtained from Cell Signaling Technology, USA. Anti-Syk rabbit polyclonal antibody was generously provided by Dr. A. Weiss. AP mouse mAb to Actin (clone AC40) and cholera toxin-HRP (CTX-HRP) to label the ganglioside GM1 (Galβ1–3GalNacβ1–4Gal (3–2αNeuAc) β1–4Glcβ1–1-Cer) were obtained from Sigma-Aldrich and Molecular Probes. AP goat anti-rabbit, goat anti-mouse and donkey anti-goat IgG (H + L) horseradish peroxidase (HRP) conjugated were from Promega (Madison, WI). Anti-mouse and anti-rabbit IgG TrueBlot™ HRP conjugated were from eBioScience; Streptavidin-HRP was from Amersham, Inc. and also from Sigma-Aldrich. Prestained SDS-PAGE Standards and ImmunoStart reagents were from Bio-Rad, USA.

Fluorescence-activated cell sorter (FACS) analysis

Surface expression of CD38, CD81 and CD19 in cells and in exosomes nanovesicles was analyzed by flow cytometry, with the antibodies indicated above, using saturating concentrations of AP

mouse mAb antibodies, compared with isotype-matched controls [18]. Analysis of exosomes by FACS was carried out as the protocol described elsewhere [19], using exosome preparations incubated with 4-μm alhehyde/sulfate latex beads (Invitrogen) [19]. Cells and exosomes were analyzed in a FACSCalibur flow cytometer (BD Biosciences); data were analyzed using CellQuest Pro (BD Biosciences) and FlowJo software (Tree Star, Inc., San Carlos, CA).

Preparation of exosome-like vesicles and electron microscopy (EM) examination

The FBS used in these experiments was ultracentrifuged overnight, $100,000 \times g$, and sterile filtered on 0.22-μm filters (GP Express PLUS Membrane, Millipore, USA) to avoid contamination with protein aggregates presented in the serum [19]. FBS supernatants were referred as *depleted-FBS* [19,20]. Namalwa (CD38$^+$) and HOM-2 (CD38$^-$) cells were washed for three times in RPMI-Hepes without FBS and placed into a new set of flasks. Cells were grown at 37 °C (1×10^6 cells/ml), for 24-h in RPMI containing 0, 5% *depleted-FBS*. Viability of the cells was determined by trypan blue exclusion (range ~90%). Exosomes were purified from the culture supernatant by differential centrifugation using standard protocols: Culture supernatants were centrifuged at low speed in sequential steps [19,21]. Cells were removed by centrifugation at $200 \times g$ for 10 min. Supernatants were collected and sequentially centrifuged twice for $1,000 \times g$ for 10 min at 4 °C. Then supernatants were passed through 0.22 μm filters. Filtrated supernatants were concentrated on VivaSpin concentrators with a Mr 50,000 MW cutoff (Vivascience, Sartorius). Concentrated supernatants were further separated by ultracentrifugation ($100,000 \times g$, 3 to 12 h, 4 °C, Sorvall AH-650 rotor). The pellet was washed in 20 mM HEPES, pH 7.2, and the microvesicles preparation were spun down by ultracentrifugation ($100,000 \times g$ for 1 h, 4 °C, Sorvall AH-650 rotor). The $100,000 \times g$ pellet was considered as *exosome preparation* and it was treated accordingly to the indicated protocols; for Western blot (WB), for immunoprecipitation (IP), or for GDP-ribosyl cyclase activity, either with 3× Laemmli sample buffer, or 1× lysis buffer with 1% Nonidet P-40 (NP-40) (Pierce), or 1% Brij 98 + 15 mM octyl D-glucoside (ODG), and in the indicated cases with the cyclase buffer. Protein concentration was determined by a micro BCA protein assay kit (Pierce, USA), using BSA as standard. For EM examination the exosome preparations, $100,000 \times g$ pellets treated as described elsewhere [19,20], and also in the legend of Fig. 1.

Detergent solubilization of cells and cell stimulation

Cells ($5–9 \times 10^7$) were washed twice in ice-cold RPMI/HEPES, resuspended in 0.45 ml of ice-cold 1× lysis buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM EGTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 20 μM phenylarsine oxide, 1 mM phenyl-methylsulfonyl fluoride, 10 mM iodoacetamide, and a mixture of small peptide protease inhibitors (Sigma Aldrich), Aprotinin, Pepstatin A, Chymostatin, Leupeptin, and Antipapain, 2 μg/ml/each) without detergent to disrupt the cells quick-frozen on dry ice, and then thawed on ice. Broken cells were homogenized on ice, by shearing through a 25-gauge needle with a 1-ml syringe, 10 times. The particulate suspension was preincubated for 4 min at 37 °C. Next 50 μl of a 10% Brij 98 (Sigma) stock solution in 20 mM HEPES, pH 7.4, was added to bring a final concentration of 1% Brij

98, and kept at 37 °C for 5 min, then centrifuged at $14,000 \times g$, for 15 min at 4 °C, the supernatant was quick-frozen on dry ice and kept at -80 °C until used [13]. In the cases indicated, cells were lysed in 1× lysis buffer containing 1% NP-40, 30 min, on ice. Nuclei were removed by centrifugation at $14,000 \times g$, for 15 min at 4 °C, as described elsewhere [18].

Stimulation of the cells was performed as described in detail elsewhere [18,22]. Cells were grown up to a density of 1×10^6/ml, centrifuged, and serum-starved in 0.1% FBS, RPMI, for 20 h. Next, cells were washed in serum free RPMI-HEPES medium and resuspended at $1–2 \times 10^7$ cells per sample or otherwise indicated, at 4 °C.

Fractionation of floating MR by sucrose gradient ultracentrifugation; Western blot (WB), immunoprecipitation (IP)

Detergent-insoluble and -soluble fractions were separated in a discontinuous sucrose gradient as described [13,18]. Samples were centrifuged for 18–20 h at $200,000 \times g$, at 4 °C, in a Sorvall AH-650 rotor. Eight sucrose gradient fractions of 0.5 ml each were collected on ice, from the top to the bottom of the gradient. Low density fractions corresponding to the 5/30% interface (fractions 2 and 3, along fraction 4) or MR, the high density soluble material corresponding to fractions 7 and 8 of the gradient or Soluble (S) and fractions 5 and 6 or intermediate fractions. Aliquots of each fraction were resolved on SDS-PAGE gels under reducing and non-reducing conditions, or otherwise indicated, and subjected to WB with specific antibodies; as described [18]. Ganglioside GM1, which migrated with the dye front on a 12.5% SDS-PAGE gel, was detected by blotting with cholera toxin-HRP conjugated following by the enhanced chemiluminescence system (ECL).

For the IP of the protein assemblies, either the exosome preparations, $100,000 \times g$ pellets, or the pooled sucrose gradient fractions, corresponding to MR, S or intermediate fractions, respectively, were previously diluted with 1× lysis buffer containing 1% Brij 98 + 15 mM ODG. IP of protein assemblies was performed by incubation of the exosomes or the pooled fractions with the specific antibodies: anti-CD38, anti-Lyn, anti-CD19, anti-CD81, anti-Hsc-70, anti-Gαi-2 and anti-actin followed by the capture of the immune complexes on protein G superparamagnetic microbeads (Miltenyi Biotec), as described [13].

GDP-ribosyl cyclase (GDPR) (NGD → cyclic GDPR) assay

GDP-ribosyl cyclase activity was measured in the exosome preparations, $100,000 \times g$ pellets, in the cell lysates prepared in cyclase buffer (referred as *cell cyclase lysates*) and in the sucrose gradient fractions corresponding to MR and S. The samples were diluted and homogenized in 10 mM Tris–HCl, pH 8, 100 μM PMSF (*cyclase buffer*) and they were incubated in 96-well black plates (no.: 265301, NUNC), in 20 mM Tris–HCl (pH 8), with or without 50 μM NGD$^+$ [23], for 60 min, at 37 °C. It was measured the cyclization of the NAD$^+$-surrogate NGD$^+$ to its fluorescent derivative cyclic-GDP-ribose [23]. The production of the cyclic-GDP-ribose was measured in a fluorescence plate reader, excitation wavelength 300 nm and emission wavelength 410 nm (Spectramax Gemini EM, with SOFTmax Pro software, Molecular Devices, USA). The enzymatic activity was calculated as relative fluorescence units (RFU), normalized by protein concentration (μg) in the sample.
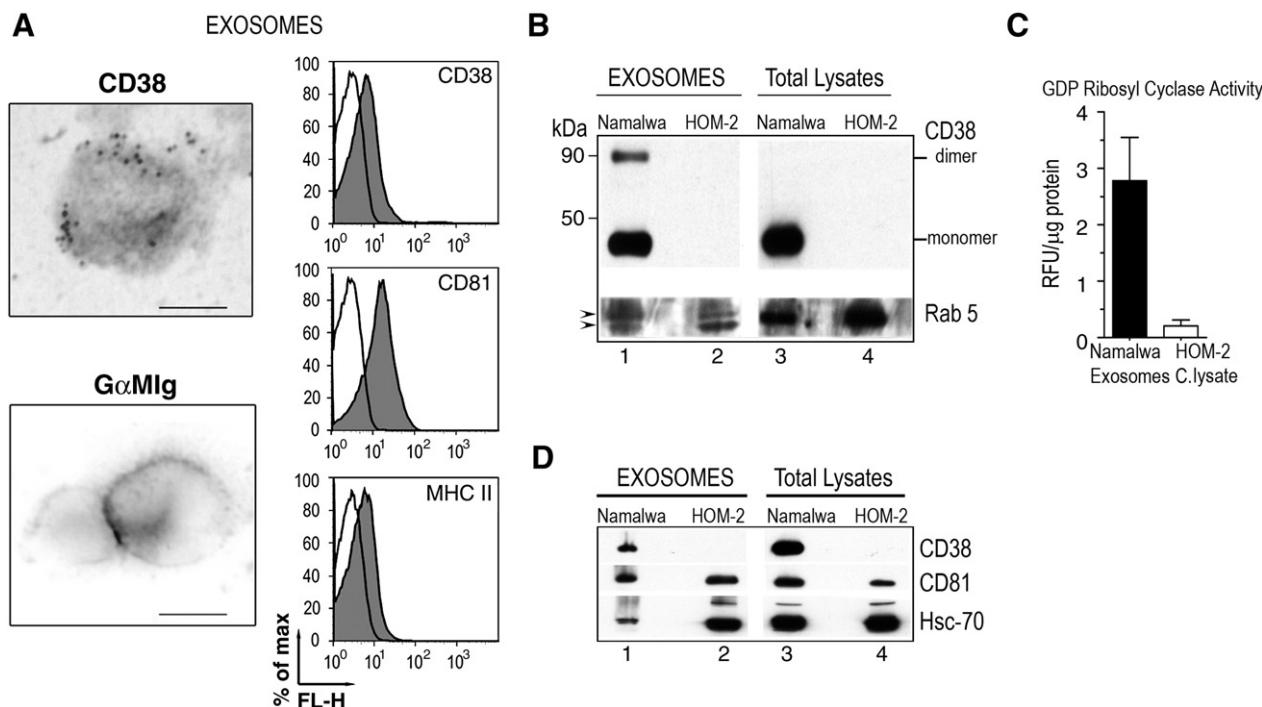
Fig. 1 – **Exosomes derived from Namalwa (CD38$^+$) B cells express enzymatically active CD38. Exosomes were isolated by differential centrifugation from culture medium of Namalwa (CD38$^+$) and HOM-2 (CD38$^-$) cells. Cells were grown at 37 °C (1×10^6 cells/ml), for 24-h in RPMI containing 0.5% *depleted-FBS*; viability was determined by trypan blue exclusion (range ~90%). The $100,000\times g$ pellet was considered as *exosome preparation* and it was treated accordingly to the indicated protocols. Protein concentration in exosomes ranged between 65 and 100 μg, in the $100,000\times g$ pellet or exosome *preparation*, obtained from 200 ml cell culture (1×10^6cells/ml). A 5 to 10 μg of protein per point was used for FACS analysis. A 2 to 4 μg of protein per point was used for GDP-ribosyl cyclase activity analysis; and 10–50 μg of protein per lane for WB analysis. (A) Left upper and lower panels, for EM examination the exosome preparations, $100,000\times g$ pellets were fixed in 2% PFA/0.125% glutaraldehyde/phosphate buffer. Treated pellets were placed on formvar nickel-coated EM grids. For the immuno-gold labeling, grids were blocked in 100 μl drops of PBS/0.5% BSA containing 100 μg/ml of human γ-globulin, (Sigma-Aldrich), then incubated with anti-CD38 mAb, HB136 (20 μg/ml, 1 h, at 37 °C) (upper panel). The grids were washed in 100 μl drops of PBS/1% BSA 5×3 min, incubated with EM grade gold labeled secondary GαMIg (H + L) antibody (no. 115-205-146, 12-nm, from Jackson, Inc.) in PBS/1% BSA (30 μg/ml, 1 h, at 37 °C) (upper and lower panels) and then washed as indicated before. The grids were stained with 2% uranyl acetate, 5 min, at room temperature and washed with doubled distilled water before EM examination (Zeiss EM10C EM, for image acquisition Kodak EM Film 4489 was used). Scale bar ~100 nm. Experiment is representative of three independent ones. (A) Right panels, FACS analysis of the surface proteins of exosome vesicles derived from Namalwa cells are shown. The exosome preparations were incubated with 4-μm beads alhehyde/sulfate latex beads (Invitrogen). After adsorbing the vesicles, the beads were subjected to 100 mM glycine in PBS for 30 min, followed by the addition of BSA (0.5% in PBS) to saturate any remaining free binding sites on the beads. Namalwa exosome vesicles were stained with specific mAb against CD38, CD81, or all Major Histocompatibility Complex class II (MHCII) HLA-DR, DP and DQ antigens (gray histograms) and compared with the respective isotype-matched control (open histograms). One out of three independent experiments is displayed. (B and D) The biochemical characterization of isolated exosomes is shown. Exosomes and total cellular lysates corresponding to the same cells were lysed in 1%-NP-40 1× lysis buffer at 4 °C, resuspended in non-reducing 3× Laemmli sample buffer and proteins were separated on 12% SDS-PAGE gels, then subjected to WB. Membranes were probed with specific antibodies for CD38, Rab-5, CD81 and Hsc-70. CD38 dimers and monomers are indicated on the right. Positions of molecular size markers in kDa are indicated on the left. (C) GDP-ribosyl cyclase activity (Relative Fluorescence Units (RFU)/μg protein) was measured in the exosome preparations from Namalwa cells ($100,000\times g$ pellets) in cyclase buffer (filled histogram) and in cell cyclase lysates from Hom-2 (CD38$^-$) cells (open histogram), prepared as indicated in Materials and methods. The enzymatic activity was determined by means of the specific in vitro fluorometric assay, using NGD$^+$ as a substrate and measuring the production of the cyclic-GDP-ribose. Results show the mean values ± SEM, $n = 3$ independent experiments.**

Membrane biotinylation

Cells were washed three times with PBS, pH 8, at 4 °C, incubated with 0.5 mg/ml Biotinamidohexanoic acid 3-Sulfo-NHS Sodium salt (Sigma-Aldrich), 30 min at 4 °C; washed three times with PBS and lysed in 1× 1% Brij 98 lysis buffer and separated in a discontinuous sucrose gradient, as described [13]. Streptavidin agarose beads were used for precipitations of biotinylated surface molecules (Sigma-Aldrich). The beads were incubated with pooled MR and S fractions, 1 h at 4 °C. Next the beads were washed with the same lysis buffer

and the adsorbed (i.e. biotinylated) proteins were eluted with 3× non-reducing Laemmli sample buffer as described [18].

Treatment with methyl-β-cyclodextrin (MβCD)

MβCD (Sigma-Aldrich) a cholesterol-binding agent was used [24]. Cells were washed and suspended in a Buffered Salt Solution (BSS): 135 mM NaC1, 5 mM KC1, 20 mM Hepes, 1 mM MgCl₂, 1.8 mM CaCl₂, 50 mM glucose, pH 7.4 with 0.5 mg/ml BSA and then treated with 20 mM MβCD (Sigma-Aldrich), in BSS at 37 °C, 30 min, before Brij-98 solubilization and then followed by fractionation in a sucrose gradient ultracentrifugation as described above [13,18].

Immunofluorescence and confocal laser-scanning microscopy (CLSM)

Immunofluorescence was performed as described in detail elsewhere [5]. Cells were plated onto Poly-L-Lysine-coated slides (PLL: 50 μg/ml; Sigma-Aldrich) (20 min at 37 °C) and fixed in 2% paraformaldehyde (PFA) (Sigma-Aldrich) for 10 min at room temperature. Cells were blocked with TNB: 0.1 M Tris–HCl, 0.15 M NaCl, 0.5% blocking reagent (Boehringer Mannheim), containing 100 μg/ml of human γ-globulin, (Sigma-Aldrich). For double staining, the cells were incubated with an AP anti-CD38 mAb, HB136, (10 μg/ml in TNB), 2 h at 37 °C, in the absence of permeabilizing agent, followed by incubation with the secondary Ab, GαMIg-Alexa 488 or Alexa 568 (Molecular Probes, A11029 and A11004, respectively, at 1/200 dilution in TNB), 1 h at 37 °C. Then washed with TNB and incubated with 1% normal mouse serum (Sigma-Aldrich) in TNB. Next, the cells were permeabilized in 0.5% Triton X-100, 5 min in TNB, washed, and incubated with an AP rabbit anti-Lyn, at 10 μg/ml in TNB, 2 h at 37 °C, next, washed again and incubated with a goat anti-rabbit Rhodamine Red-X Ab (Molecular Probes: R-6394, 1/500 dilution in TNB) 1 h at 37 °C. Ganglioside GM1 was detected with Alexa 488-labeled CTX-B (10 μg/ml) (Molecular Probes). Control isotype antibodies were included in all experiments. After mounting, CLSM images were acquired with a Leica TCS-SP5-CLSM, equipped with Ar and He–Ne laser beams and attached to inverted Epi-fluorescence microscope DMI 6000CS, it was used either the HCX PL APO CS 100× 1.4 oil objective, or the 63× 1.40–0.60 oil objective or the 40×/1.25–0.75 oil objective. Serial fluorescence and DIC images were obtained simultaneously as described [5]. Positive image masks of significant colocalization of two fluorophores were generated and analyzed from the image fluorogram data sets by defining the specific areas—regions of interest (AOI) with a bounding box of the fluorogram using a Leica confocal software package (Leica LAS-AF) [5].

Statistical analysis and data presentation

Densitometric analyses were done using the Quantity One 1-D Analysis Software Version 4.4 (Bio-Rad). Experiments were repeated at least twice, and usually three or more times. Data are presented as the mean ± SEM. Statistical analyses were performed using the Student's t-test, when appropriate. Probability (P) values of <0.05 were considered statistically significant. The Graph Pad Prism Software, version 4.02, was used (Inc. San Diego, CA).

Results

Exosomes derived from Namalwa (CD38⁺) B cells express enzymatically active CD38

Exosomes released into the culture media were isolated by differential centrifugation, and their morphology was examined by EM (Fig. 1A left, upper and lower panels). To visualize CD38 in isolated exosomes, immunogold labeling was utilized in combination with the anti-CD38 mAb, HB136. The detection of colloidal gold on the outer layer of the exosomes confirmed the presence of CD38.

FACS analysis showed that exosomes coated on aldehyde/sulfate latex beads displayed CD38, tetraspanin CD81 molecules, and the MHC class II molecules (Fig. 1A right, upper, middle and lower panels, respectively). The phenotypic analysis of the exosomes was also carried out by WB. Experiments using isolated exosomes from Namalwa cells revealed the presence of CD38 molecules in exosome vesicles (Fig. 1B and D, lane 1). Structurally, exosomic CD38 appeared as monomeric and dimeric forms (Fig. 1B, lane 1), and the monomeric form was the primary version in total cell lysates (Fig. 1B, lane 3). CD38 dimers were reported in human [16] and mouse B lymphocytes [25], and dimers were also confirmed by crystal studies [26]. As a control, exosomes were also isolated from the HOM-2 human lymphoblastoid B cell line because HOM-2 cells do not express surface CD38 molecules as shown by WB (Fig. 1B and D, lane 4) and by FACS (Supplemental Fig. S1A). CD38 was not detected in exosomes isolated from HOM-2 cells (Fig. 1B and D, lane 2); however, exosome vesicles from these cells were characterized by other exosome associated proteins [7]. Rab5, a small GTPase that controls fusion events in the endosomal system [27], and the stress and tetraspanin proteins Hsc-70 and CD81 were found in exosomes released from Namalwa and HOM-2 cells (Fig. 1B and D, lanes 1 and 2).

Next, we analyzed the enzymatic activity of CD38 molecules in isolated exosomes. To this end, GDP ribosyl cyclase activity was measured in exosomes. GDP ribosyl cyclase activity was determined via a specific in vitro fluorometric assay that uses NGD⁺ as a substrate to produce cyclic-GDP-ribose [23]. Fig. 1C shows the GDP ribosyl cyclase activity produced by CD38 molecules present in the 100,000×g pellet of isolated exosome vesicles (2.78 ± 0.77 RFU/μg protein in the sample, n = 3 independent experiments). The results indicate that cyclic-GDP-ribose was produced after incubating Namalwa exosomes with the substrate NGD⁺. In contrast, cyclic-GDP-ribose was not generated from either CD38⁻ HOM-2 cell cyclase lysates (0.21 ± 0.10 RFU/μg in protein, n = 3 independent experiments) or exosomes derived from the HOM-2 cell line (not shown).

CD38 in exosomes is associated with signaling complexes containing CD81, Hsc-70 and Lyn

We identified signaling molecules associated with CD38 in exosomes. The localization of CD38 molecules in critical membrane domains and the formation of supramolecular complexes are necessary conditions for the transduction of CD38-mediated signals [1,13,16,18,28]. CD38 molecules were immunoisolated from exosome lysates prepared in 1× lysis buffer containing 1% Brij

98 + 15 mM ODG with OKT10 mAb bound to MACS protein-G-microbeads. The retrieved proteins were identified by WB. ODG was utilized because harsher detergent conditions were necessary to better solubilize proteins without compromising specific protein–protein and protein–lipid complexes. In addition to monomeric and dimeric forms of CD38, CD38 immunoprecipitates contained CD81 (Fig. 2A, upper left panels). Reciprocal experiments performed with the anti-CD81 mAb showed that CD38 and CD81 were bi-directionally associated in exosomes (Fig. 2A, upper center panels).

CD38 immunoprecipitates contained a 70 kDa protein, which was identified as the stress protein Hsc-70 (Fig. 2A, lower left panels). Hsc-70 was also associated with CD81 (Fig. 2A, lower center panels). CD38 protein complexes in exosomes also included a 53–56 kDa molecule, which was identified as the Src-family tyrosine kinase Lyn (Fig. 2A, lower left panels). The anti-Lyn polyclonal antibody (Lyn 44, SC15) recognized an additional band of a molecular weight lower than conventional Lyn. This low molecular weight Lyn is reminiscent of the low molecular weight Lyn found in exosomes secreted by Daudi cells, likely resulting from caspase-3-like activity [6]. This finding appears to be peculiar to Daudi cells because neither Namalwa exosomes nor CD38-immunoprecipitates contain caspase-3 activity (not shown). Immunoprecipitation with an anti-Hsc-70 Ab indicated that Lyn co-immunoprecipitates with Hsc-70 (Fig. 2B, left panels). Moreover, Lyn immunoprecipitation isolated Lyn and Hsc-70 (Fig. 2B, center panels) but not CD38 (not shown). To test if Lyn and Hsc-70 protein complexes are formed in the absence of CD38, Lyn was immunoprecipitated from exosome lysates derived from (CD38$^-$) HOM-2 cells. The results indicated that Lyn immunocomplexes contained Lyn and Hsc-70 proteins even in the absence of CD38 (Fig. 2C, left panels).

These results demonstrated that exosomes derived from lymphoblastoid B cells contained protein complexes that included monomeric and dimeric CD38 molecules, along with CD81 tetraspanin, the tyrosine kinase Lyn and Hsc-70. The association between CD38 and CD81 in isolated exosomes was bi-directional, and similar interactions were also observed between Lyn and Hsc-70.

CD38 is highly localized in MR isolated from human B cell lines

The mechanism for the specific sorting of proteins in exosome membranes is not completely known; however, MR-associated proteins are sorted into exosomes [6]. We aimed to isolate and characterize MR microdomains from CD38$^+$ Namalwa and Raji cell lines and test whether CD38 in MR interacts with similar protein complexes as those observed in exosomes. A flotation assay based on resistance to solubilization by Brij 98 at 37 °C [13] and buoyancy at low density fractions of a bottom-loaded discontinuous sucrose gradient (steps of 5%, 30%, and 40% sucrose) was utilized to isolate the complexes. MR (fractions 2, 3 and 4) and non-MR or S fractions (7–8) were tested for the presence of CD38 (Fig. 3A and C). MR contained approximately 2% of total proteins found in the whole sucrose gradient but were highly enriched in proteins known to be associated with MR (Supplemental Fig. S1B).

CD38 localized to MR (Fig. 3A and C). Densitometric analysis of WBs from different experiments using Namalwa (Fig. 3B) and Raji cells (Fig. 3D) demonstrated that ~50% of total CD38 localized in MR, whereas CD38 present in S fractions corresponded to ~30% of
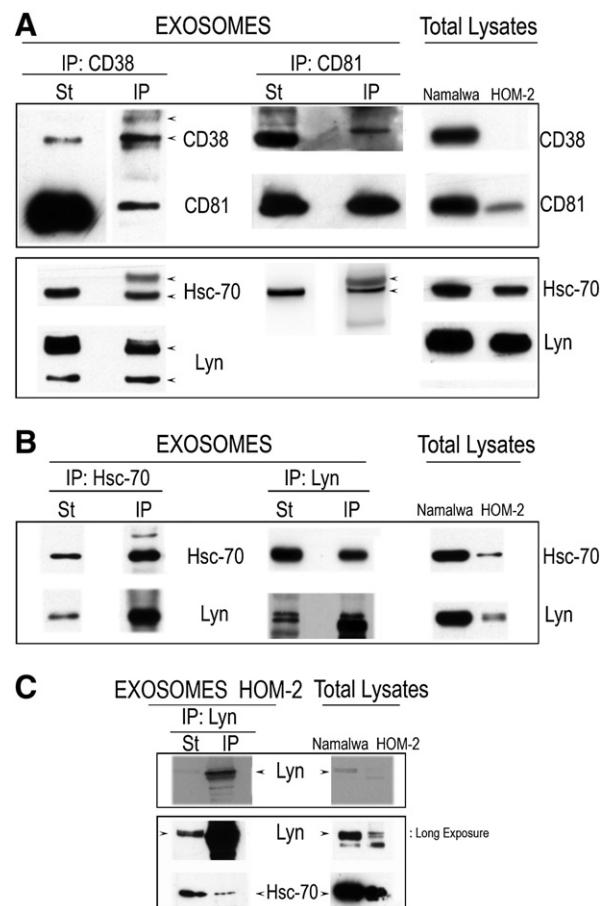

Fig. 2 – CD38 in exosomes is associated with signaling complexes containing CD81, Hsc-70 and Lyn. (A) CD38 and tetraspanin CD81 were immunoisolated from exosomes derived from Namalwa cells. Exosomes were lysed in 1× lysis buffer containing 1% Brij 98 plus 15 mM octyl D-glucoside (ODG) and subjected to immunoprecipitation (IP) with specific mAbs for CD38 (left panels) and CD81 (middle panels) bound to μMACS protein-G-microbeads. The retrieved proteins were eluted with non-reducing 3× Laemmli sample buffer, loaded onto 12% SDS-PAGE gels and subjected to WB with the indicated antibodies. Aliquots of the exosome lysates before the IP procedure (named as starting material or St) were also loaded onto the gels. Next, the membranes, as portrayed on the panels were stripped and probed again with the indicated antibodies. CD38 and CD81 IP also contained the Hsc-70 protein. In addition CD38 IP contained the tyrosine kinase Lyn. (B) Hsc-70 and Lyn proteins were also specifically immunoprecipitated from the exosome lysates prepared as in A, with anti-Hsc-70 and anti-Lyn antibodies, respectively. The IP material were eluted with reducing 3× Laemmli sample buffer and subjected to a similar procedure as described in A. The membranes were probed with the indicated antibodies plus a rabbit IgG TrueBlot™ or a mouse IgG TrueBlot™ HRP-conjugates antibodies, respectively. (C) Lyn was immunoprecipitated from exosomes derived from Hom-2 (CD38$^-$) cells; exosome lysates were prepared as in panel A. The samples were subjected to the same procedure as described in panel B. Aliquots of total cell lysates prepared in 1× lysis buffer containing 1%-Brij-98 plus 15 mM ODG were analyzed also in the gels (A, B and C right panels).
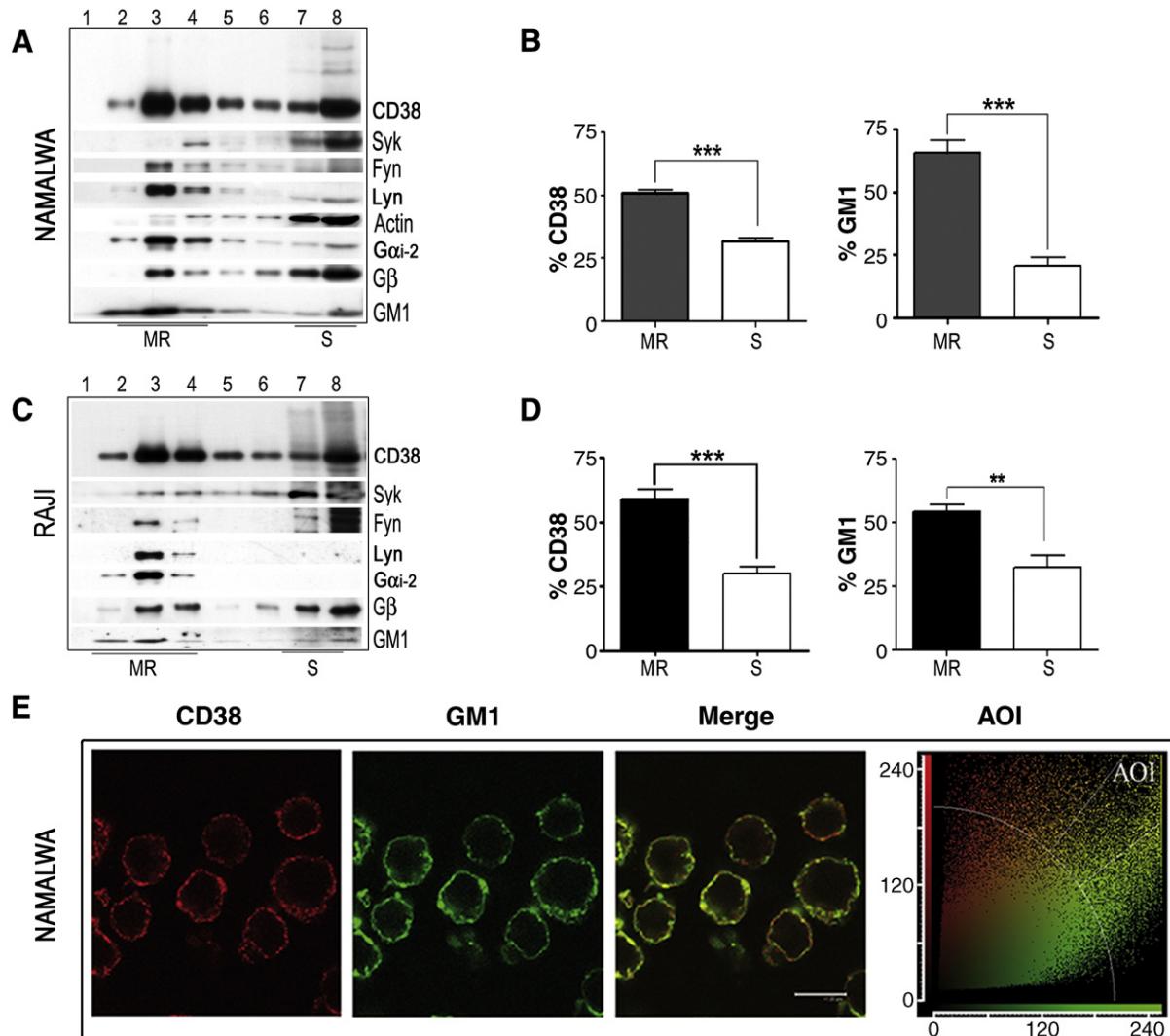
Fig. 3 – CD38 is highly localized in MR isolated from human B cell lines. (A and C) Namalwa and Raji cells were lysed at 37 °C in 1× lysis buffer containing 1% Brij 98, and fractionated on a sucrose gradient. Eight fractions of 0.5 ml were collected from the top to the bottom of the gradient; 18-μl aliquots of each fraction of the gradient were diluted with 9 μl of non-reducing 3× Laemmli sample buffer. The resulting 27 μl was resolved on 12.5% SDS-PAGE. Gels were transferred to PVDF and subject to WB with the antibodies indicated on the right. Ganglioside GM1 was detected with cholera toxin-HRP conjugated. Representative experiments are shown from more than six independent ones. A panel, the distribution profile of the indicated proteins along the fractions of a sucrose gradient from Namalwa cells is shown. Densitometric analyses of several WB from Namalwa sucrose gradients indicated that the intracellular membrane-bound and raft resident tyrosine kinases Lyn ($89.59 \pm 6.66\%$, $P < 0.0001$, $n = 4$) and Fyn ($60.29 \pm 16.22\%$, $n = 2$), and the G protein subunit Gαi-2 ($75.77 \pm 1.18\%$, $P < 0.0001$, $n = 4$), were partitioned into low density fractions corresponding to the 5/30% interface (fractions 2, 3 and 4) and referred to in the figure as Membrane Raft (MR), which was consistent with their residency in the glycolipid-enriched membranes. In contrast, the high density soluble material corresponding to fractions 7 and 8 and referred to as Soluble (S) contained ~$7.44 \pm 3.93\%$ of total Lyn; ~$17.1 \pm 12.2\%$ of total Fyn, and ~$15.73 \pm 1.07\%$ of total Gαi-2. Also ~$32.85 \pm 3.4\%$ ($P = 0.02$, $n = 4$) of the total Gβ; a smaller percentage of Syk tyrosine kinase, along with the cytoskeleton protein actin, were detected in MR. C shows the distribution profile of the indicated proteins along the fractions of a sucrose gradient from Raji cells. (B and D) Densitometric analyses of the several WB are shown corresponding to CD38 and GM1 from Namalwa and Raji sucrose gradients, respectively. The distribution between MR (filled histograms) and S fractions (open histograms) is represented. All the densitometric data in MR and S pools are presented as percentage of the sum of all sucrose gradient fractions values (fractions 1–8). Densitometric analyses were carried out with the Quantity One 1-D Analysis Software Version 4.4 (Bio-Rad). Data are the mean ± SEM. Statistical analyses were performed using the Student's t-test to compare the values on the MR versus on the S. P values: **$P < 0.01$, ***$P < 0.001$ (GraphPad Prism Software, v 4.02). (E) Colocalization of CD38 with the ganglioside GM1 was analyzed by CLSM. Representative images are shown. CD38 (red) was immunostained with HB136 anti-CD38 mAb (10 μg/ml in TNB), followed by a GαMIg-Alexa 568 labeled secondary antibody (Molecular Probes). Ganglioside GM1 (green) was detected with CTX-B-Alexa 488 labeled (10 μg/ml in TNB). CLSM images were acquired with a Leica TCS-SP5 CSLM, using HCX PL APO CS 100× 1.4 oil objective. CD38 colocalized with GM1 resulting in yellow areas (Merge). Scale bar: ~11 μm. A semiquantitative assessment of the level of colocalization was done by analyzing the colocalization scatter plot and generating a mask of the area of interest (AOI; right panel).
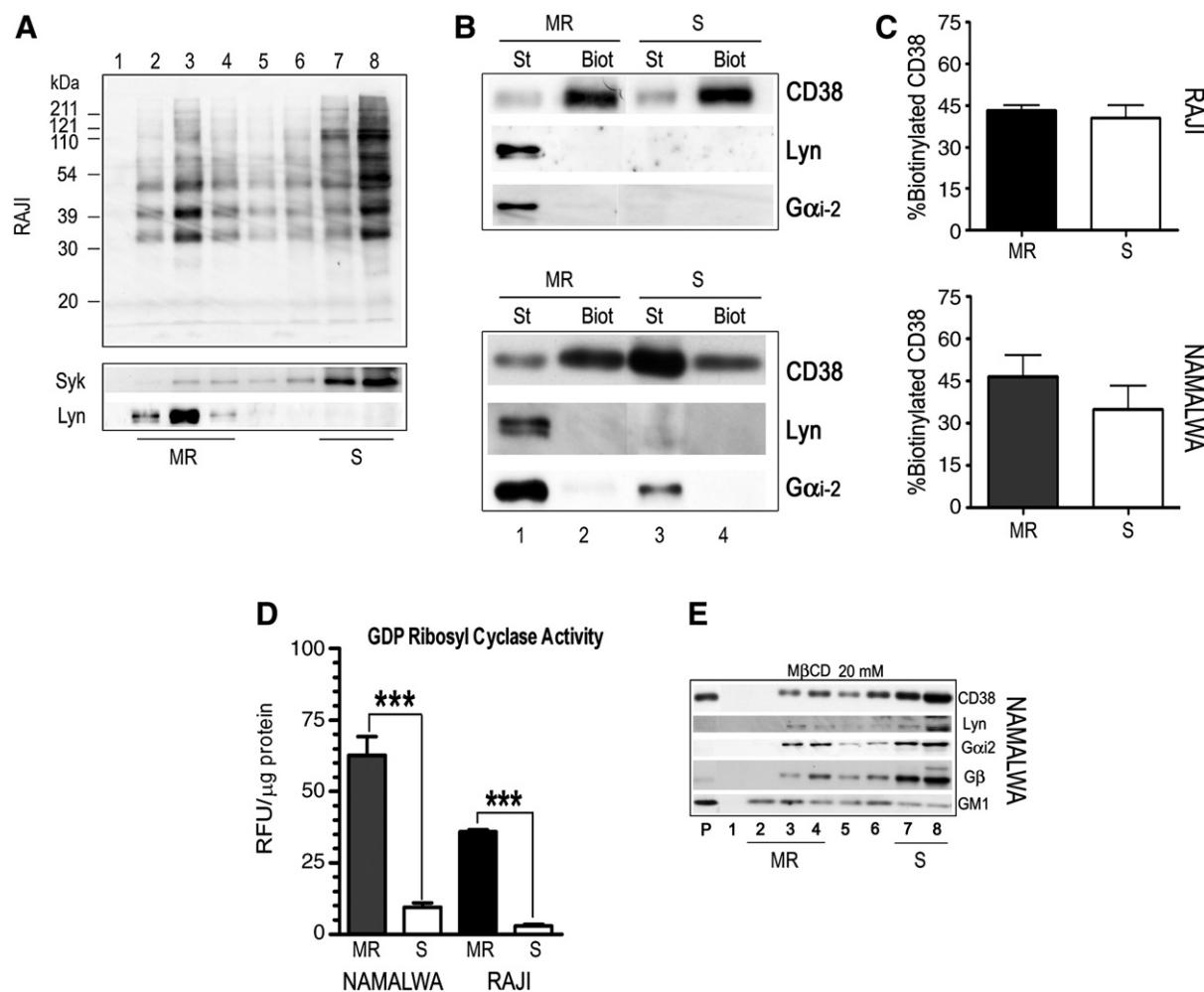
Fig. 4 – Cell surface distribution and enzymatic activities of CD38 in MR. (A, B and C) Cell surface proteins of Namalwa and Raji cells were labeled with Biotinamidohexanoic acid 3-Sulfo-NHS sodium salt. The cells were lysed in 1% Brij 98-lysis buffer and fractionated on a sucrose gradient, as described in Fig. 3. (A) Aliquots (15 μl) of each fraction of a sucrose gradient from Raji cells, in non-reducing 3× Laemmli sample buffer, were resolved on 12.5% SDS-PAGE gel, transferred to PVDF and the distribution of the biotinylated proteins along the gradient was detected with streptavidin-HRP. Molecular size markers in kDa are indicated on the left (upper panel). The membrane was stripped and the distribution along the gradient of the non-biotinylated Syk and Lyn proteins was analyzed by WB (lower panels). The antibodies are indicated on the left. (B) The biotinylated proteins were pulled-down with streptavidin-agarose beads from MR and S fractions, corresponding to Raji and Namalwa sucrose gradients, respectively. Biotinylated proteins were eluted from the beads with non-reducing 3× Laemmli sample buffer, resolved on a 12.5% SDS-PAGE and subjected to WB with HB136 anti-CD38 mAb (panels are indicated by CD38). Aliquots of the starting material (St), before the pull-down experiments, corresponding to the MR and to the S fractions were loaded in the gels. The non-biotinylated intracellular proteins, Lyn and Gαi-2 were analyzed by WB, as controls. (C) Densitometric analyses of the CD38 WB from different experiments like those represented in panel B are shown. The proportion of biotinylated CD38 pulled down from MR and S fractions, respectively, is represented. (D) CD38 GDP-ribosyl cyclase activity (RFU/μg protein) was measured in MR and S fractions from several sucrose gradients corresponding to Namalwa and Raji cells, respectively (***$P < 0.001$ $n = 5$ independent experiments in each cell line). Statistical analyses were performed using the Student's t-test to compare the values in MR (filled histograms) versus in S fractions (open histograms). (E) Namalwa cells were treated with MβCD 20 mM (30 min, at 37 °C), then solubilized in 1% Brij 98-lysis buffer and fractionated on a sucrose gradient, as in Fig. 3. The effect of the MβCD-treatment on the distribution of the proteins CD38, Lyn, Gαi2, and Gβ, and the ganglioside GM1, along the sucrose gradient fractions, was analyzed by WB. The antibodies are indicated on the right. A representative experiment is shown from more than four independent ones.

total CD38 ($P < 0.0001$, $n = 11$ and $P < 0.0001$, $n = 6$ independent experiments in each cell line, respectively). The intermediate fractions 5–6 contained the remaining CD38 molecules. A similar distribution pattern of the ganglioside GM1 between MR and S fractions was observed (Fig. 3B and D, right panels) ($P < 0.0001$,

$n = 6$ and $P = 0.0068$, $n = 4$ independent experiments in each cell line, respectively). Colocalization between CD38 and GM1 was analyzed in Namalwa cells by CLSM. A semi-quantitative assessment of the level of colocalization was performed by analyzing the colocalization scatter plot and by generating a mask of the area of

Table 1 – Disruption of MR in Namalwa cells significantly altered the partitioning of CD38 and GM1.

	MR[a]		S[a]	
	NT[b]	MβCD-treated	NT	MβCD-treated
CD38	53.02 ± 1.30[c]	22.99 ± 2.17[c]	31.58 ± 2.90[d]	54.01 ± 4.24[d]
GM1	72.02 ± 3.4[e]	40.82 ± 5.10[e]	18.70 ± 4.80[f]	19.99 ± 2.77[f]

[a] Cells were lysed in 1% Brij 98 followed by a sucrose gradient centrifugation, eight fractions of 0.5 ml were collected, and aliquots of each fraction were analyzed by Western blot for the indicated proteins. Densitometric data on MR and S pools are presented as percentage of the sum of all sucrose gradient fractions. Data are presented as the mean ± SEM.

[b] NT: non-treated.

[c] NT versus the MβCD-treated CD38 values in the MR pool. $P \leq 0.0001$, $n = 5$ independent experiments. Student's t-test.

[d] NT vs. MβCD-treated CD38 values in the S pool. $P = 0.0024$, $n = 5$ independent experiments. Student's t-test.

[e] NT vs. MβCD-treated GM1 values in the MR pool. $P = 0.0085$, $n = 5$ independent experiments. Student's t-test.

[f] NT vs. MβCD-treated GM1 values in the S pool. The differences were not statistically significant.

interest (AOI) (Fig. 3E) (colocalization rate ~31.39%; Pearson's correlation 0.74, and overlap coefficient 0.84).

Cell surface expression and enzymatic activity of CD38 in MR

The proportion of cell surface CD38 molecules recruited to MR was examined. Cell surface proteins were selectively labeled with sulpho-NHS biotin in Raji and Namalwa cells. A representative profile of the distribution of biotinylated proteins along a sucrose gradient is shown in Fig. 4A (upper panel). Biotinylated proteins were isolated from MR and from S fractions with streptavidin-agarose beads, and a WB was performed with the specific anti-human CD38, HB136 mAb (Fig. 4B, CD38 is indicated in the panels). In both cell lines, densitometry of the WB indicated that ~44% of the surface CD38 was detected in MR. Also, ~38% of surface CD38 was located in the S fractions ($n = 2$ independent

Table 2 – Disruption of MR in Namalwa cells significantly reduced CD38 associated cyclase activity.

MR[a]		S[a]	
NT[b]	MβCD-treated	NT	MβCD-treated
65.55 ± 6.75[c]	16.40 ± 3.20[c]	6.10 ± 0.50[d]	11.58 ± 0.29[d]

[a] Cells were lysed in 1% Brij 98 followed by a sucrose gradient centrifugation, and eight fractions of 0.5 ml were collected. In pooled sucrose gradient fractions, corresponding to MR and S pools GDP ribosyl cyclase activity of CD38 was determined by means of the in vitro fluorometric assay using NGD$^+$ as a substrate. Data are presented as the mean ± SEM of RFU/μg protein, where RFU stands for Relative Fluorescence Units.

[b] NT: non-treated.

[c] NT versus MβCD-treated values for GDP cyclase activity in the MR pool. $P = 0.0002$, $n = 5$ independent experiments. Student's t-test.

[d] NT versus MβCD-treated values for GDP cyclase activity in the S pool. $P < 0.0001$, $n = 5$ independent experiments. Student's t-test.

experiments, in each cell line) (Fig. 4C, upper and lower panels). The intermediate fractions, 5–6, contained the remaining surface CD38 molecules (~18%). The intermediate fractions are reminiscent of the high density-MR described to be involved in the endocytosis of mature MCH class I molecules in B cells [15]. Altogether, light dense-MR (top of the gradient) and high dense-MR (or intermediate fractions) contained ~62% of cell surface CD38 molecules. The intracellular MR proteins Lyn and Gαi-2 were not detected with the pull-down of biotinylated proteins (Fig. 4B, upper and lower panels, lanes 2 and 4), which is indicative of the specificity of the cell surface labeling procedure.

The enzymatic activity of CD38 was analyzed in MR and S fractions from Namalwa and Raji cells. GDP ribosyl cyclase activity of CD38 was determined by an in vitro fluorometric assay that uses NGD$^+$ as a substrate (Fig. 4D) and by measuring cyclic-GDP-ribose [23]. Fig. 4D shows the GDP ribosyl cyclase activity of CD38. In both cells lines, the GDP ribosyl cyclase activity was significantly higher in the MR fractions compared to the S fractions. These results indicate that CD38 in MR was enzymatically active.

We also investigated whether the MR association of CD38 was dependent on cholesterol. Therefore, we examined the effect of MβCD, the cholesterol-binding agent that disrupts MR [24], on the partitioning of CD38 and its enzymatic activity. After MβCD-treatment of Namalwa cells, the distribution of CD38 changed along the sucrose gradient fractions. The prevalence of CD38 shifted from the MR towards the intermediate and S fractions (Fig. 4E). Densitometric analyses of several WBs are indicated in Table 1. Similar results were also observed for Lyn and Gαi-2 proteins [29] (Fig. 4E). Localization of GM1 decreased in MR after MβCD treatment: GM1 was re-distributed between the MR, the intermediate (5 and 6) (~9. 28% of total GM1 in untreated cells vs. ~39.19% of total GM1 in MβCD-treated) and the S fractions (Table 1). Moreover, disruption of MR in Namalwa cells significantly reduced CD38-MR associated cyclase activity, whereas the CD38 cyclase activity in S fractions increased (Table 2). These results propose a positive role of the MR micro-environment in CD38 enzymatic activity.

Receptor activity and translocation of CD38 to MR in B cells

The participation of PI 3-kinase in CD38-mediated signaling in immature B cells and T cells is well-documented [13,18,30,31]. In this study, to determine if CD38 is coupled to Akt activation (a PI 3-kinase target) in B cells, we assessed the phosphorylation status of Akt upon CD38 ligation. To this end, cells were stimulated with the anti-CD38 mAb IB4 followed by cross-linking with the F(ab′)2 fraction of a secondary antibody. An anti-phospho-Akt (Ser 473)-specific antibody was used to probe total cell lysates, revealing CD38-mediated Akt/PKB activation. Phosphorylation at Ser 473 was observed 5 min after CD38 engagement (Fig. 5A), which coincided with increased translocation of CD38 to the MR, as shown in Fig. 5B. Next, the blot was stripped and re-probed with an anti-Akt-specific antibody that detects total Akt (phosphorylation state-independent). Akt mobility on SDS-PAGE gels shifted 5 min after mAb ligation (Fig. 5A) and is attributable to the phosphorylation at Thr 308 and Akt activation [18].

Activation of Erk was analyzed using an anti-diphospho-Erk antibody (Thr 202/Tyr 204). Dual phosphorylation at these sites correlated with Erk activation. CD38 ligation by IB4 also induced significant activation of Erk-1 and Erk-2 in serum-starved

Fig. 5 – Receptor activity and translocation of CD38 to MR in B cells. (A) Namalwa cells were serum-starved for 20 h and left unstimulated (NT; lane 1), mock-stimulated with the secondary F (ab′) 2 GαMIg antibody alone (lane 2), or stimulated for 1 and 5 min at 37 °C, with the anti-CD38 mAb IB4 + GαMIg (lanes 3 and 4). After stimulation cells were lysed in ice-cold 1% NP-40 1× lysis buffer. Post-nuclear supernatants were separated on 10% SDS-PAGE gels under reducing conditions and subjected to WB. The upper blot was probed with the anti-phospho-Akt-Ser473. Next the membrane was stripped and re-probed with the anti-total Akt polyclonal antibody. Lower blot was probed with the anti-diphospho-Erk mAb (pErk-Thr202/Tyr204). Next the filter was stripped and re-probed with the anti-total-Erk (p42 and p44) polyclonal antibody. Densitometric analysis of Erk and Akt phosphorylation levels are shown (open histograms). **(B)** Serum-starved Namalwa (left), or Raji cells (right) were stimulated (as in panel A) for 5 min (filled histograms). The cells were lysed in 1× lysis buffer containing 1% Brij 98 and subjected to sucrose gradient fractionation, and gradient fractions were separated on 12% SDS-PAGE under non-reducing conditions, as described in Fig. 3. The distribution of CD38 along the sucrose gradient was analyzed by WB with anti-CD38 mAb (HB136). Densitometric analyses of the WB were carried out, to assess CD38 distribution into MR and S fractions before and after CD38 stimulation. Data are presented (mean ± SEM). The experiments were repeated twice.

Namalwa cells (Fig. 5A), which was also concurrent with increased CD38 translocation to MR.

Signaling partners of CD38 in MR

In exosomes, CD38 interacts with CD81, Hsc-70 and Lyn. Similar molecular interactions were also investigated in isolated MR. The first candidate to be considered was tetraspanin CD81. In normal and neoplastic B lymphocytes, capping of CD38 induces co-capping of CD81 and vice versa [16]. WB was used to examine the distribution of CD81 along the fractions of a sucrose gradient from 1% Brij 98 lysates

of CD81+ Namalwa cells (Fig. 6A). Densitometry analysis indicated that CD81 is located in both the MR and the S fractions. To test if an association occurs between CD38 and CD81 in MR, CD38-protein complexes were immunoisolated from the Brij 98-MR and the Brij 98-S fractions. The results indicated that CD81 co-immunoprecipitated with CD38 from the MR and, to a lesser extent, from the S fractions (Fig. 6B). Reciprocal immunoprecitations were performed using an anti-CD81 mAb (Fig. 6C, upper panel). CD38 was detected in CD81-immunoprecipitates from MR and from the S fractions (Fig. 6C, middle panel). Therefore, CD38/CD81 complexes are present in the MR and S fractions, and the CD81 protein complex isolated from MR and S fractions also contained Hsc-70 (Fig. 6C, lower panel).

The association in MR between CD38 and Hsc-70 was evaluated using a proteomics approach [32] (Supplemental Results and Supplemental Fig. S2). The proteins retrieved with the CD38-immunoprecitates from MR were eluted and subjected to two dimensional electrophoresis. Peptide mass fingerprinting (PMF) analysis of a 70 kDa spot revealed that 17 out of 26 tryptic peptide sequences matched the human Hsc-70 amino acid sequence (HSP7C-HUMAN, Swiss-Prot accession No. P11142; Mass (kDa)/ pI theoretical: 71.08/5.37; Mascot Score: 205/Expect: 5.5e-17; sequence coverage 39%). Following the same procedure, two additional spots were identified, a 40 kDa band that matched to the human actin cytoplasmic-2 amino acid sequence and another 50 kDa band that matched to the human tubulin alpha-1B chain (Supplemental Results, Supplemental data 1 and data 2, and Fig. S2). Co-immunoprecipitation experiments of CD38 and Hsc-70, using specific Abs for each protein, followed by WB analysis further indicated that Hsc-70 and CD38 associated bi-directionally only in MR (Supplemental Fig. S3B and C). The actin/CD38 relationship in MR was also verified by co-immunoprecipitation and WB analyses (Supplemental Fig. S3E and F).

CD38 tends to cluster with professional signaling molecules [1,33]. CD19 is laterally associated with surface CD38 in normal B lymphocytes and in B-CLL cells [1,16]. Sensitivity to cholesterol depletion indicates the involvement of MR. Therefore, a potential candidate to be considered in investigating the signaling complexes of MR-CD38 was CD19 [16,31]. The distribution of CD19 in the fractions of a sucrose gradient from 1% Brij 98 lysates of CD19+ Namalwa cells was examined by WB (Fig. 6D). Densitometry analysis indicated that CD19 is located in both MR and S fractions. CD19 displayed a bi-directional association with CD38 in the MR fractions but not in the S fractions (Fig. 6E and F). The CD38/CD19 complex isolated from MR also included Lyn, a 53–56 kDa protein member of the Src-family tyrosine kinase (Fig. 6F, lower panel).

The results indicated that Lyn and CD38 are members of the CD19 protein complex in MR (Fig. 6F); however, it was still unclear if CD38 directly associated with Lyn in MR. To explore this possibility, CD38 was immunoisolated from Brij 98-MR, from the intermediate (5–6) fractions and from the S fractions and the retrieved proteins were analyzed by WB (Fig. 7A). Lyn was co-immunoprecipitated with CD38 from MR but not from the other fractions (Fig. 7A, second panel). Lyn was also co-immunoprecipitated with CD38-MR upon CD38 cross-linking with the anti-CD38 mAb, IB4. Moreover, the Lyn protein associated with CD38-MR was tyrosine phosphorylated; however, no major changes in the levels of Lyn tyrosine phosphorylation were observed following CD38 engagement with the specific antibody (data not shown). CD38 and Lyn colocalization was examined by CLSM. The
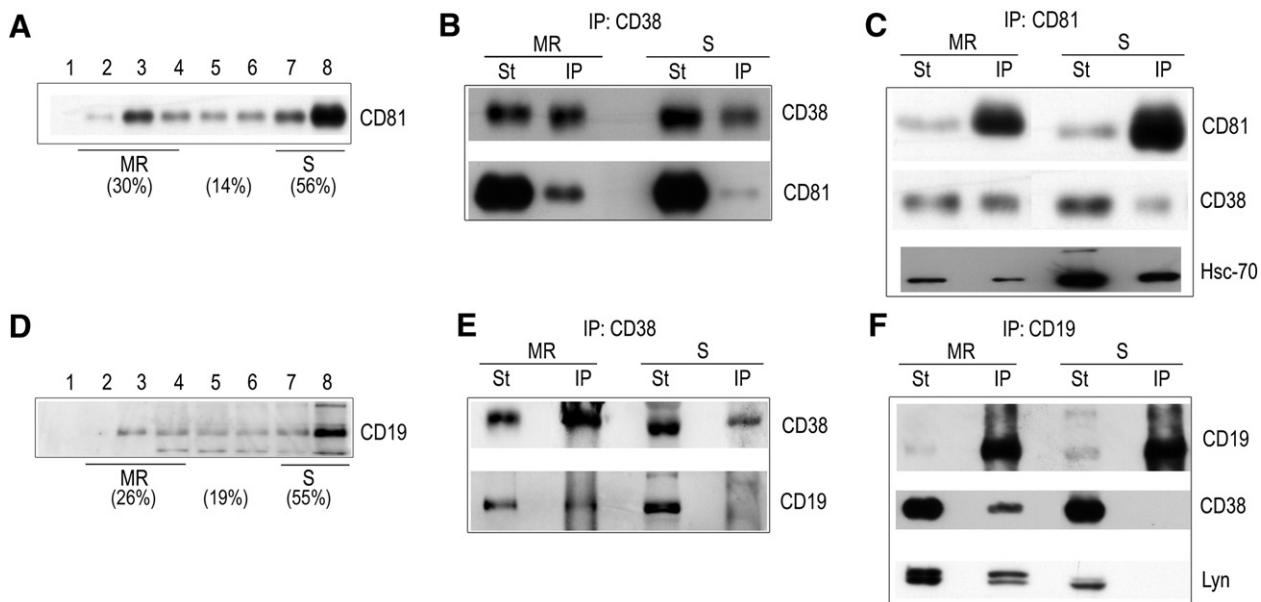
Fig. 6 – CD38 is associated with CD81 and CD19 receptors in MR. Namalwa cells ($5–9 \times 10^7$ cells/ml) were lysed in 1× lysis buffer containing 1% Brij 98, at 37 °C. MR and S fractions were isolated by sucrose gradient centrifugation, as in Fig. 3. (A and D) CD81 and CD19 distribution profiles along the sucrose gradients fractions were analyzed by WB. Representatives WB are showed. Densitometric analyses of the blots are indicated in a parenthetical. (B, C, E and F) CD38, CD81 and CD19 were immunoisolated from MR and S fractions of the sucrose gradients from Namalwa cells: MR and S pools were diluted in 1× lysis buffer containing 1% Brij 98 plus 15 mM ODG and subjected to immunoprecipitations with specific mAbs to CD38 (B and E panels), CD81 (C panels), or CD19 (F panels), respectively. The retrieved proteins were eluted with 1×-XT Bio-Rad Sample Buffer. Immunoprecipitates (IP) were separated on 4–12% Bis-Tris-SDS-PAGE gels, under non-reducing (B, C, E and F) and reducing conditions (C, panel corresponding to WB with anti-Hsc-70) and blotted with specific antibodies indicated on the right of the panels. Aliquots of the pooled MR or S fractions, before the IP procedure (named as starting material or St) were also analyzed.

colocalization areas of CD38 and Lyn appeared as yellow (Merge) or white (positive image or Mask). A representative experiment is shown in Fig. 7B. The level of colocalization was measured by analyzing the colocalization scatter plot and generating a mask of the area of interest (AOI) (Fig. 7B). Analysis of the representative CLSM images indicated that the colocalization rate of CD38 with Lyn was ~46.63% (Pearson's correlation 0.80 and overlap coefficient of 0.85). No colocalization was observed with the isotype-matched mAb (Supplemental Fig. S4).

We also observed that CD38 complexes immunoprecipitated from MR included a 41 kDa band, which was identified as the Gαi-2 protein subunit; however, the Gβ subunit and the Fyn kinase did not associate with CD38 (Fig. 7A). The association of Lyn and Gαi-2 with CD38 was assessed by reciprocal immunoprecitation experiments. CD38 and Gαi-2 were detected in Lyn-immunoprecipitates from MR fractions (Fig. 7C) but not from S fractions. Likewise, CD38 and Lyn were detected in Gαi-2 immunoprecipitates from MR (Fig. 7D).

These observations indicate that CD38 is part of a supramolecular signaling complex that includes the CD81 and CD19 receptors. The contribution of all the components of the complex varies in terms of function and structure. The CD38/CD81 complex is prevalently, although not exclusively, observed in MR. Moreover, CD38 can establish a vast array of molecular associations in MR and potentially contribute to different signaling complexes through the tyrosine kinase Lyn, the heterotrimeric G protein subunit Gαi-2, the heat shock protein Hsc-70 and actin.

Discussion

The original observation of this work is that full-length CD38 inserted in the cell membrane is expressed on the surface of exosomes derived from human B cells, and exosomic CD38 conserves its enzymatic activity (Fig. 1D). Exosomic CD38 molecules are presented either monomers (~45 kDa) or in form of dimers (~90 kDa), this structural organization has already been reported [16,25,26]. Exosome vesicles are generated by several cell types through the fusion of the plasma membrane with multi-vesicular bodies (MVBs) [7,34]. Our group reported that CD38-containing recycling endosomes are recruited to the immunological synapse upon TCR engagement, where they fuse with the plasma membrane [5]. Furthermore, MVBs containing CD38 that fused with the plasma membrane were observed by EM [35]. Additional evidence for this phenomenon is supported by non-human models. For example, endogenous ADP-ribosyl cyclases in sea urchin eggs are located in the lumen of acidic, exocytic vesicles, where they are active [36]. The exosomal CD38 pool is also entirely distinct from soluble CD38 found in several biological fluids, including culture supernatants [1]. Soluble CD38 is a ~39 kDa molecule that adheres to the cell surface without membrane insertion.

MR may support the sorting of proteins associated with exosomes. CD38 is expressed by exosomic vesicles; therefore, we studied CD38 associated with MR. MR were extracted from Brij 98 cell lysates at
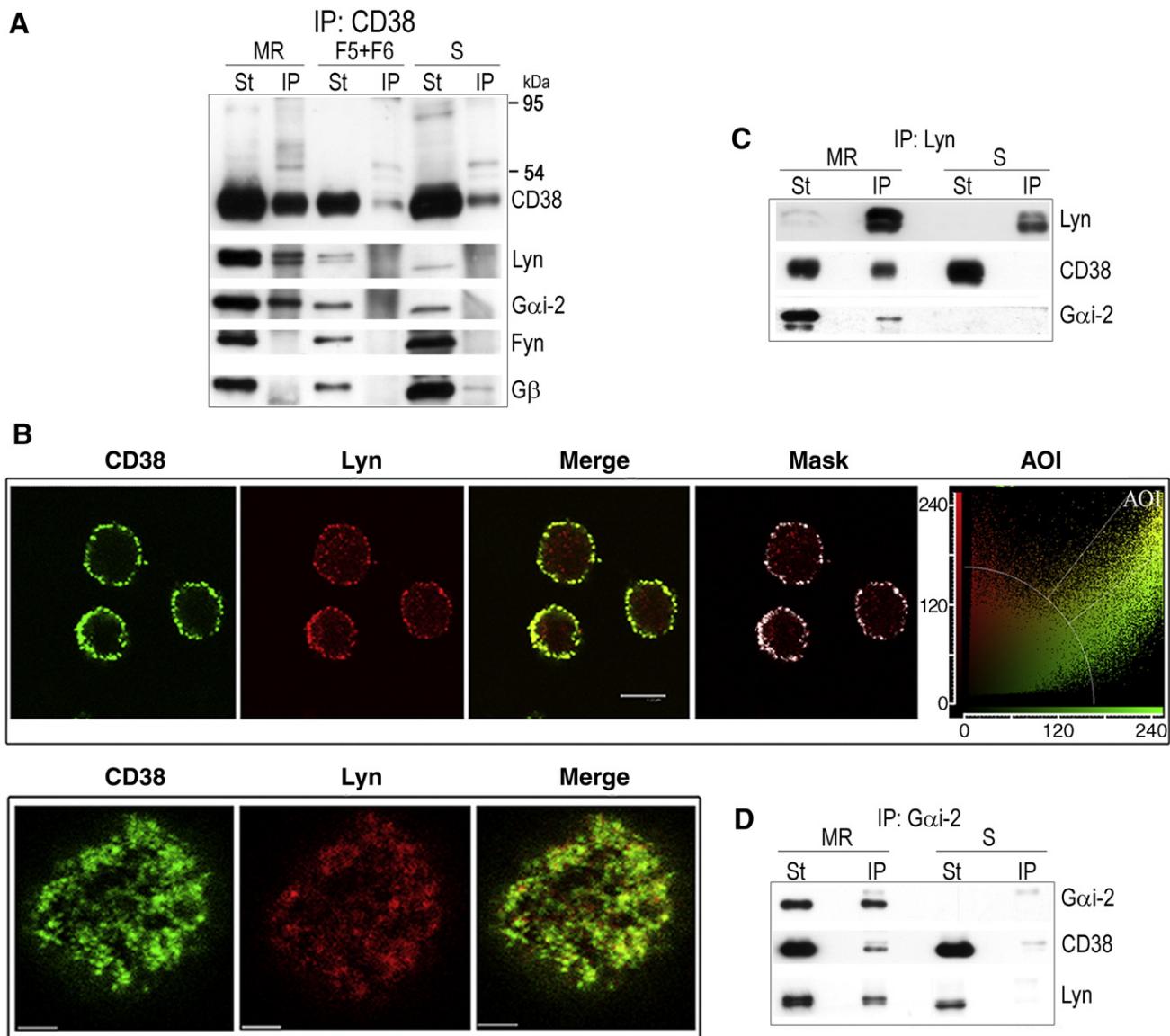
Fig. 7 – CD38 in MR is associated with the tyrosine kinase Lyn and the heterotrimeric G protein subunit Gαi-2. (A, C, and D), Namalwa cells were lysed as described in Fig. 3. MR, S, and intermediate (5–6) fractions were isolated by sucrose gradient centrifugation, as described in Fig. 3. A 1× lysis buffer containing 1% Brij 98 plus 15 mM ODG was added to the indicated fractions and they were subjected to IP, either with: anti-CD38 mAb, anti-Lyn, or anti-Gαi-2 as described in Fig. 6. Immunoprecipitated proteins were separated on 12% SDS-PAGE gels under non-reducing conditions, and blotted with the antibodies indicated on the right of the panels. (B) Colocalization of CD38 with Lyn in Namalwa cells was examined by CLSM: CD38 was immunostained with HB136 anti-CD38 mAb (10 μg/ml in TNB), followed by a GαMIg-Alexa 488 labeled secondary antibody (Molecular Probes), as described in Fig. 3E (more details in Materials and methods). Lyn was stained with an anti-Lyn (44, sc-15), (10 μg/ml in TNB), followed by a goat anti-rabbit Rhodamine Red-X labeled Ab (Molecular Probes) secondary antibody. Upper four panels, the colocalization between CD38 and Lyn were indicated by the resulting yellow image (Merge), and it was quantitated by analyzing the colocalization scatter plot and generating a Mask image (fourth panel) of the area of interest (AOI). CLSM images were acquired with a Leica TCS-SP5 CSLM, using HCX PL APO CS 100× 1.4 oil objective. Scale bar: ~7 μm. Lower three panels show the upper surface/stack of a Namalwa cell stained with the indicated antibodies against CD38 (green) and Lyn (red), respectively. Yellow images are indicative of colocalization between both proteins (Merge). Images were acquired with the Leica TCS-SP5 CSLM, using a HCX PL APO CS 63× 1.40–0.60 oil objective. Scale bar: ~2.47 μm.

37 °C, a condition that preserves tetraspanin–tetraspanin and tetraspanin–protein complexes and their ability to localize to low-density fractions in sucrose gradients [37]. These characteristics and other physical and functional characteristics of Brij 98 MR [11,13] render

them distinct from classical Triton X-100 rafts and more similar to tetraspanin-enriched microdomains (TEMs) [38]. CD38 appears to be more abundant and enzymatically active in MR compared to S fractions, and the disruption of MR alters the CD38 partition and

enzymatic activity. Furthermore, cross-linking with CD38-specific mAb induces Akt/PKB and Erk activation, CD38 oligomerization and increased CD38 resistance to detergent solubilization that is one of the hallmarks of proteins associated with MR.

Next, we identified the molecules associated with CD38. Membrane organization and lateral interactions with other cell surface signaling molecules are crucial for CD38-mediated signals [1,13,18]. In exosomes, CD38 interacts with CD81, Hsc-70 and Lyn. Similar molecular interactions are observed in MR, which host the majority of CD38 in the B cell lines analyzed. CD38/CD81 interactions occur in exosomes and in MR in the presence of 1% Brij 98 lysis plus 15 mM ODG, which further improves solubility (Figs. 2A and 6B, C). CD38/CD81 interactions were also detected in the pool of S fractions isolated from B cells lysed in 1% Brij 98 at 37 °C (Fig. 6B and C). The S fractions correspond to fully solubilized membrane proteins and cytosolic components. However, these interactions are not maintained when exosomes are lysed in 1% NP-40 at 4 °C (not shown), a condition that also disrupts tetraspanin–protein complexes [37]. These results support the conclusion that CD38/CD81 complexes are not artifacts resulting from incomplete solubilization but represent discrete units of moderate size that can be fully solubilized. In human monocytes, tetraspanin CD9 has been reported to be associated with CD38 both in raft and nonraft compartments [28]. CD38/CD81 complexes likely rely upon direct protein–protein interactions; therefore, lipids may exert a role in the stabilization of the CD38/CD81 complexes due to their prevalence in exosomes and MR as opposed to the S fractions.

The results also show that the reciprocal association between CD38 and CD19 occurs exclusively in isolated MR of mature B cells. CD19 is laterally associated with surface CD38 in normal B lymphocytes and in B-CLL cells [16,39]. Sensitivity to cholesterol depletion indicates the involvement of MR. Moreover, polar aggregation of CD38 in normal and neoplastic B cells includes CD81 and vice versa [1,16,39]. CD19 and CD81 are likely direct partners because their interactions are maintained in stringent detergent conditions and/or captured by covalent crosslinking [38]. Furthermore, CD81 likely serves as the physical link between CD38 and CD19.

The intracellular Hsc-70 chaperone [40] is another component of the CD38/CD81 supramolecular complex observed in exosomes and in MR. Hsc-70 is constitutively expressed in exosomes and is involved in protein sorting in MVBs [21]. The complexes associated with CD38 in isolated exosomes are rich in Hsc-70 and Lyn (Fig. 2B). The association of Hsc-70 with CD38 appears to be independent of CD38; indeed, Lyn immunoprecipitated from Namalwa CD38$^+$ B cell exosomes retrieves Lyn and Hsc-70 but not CD38. Lyn/Hsc-70 complexes are also detected in the CD38$^-$ HOM-2 exosomes (Fig. 2C). The absence of CD38 in Lyn immunoprecipitates from Namalwa cell exosomes is rather surprising because Lyn displays a bi-directional association with CD38 in MR (Fig. 7). MR contains a high concentration of tyrosine kinases such as Lyn [41]. Assuming that the CD38/Lyn/Hsc-70 interactions are of low stoichiometry, differences in the relative concentration of these proteins in exosomes compared to MR may explain these results. CLSM imaging reveals that CD38 expression on the surface of the Namalwa cells has a patchy appearance. The co-precipitation of CD38 with Lyn in MR, even in the absence of crosslinking with anti-CD38 mAbs, suggests the presence of preformed CD38 clusters that interact with Lyn in MR (Fig. 7B). Therefore, Lyn is part of a supramolecular signaling complex that includes CD38 and BCR/CD19/CD21/CD81

[41,42]. Another member of the CD38/Lyn complex is Gαi-2 (Fig. 7A, C and D). The mechanism of association between Gαi-2 and CD38 in MR is currently unknown. In epithelial cells, clusters of CD59, a glycosylphosphatidylinositol-anchored receptor, recruit Lyn and Gαi2 via protein–protein and raft (lipid–lipid) interactions. Lyn is most likely activated by the Gαi2 binding in the same CD59 cluster, which induces the binding of the CD59 cluster to F-actin, resulting in CD59 transient immobilization, which is crucial for the further recruitment and activation of PLC-γ2 [43]. Therefore, in B cells, interactions between CD38, Lyn and Gαi-2 in MR may be functionally relevant and represent a bridge with actin or other cytoskeletal proteins. This model has been reinforced by the finding that the CD38 supramolecular complex observed in MR also contains actin (Supplementary Figs. S2 and S3).

The original hypothesis concerning MR has recently been modified in light of the key roles now attributed to selected membrane proteins in capturing and stabilizing intrinsically unstable lipid domains [8,41,44–46]. CLSM imaging reveals that CD38 colocalized with ganglioside GM1. CD38 may be capable of capturing and stabilizing MR by interacting with negatively charged polar groups of sphingolipids [26] and complex gangliosides present in MR [47]. As a consequence of undergoing palmitoylation [38], tetraspanins can also associate with cholesterol and gangliosides [48]. Indeed, exosomes contain large amounts of cholesterol, sphingomyelin, ganglioside GM3 [49] and lipids that are typically enriched in MR and/or TEMs. The presence of CD38 in these structures in B lymphocytes may be important for cADPR-, ADPR-, and NAADP-dependent Ca^{2+} signaling, as shown in other cell systems [1,50].

In conclusion, the novelty of this study is the unexpected presence of CD38 on the surface of exosomes released by cells of B lymphocytic origin, which may be regulated by the previous incorporation of CD38 into MR- or tretaspanin-protein signaling networks. These findings create new perspectives regarding the roles of the molecules expressed by exosomes in the regulation of homo- and heterotypic intercellular interactions, which is particularly significant in closed environments. Also, CD38$^+$-exosomes may carry elements capable of modifying the immune response.

Contribution

Conflict-of-interest disclosure

Authors declare no competing financial interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2010.05.032.

R E F E R E N C E S

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Anexo II: *Trabajos no publicados*

Increased CD38 expression in T cells and high blood plasma levels plasma levels of anti-CD38 IgG autoantibodies identify two different subsets of patients with systemic lupus erythematosus

Clinical and immunological evaluation of a patient affected with Hemophagocytic Lymphohistiocytosis associated with Leishmania infection

Deficient activation of peripheral blood T cells from Pemphigus patients upon stimulation with a mixture of superantigens

Increased CD38 expression in T cells and high blood plasma levels of anti-CD38 IgG

autoantibodies identify two different subsets of patients with systemic lupus erythematosus.

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ABSTRACT

Anti-CD38 IgG autoantibody plasma levels and CD38 expression on T cells were significantly higher in systemic lupus erythematosus (SLE) patients than in Normal donors. However, CD38 expression in SLE T cells correlated with the plasma levels of a number of cytokines, including Type 1 and Type 2 cytokines, and was more prevalent in clinically active SLE patients, whereas elevated anti-CD38 autoantibodies correlated with moderate increased plasma concentration of IL-10 and IFN-γ and was more frequent in clinically quiescent SLE patients. These data suggest that increased CD38 expression in SLE T cells could be the consequence of the action of proinflammatory cytokines such as TNF-α and IFN-γ, and indicative of SLE patients with a more active disease, and overt abnormal profile of circulating cytokines, whereas the presence of anti-CD38 autoantibodies in plasma could be indicative of SLE patients with a relatively well-controlled disease in whom the immunological abnormalities are in remission.

Keywords: Autoantibodies; CD38; Cytokines; Disease Activity; Systemic Lupus Erythematosus; T cells.

1. Introduction

CD38 is a ubiquitous protein expressed in myriad cells and tissues [1-3]. It is a member of a gene family that has the ability to convert NAD into cADPR [4]. In addition to its ADP-ribosyl cyclase enzymatic function, CD38 also catalyzes a transglycosylation reaction exchanging the terminal nicotinamide group of the substrate NADP with nicotinic acid resulting in the production of NAADP [5]. Both cADPR and NAADP are Ca^{2+}-mobilizing messengers thought to be important in various cellular signaling events [6, 7].

Increased CD38 expression in different cell types has been associated with a number of human diseases [8, 9]. Thus, CD38 represents a reliable negative prognostic marker in chronic lymphocytic leukemia (CLL) [10]. However, CD38 expression in CLL B cells also affects expansion and proliferation of the neoplastic clones, and it is, therefore, considered as part of a network sustaining growth and survival of CLL cells [11].

In SLE patients increased percentages of B cells positive for CD38 have been consistently shown by different groups [12-14]. Active-SLE patients have circulating CD38^{bright} Ig-secreting cells that are not found in normal individuals [12]. This plasma cell subset disappears from circulation during treatment with humanized anti-CD154 mAb, and it is associated with decreases in anti-double-stranded DNA (anti-dsDNA) Ab levels, proteinuria, and SLE disease activity index [12]. Moreover, in SLE patients with active disease, B cells expressing high levels of CD38 spontaneously produce IgG class anti-dsDNA *in vitro*, whereas persistence of CD38^{+} B cells during periods with clinically quiescent disease seems to underly hypergammaglobulinaemia but not anti-dsDNA production [14]. Likewise, T cells from active SLE patients over-express CD38 [15-17], which might be playing a role in modulating TCR signaling [17].

It has been postulated that CD38 may play a role in the pathogenesis of diabetes mellitus, which is likely related to increased anti-CD38 autoantibodies detected in Japanese type 2 [18], as well as Caucasian type 1 and 2 diabetic patients [19]. Autoantibodies to CD38 are also detected in patients with chronic autoimmune thyroiditis and Graves' disease [20]. However, no similar studies have been carried out in other autoimmune diseases, such as SLE.

The aim of the present study is to define the prevalence of autoantibodies to CD38 in SLE subjects, using an immunoenzymatic assay suitable for large-scale screening, and to explore the association of these autoantibodies with CD38 surface expression in T cells, cytokine production and SLE characteristics.

2. Methods

2.1. Patients

A total of 69 SLE consecutive patients (61 female, 6 male) attending the outpatient clinic were selected. No exclusion criteria were used for the selection of patients in order to obtain a heterogeneous sample representative of a broad spectrum of clinical and laboratory phenotypes. All SLE patients fulfilled the American College of Rheumatology criteria for SLE [21]. Disease activity was measured using the SLE Disease Activity Index (SLEDAI) [21]. The SLE patients had a median SLEDAI of 2 (range: 0 to 20). A total of 71 Normal control subjects were selected at the local blood bank (43 female, 28 male). All patients and Normal controls were Caucasians. The study protocol was approved by the Hospital Clínico San Cecilio, and CSIC Review Board and Ethics Committees. Written informed consent was obtained from all participating patients and volunteers according to the Declaration of Helsinki.

2.2. Blood plasma samples

Blood was collected by the BD Vacutainer system into K$_2$-EDTA tubes (BD Diagnostics, NJ, USA) and plasma was separated from cells by density gradient centrifugation over HISTOPAQUE®-1077 (Sigma-Aldrich, St. Louis, MO). The supernatant was collected, checked for the absence of cells by light microscopy, and fractionated in aliquots that were stored at – 80ºC [17].

2.3. Recombinant CD38 proteins

The recombinant His-tagged GST-CD38 fusion protein used as the target antigen in the ELISA and Western blots experiments was kindly obtained from Prof. C. F. Chang at the National University of Singapore [22]. The other recombinant CD38 protein used in Western blot experiments (rCD38) lacks the four putative N-linked glycosylation sites and the intracellular and transmembrane spanning regions and was produced as a soluble, enzymatically active CD38 in yeast [23].

2.4. Analysis of anti-CD38 autoantibodies by enzymatic immunoassay

Anti-CD38 IgG and IgM concentrations were measured by ELISA. Briefly, the His-tagged GST-CD38 fusion protein (Fr #20) was added to 96-well polystyrene plates (Nunc, Roskilde, Denmark) in coating buffer (0.05 M Na$_2$CO$_3$/NaHCO$_3$, pH 9.6) and incubated for 16 hours at 4°C. Plates were then washed twice with phosphate buffered-saline (PBS) containing 0.1% Tween 20 (PBS/Tween) and blocked for 2h with 5% BSA in PBS. Plasma samples were diluted 1:4 in PBS/Tween and 100 µl was added to each well and incubated for 2 hours at 37ºC. Following four washings with PBS/Tween, horseradish peroxidase-conjugated goat anti-human IgG (Sigma-Aldrich, St. Louis, MO) anti-serum was added and incubated for 1h. Plates were then washed 6 times and developed with 100 µl o-phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich, St. Louis, MO). Substrate color development was stopped by 0.5 M sulfuric

acid and absorbance was measured at 450 nm using a VersaMax tunable microplate reader (Molecular Devices, Chicago, IL) with the results expressed in optical density units (ODU). For IgM measurement, plasma samples were incubated as before and detected with purified goat anti-human IgM (Sigma-Aldrich, St. Louis, MO) followed by HRP-conjugated rabbit anti-goat IgG (Sigma-Aldrich, St. Louis, MO). The development was as described above. Validation of both assays was done in-house. Control plasma derived from two Normal controls and two SLE patients were added to each assay to determine inter-assay variation, which was always less than 15%. The intra-assay variation of triplicates was always less than 5%. A positive control was performed using the rabbit polyclonal IgG against human CD38 (H-170) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) raised against an epitope corresponding to amino acids 1-170 mapping at the amino terminus of human CD38. This was followed with washing and incubation with the secondary antibody (goat anti-rabbit IgG) conjugated with horseradish peroxidase (Promega Co., Madison, WI). Other antibodies used were HB136, OKT10, and OKT3 mAbs.

2.5. Specificity analysis of anti-CD38 aAbs by Western blot

For Western blot analysis, GST-CD38, or rCD38 were run on 4-12% NuPAGE Bis-Tris gels (Invitrogen), loading 0.5 μg of protein in each lane. Migrated proteins were then transferred to polyvinilidene difluoride (PVDF) membrane with a semi-dry transfer apparatus (Hoefer Pharmacia Biotech, San Francisco, CA) in Tris-glycine buffer containing 20% methanol and 0.035% SDS at 0.8 mA/cm^2. Membranes were saturated with 3% nonfat dry milk in Tris-buffered saline with Tween-20 (TNAT) (10 mmol/l Tris [pH 7.4], 100 mmol/l NaCl, and 0.1% Tween-20) and incubated with plasma of patients diluted 1: 100, or 1:1,000 in 5% milk TNAT. Following four washings with PBS/Tween, horseradish peroxidase-conjugated goat anti-human

IgG (Sigma-Aldrich, St. Louis, MO) anti-serum was added and incubated for 1h. The reaction was visualized using enhanced chemiluminiscence reagents (Bio-Rad) and exposure to X-ray film and/or to imaging using the ChemiDoc XRS System (Bio-Rad).

2.6. Identification of GST-CD38 by mass spectrometry

In selected experiments GST-CD38 was run on SDS-PAGE as above and gels were stained with SYPRO Ruby overnight as described [24]. Bands were excised and in-gel digested with trypsin (Promega, Madison, WI) using a Digest MSPro (Intavis, Koeln, Germany) following standard procedures. Briefly, gel spots were washed with water and 20 mM ammonium bicarbonate pH 7.8, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with trypsin (12.5 ng/µL) for 16 h at 37ºC. Tryptic peptides were extracted with acetonitrile/water 0.25% TFA. Extracts were evaporated to dryness by vacuum and redisolved in 5 µL ACN/H_2O 1/1 v/v with 0.1% TFA.

Samples were analyzed by LC-MS/MS using a linear LTQ ion trap equipped with a microESI ion source (ThermoFisher, San Jose, CA). Each extract was diluted up to 40 µL with 1% formic acid. Samples were loaded in a chromatographic system consisting of a C18 preconcentration cartridge (Agilent Technologies, Barcelona, Spain) connected to a 10 cm long, 150 µm i.d. Vydac C18 column (Vydac, IL, USA). The separation was done at 1 µL/min with a 60 minute acetonitrile gradient from 3 to 40% (solvent A: 0.1% formic acid, solvent B: acetonitrile 0.1% formic acid). The HPLC system was composed of an Agilent 1200 capillary pump, binary pump, a thermostated microinjector and a micro switch valve. The LTQ instrument was operated in the positive ion mode with a spray voltage of 2 kV. The full MS scan range was m/z 400-2000. The spectrometric analysis was performed in an automatic dependent mode,

acquiring 8 MS/MS scans of the most abundant signals in each full scan. Dynamic exclusion was set to 1 min to avoid the redundant selection of precursor ions.

MS/MS spectra were searched using SEQUEST (Bioworks v3.3, ThermoFisher, San Jose, CA) with the following parameters: peptide mass tolerance 2 Da, fragment tolerance: 0.8 Da, enzyme: trypsin with a maximum of two missed cleavages, static modifications: cysteine carbamidomethylated (+57 Da), dynamic modifications: methionine oxidation (+16 Da). The Uniprot database (Human Swiss-Prot release 14.8, taxonomy: human with GST added) to which its reversed copy was appended was used for the search. Identifications were filtered with Bioworks scores Sf > 0.6 and P(pep) < 0.005 and were manually validated.

2.7. Cytokine assay

The Bio-Plex Precision Pro Human Cytokine 10-Plex kit assay (Bio-Rad, Hercules, CA) was used to simultaneously test 10 cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IFN-γ and TNF-α. Assays were performed according with manufacturers' protocol. Analyses of experimental data were performed using five-parameter logistic curve fitting to standard analyte values.

2.8. Flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) or purified T cells were analyzed for surface expression of CD3, CD4, CD8, CD38, CD19 and CD56 by single or double-staining using FITC-, or PE-labeled anti-CD3, anti-CD38, anti-CD19, and anti-CD56 mAbs in the relevant combinations. Compensation settings were adjusted using single-stained PBMC, or T cell samples. Isotype-matching labeled antibodies were used to calculate the nonspecific staining. PBMC and lymphocytes were gated according with their forward and scatter characteristics [17]. Two color immunofluorescence analysis was performed on either a FACScan, or a FACScalibur

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flow cytometer (BD Biosciences, San Jose, CA), using the CellQuest Pro (BD Biosciences), and FlowJo (Tree Star, Inc. San Carlos, CA) software.

2.9. Statistical analysis

Values are given as the mean ± standard error (SE), unless otherwise indicated. Comparisons between two groups were performed with the Student's *t* test for normally distributed variables and the Mann-Whitney *U* test for non-normal variables. Each individual marker was evaluated by a receiver-operating characteristic (ROC) curve, the area under the ROC curve (AUC), and sensitivity at 95% specificity (ROC [0.005]). Fisher's exact test was used to compare two proportions. The one-way ANOVA test was used to compare mean values of three or more groups. In some occasions the Kruskal-Wallis nonparametric test was used to compare medians instead. If the mean values were statistically significant by the ANOVA test, then the Tukey-Kramer test was used to compare all pairs of groups. Differences were considered statistically significant for *P* values < 0.05. Analysis of the data was done using the GraphPad Prism version 5.01 software (GraphPad Software, Inc., San Diego, CA).

3. Results

3.1. Distinct immunoreactivity of SLE plasmas to GST-CD38 recombinant protein revealed by Western blotting and ELISA

The extracellular domain of human CD38, beginning with Pro 44, was expressed as a 6× His-containing GST fusion protein. The fusion protein was purified by Ni-NTA affinity chromatography followed by gel filtration via a Superdex 200 (S200) column. Two major bands at 85, and 56 kDa were detected when S200 fractions #15 to #21 were subjected to SDS-PAGE under reducing conditions and stained with Coomassie blue [22]. The 85 kDa protein was predominant in the first 2 fractions, and the 56 kDa in the last 3 fractions, whereas fractions #17

and #18 contained similar amounts of both proteins. At that point, and in contrast to the 56 kDa protein, the 85 kDa band (p85) was not clearly identified by peptide mass fingerprinting analysis [22]. In an attempt to identify p85, S200 Fr #19 was run on SDS-PAGE in parallel with purified GST (Sigma) and the gel was stained with Sypro-Ruby. Three bands at 110, 85 and 56 kDa were detected (Fig. 1A). The 56 kDa band showed the expected molecular weight of GST fused with the nonglycosylated monomeric form of the extracellular domain of CD38 [22]. By using 2-mercaptoethanol instead of DTT as reducing agent the 110 kDa band completely disappeared (data not shown), suggesting that this band may correspond to dimers of the 56 kDa band. By using tandem mass spectrometry the three bands were unambiguously identified as GST-CD38 (Supplemental Table 1).

To test the immunoreactivity of the SLE plasmas to the GST-CD38 recombinant protein, a pilot experiment was performed in which the plasma from the SLE patient P23.2 diluted at 1:100 was immunoblotted against S200 Fr #19. As a control, equal amounts of another recombinant CD38 protein (termed rCD38), which is not fused to GST, nor to 6 x His tag, were run in parallel with Fr #19 (0.6 µg/lane). As shown in Fig. 1B, when samples were run under non-reducing conditions, the major band highlighted by P23.2 SLE plasma displayed an apparent molecular weight of ~85 kDa (lane 1), corresponding to the middle band detected by Ponceau S staining of GST-CD38 (lane 7). P23.2 plasma reacted to a lesser extent with the 56 kDa monomeric form of GST-CD38 (lane 1), which is the major band of Fr #19 stained with Ponceau S (lane 7), and with the 30 kDa rCD38 (lane 2), whereas no reactivity was seen with the 110 kDa GST-CD38 form (lane 1).

Densitometric analysis of Sypro Ruby stained gels indicated that about 20% of Fr #19 was comprised of p85 (data not shown). The cause of the abnormal mobility of p85 GST-CD38

on SDS-PAGE is unknown, although an unidentified posttranslational modification (PTM) could be added to GST-CD38 along its expression in insect cells. In favor of this interpretation is the fact that p85 show distinct epitopes not shared with the unglycosylated 56 kDa and 110 kDa GST-CD38 forms, nor with rCD38, which is also non-glycosylated since it lacks the four N-linked glycosylation sites of human CD38 [23]. Thus, if Fr #19 was treated with the reducing agent DTT, which breaks disulfide bridges, then the immunoreactivity of P23 SLE plasma to the 85 kDa band was conserved, whereas the reactivity against the 56 kDa band was lost (Fig. 1C, lane 1). In contrast, p85 was not recognized by the polyclonal anti-CD38 antibody H-170, while both the 56 kDa and the 110 kDa GST-CD38 forms were readily detected by this antibody (Fig. 1B, lane 3). On the other hand, H-170, which was raised against an N-terminal CD38 synthetic peptide (amino acids 1-170 of human CD38), weakly reacted with rCD38 (Fig. 1B, lane 4), despite the fact that similar amounts of 56 kDa GST-CD38 and 30 kDa rCD38 were detected by Ponceau S staining (Fig. 1B, lanes 7 and 8). In contrast, #77, a polyclonal rabbit antiserum raised against rCD38, readily recognized either rCD38 or the 56 kDa and 110 kDa GST-CD38 forms, but not p85. Therefore, the epitopes recognized by P23.2 SLE plasma on p85 are not detected by H-170 or #77 anti-CD38 antisera, which are both raised against non-glycosylated polypeptide chains.

Since the three GST-CD38 forms of Fr #19 contain GST, and the 56 kDa form is more abundant than p85, the blotting experiments of Fig. 1B and 1C suggested that the reactivity of the SLE plasma was not directed against GST. To further confirm this, S200 Fr #19 was run on SDS-PAGE under reducing conditions in parallel with an equal amount of purified GST and blotted against the plasma of two Normal controls and one SLE patient at 1:1,000 dilution. As shown in Fig 1D, neither of the plasmas analyzed reacted with the 26 kDa GST (Fig. 1D, lanes 2,

4, and 6). In contrast, the plasma from C25 and P43 SLE reacted with the 85 kDa GST-CD38 (lanes 1 and 5, respectively), despite the fact that it was 5-fold less concentrated than GST or 56 kDa GST-CD38. The C26 Normal plasma gave no signal with the 85 kDa band (lane 3).

Given the unexpected immunoreactivity of p85 GST-CD38 with SLE and Normal plasmas, the following experiments were performed with fraction S200 Fr #20, which was 95% pure and had at least 4-fold less p85 content than Fr #19 [22]. First, Fr #20 was immobilized to 96-well plates at a similar concentration (0.5 μg/well) of that used in the Western blot experiments described above (0.6 μg/lane), and probed with the rabbit polyclonal anti-CD38 Ab, H-170, or with two different anti-CD38 mAbs, HB136 and OKT10. An anti-CD3 mAb, OKT3, was used as a negative control. As shown in Fig. 1E, the reactivity obtained with the polyclonal anti-CD38 antibody H-170 was evident. On the contrary, the anti-CD38 mAbs HB136 and OKT10 exhibited a binding to the target molecule that was similar to that of the negative control OKT3. Note that HB136 and OKT10 mAbs recognize conformational epitopes on CD38 [25] at the C-terminal portion of CD38 (amino acids 273-285) [26], whereas it is likely that the anti-peptide antibody H-170 preferentially recognizes linear epitopes on CD38.

Fr #20 was also used to test whether this fraction showed similar immunoreactivity with plasmas (from SLE patients or Normal controls) than that shown by Fr #19. Fig. 1E shows the ODU reading (IgG binding to Fr #20) obtained with the plasma from three different SLE patients, and from two Normal donors. SLE plasmas displayed 3-9-fold more IgG binding to Fr #20 than that shown by Normal plasmas. In particular, P43 SLE showed an 8.8-fold difference with C25 (3.18 ± 0.03 ODU vs 0.43 ± 0.006 ODU). In contrast, the Western blot experiments showed that P43 and C25 had similar immunoreactivity to Fr #19, which was in fact mainly directed against p85 (Fig. 1D). Note, however, that the 56 kDa GST-CD38 form was also

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recognized by SLE plasmas as long as Fr #19 was not reduced with DTT (Fig. 1B, lane 1). Western-blot analyses require the presence of SDS, either during protein separation by SDS-PAGE or during protein transferring to PVDF membranes. It is likely that along these processes epitopes sensitive to ionic detergents and reducing agents were lost in 56 kDa GST-CD38, making this protein virtually undetectable to plasmas otherwise positive for anti-CD38 autoantibodies. In contrast, in the ELISA setting, Fr #20 was attached to the plastic wells in the absence of DTT, or any ionic detergent as SDS, making it more suitable to be recognized by autoantibodies that specifically recognize epitopes sensitive to those reagents.

The MaxiSorp surface of the ELISA microplates used in this study is a modified, highly charged polystyrene surface with high affinity to molecules with polar or hydrophilic groups. The surface has a high binding capacity for proteins, including globular antibodies in proper orientation. In contrast the PVDF membrane used for Western blotting is highly hydrophobic and has high affinity to molecules of a more hydrophobic character. Our results suggest that upon binding to the highly hydrophilic surface of the plastic wells, GST-CD38 adopts a better orientation, which reduces problems with antibody recognition of CD38 due to masking of epitopes.

In summary, the stronger immunoreactivity of SLE plasmas to Fr #20 than to Fr #19 seems to be related, at least in part, to the lower p85 content in Fr #20, and to a more efficient recognition of the 56 kDa GST-CD38 protein upon attachment to plastic surfaces. The ELISA procedure seems to be more sensitive and reproducible and as specific as the Western blot assay, with the advantage of a format more amenable to large scale studies [27].

3.2. Increased levels of anti-CD38 IgG autoantibodies in blood plasma from SLE patients

Between the representative plasma samples shown in Fig. 1E, a whole range of intermediate reactivities were obtained with plasma from other SLE patients and Normal controls. Since our control group was composed of only 61% females versus 91% of the SLE patients, we first tested whether there were differences in anti-CD38 IgG levels between females and males in the control group. Although the mean value for anti-CD38 IgG levels was slightly higher in females (0.82 ± 0.03, n = 43) than in males (0.77 ± 0.04, n = 28), these differences were not statistically significant ($P = 0.3466$, t-test). Therefore, we used the whole control group in the following assays.

Fig. 2A shows the distribution of anti-CD38 IgG levels among the Normal donors and SLE patients. The anti-CD38 IgG mean value was significantly higher in SLE (0.97 ± 0.06; n = 69) than in Normal donors (0.80 ± 0.03; n = 71, $P = 0.0071$, t-test). SLE patients were segregated according with their SLEDAI scores, a validated measure of global disease activity [21], in two groups: clinically quiescent (SLEDAI = 0, n = 26), and clinically active SLE (SLEDAI > 0, range: 2-20, median: 4, n = 43). In both SLE groups the mean values of anti-CD38 IgG levels were significantly higher than in Normal controls (1.01 ± 0.07; $P = 0.0012$, and 0.95 ± 0.08; $P = 0.0386$, respectively, t-test).

Anti-CD38 IgG levels differentiate very well between SLE patients and Normal controls, as confirmed by the ROC analysis. The area under the ROC curve (AUC) for anti-CD38 IgG was 0.60 ± 0.05 (mean $\pm$ SE; $P = 0.0374$). At a cut-off value of 1.25 ODU, anti-CD38 IgG revealed a sensitivity of 13.04% and a specificity of 97.2% to distinguish SLE from Normal donors. The corresponding positive likelihood ratio (LR) was 4.63. On the basis of this cut-off value, 13% of all SLE patients tested were positive for anti-CD38 IgG autoantibodies versus 2.8% of Normal controls ($P = 0.0297$, Fisher's exact test). These differences were even higher when the anti-

CD38 IgG levels of the quiescent SLE group were compared with those of the Normal control group. The AUC was 0.67 ± 0.07; $P = 0.009857$, showing that 19.2% of the SLE patients with SLEDAI = 0 were positive for anti-CD38 IgG autoantibodies ($P = 0.0140$, Fisher's exact test). In the active SLE group (SLEDAI > 0), the number of positive SLE patients for anti-CD38 IgG autoantibodies dropped to 9.3%. Therefore, anti-CD38 autoantibodies seemed to be more prevalent in SLE patients with inactive disease than in SLE patients with active disease, although the differences between them were not statistically significant. The low number of SLE patients with a high SLEDAI score (>6) precluded a definitive conclusion. In summary, the anti-CD38 IgG test had enough power to distinguish between SLE patients and Normal controls, and in particular to distinguish between clinically quiescent SLE patients and Normal controls.

Fig. 2B shows the distribution of anti-CD38 IgM levels among the Normal donors and SLE patients. There was no significant difference in anti-CD38 IgM mean levels between SLE patients and Normal donors ($P = 0.5568$). Likewise, clinically active SLE patients showed similar anti-CD38 IgM antibodies levels to those of the quiescent SLE patients or of the Normal donors control group. No correlation was found between anti-CD38 IgG and anti-CD38 IgM levels in any of the groups tested (data not shown).

3.3. Distinct blood plasma cytokine profile in anti-CD38$^+$ SLE patients.

In PBMCs, binding of agonistic anti-CD38 mAbs induces release of pro-inflammatory cytokines, i.e., IL-1, IL-6, and TNF-α [28], and similar effects may be triggered by anti-CD38 autoantibodies. On the other hand, increased levels of either proinflammatory, or anti-inflammatory cytokines in blood plasma or serum from SLE patients have been reported earlier [29]. We used multiplex bead array technology to simultaneously measure the levels of 10 different cytokines in blood plasma from SLE patients and Normal controls that had previously

tested for anti-CD38 IgG autoantibodies. As shown in Table 1, SLE patients had increased

plasma levels of both Type 1 (IL-12, IFN-γ, and TNF-α) and Type 2 (IL4, IL-6, IL-10, and IL-

13) cytokines as compared with Normal controls. Moreover, IL-2 plasma levels were also

elevated. IL-2 is considered a Type 0 cytokine because contributes to the expansion of all T cell

types including effector Th1, Th2, Th17 cells, memory CD8$^+$ T cells, and regulatory T cells [30].

IL-2 can also contribute to the expansion of CD25$^+$ B cells, and indeed IL-2 is required for

polyclonal Ig production by committed human B cells [31]. Neither IL-1β, nor IL-5 plasma

levels were significantly elevated in SLE patients (Table 1).

The above data also indicated that there was a notorious imbalance between the

concentration of some Type 2 cytokines such as IL-6, IL-10, IL-13, and the concentration of

Type 1 cytokines such as IL-12, TNF-α, or IFN-γ. The shift toward Type 2 cytokines was better

illustrated by determining the ratio of the concentration of these cytokines to that of IFN-γ in

each individual sample (Fig. 3A). The mean ratios for Type 2 cytokines ranged from 13.2 for IL-

6/IFN-γ to 4.5 for IL-13/IFN-γ. The only exception to this profile for Type 2 cytokines was IL-4,

which had similar mean levels to IFN-γ, and showed a mean IL-4/IFN-γ ratio of 1.32. In contrast,

the mean ratios for Type 1 cytokines ranged from 0.83 for IL1-β/IFN-γ to 1.43 to IL-12/IFN-γ.

Note also that the IL-2/IFN-γ ratio was 12.8. In summary, these data strongly suggest the

predominance of Type 2 over Type 1 cytokines in SLE plasmas.

To test whether anti-CD38 IgG and cytokine levels were correlated or not, SLE patients

were split in two groups: anti-CD38 IgG positive (anti-CD38$^+$, above the cut-off value of 1.25)

and anti-CD38 IgG negative (anti-CD38$^-$, below 1.25). A significant positive correlation was

found between anti-CD38 IgG autoantibodies and IL-10, or IFN-γ plasma levels (Table 2).

Moreover, in the anti-CD38$^+$ group, there was a significant increase in 9 out of 10 cytokines

tested in the 2-5-fold increase range above the mean control values (Fig. 3B). In contrast, in the anti-CD38^{-} group there was no correlation of anti-CD38 IgG levels with any of the cytokines tested (Table 2). This group was heterogeneous in terms of cytokine plasma levels and they could be separated in two additional groups according with a cut-off value of 21 pg/ml for IL-10 (mean + 2SD of normal values). Those anti-CD38^{-} SLE patients with IL-10 plasma levels > 21 pg/ml had also the highest plasma levels of all cytokine tested (12-22-fold increase above mean control values) (Fig. 3C), whereas the anti-CD38^{-} SLE patients with IL-10 plasma levels < 21 pg/ml showed a similar cytokine profile than the control group, with the exception of IL-2 and IL-6, which were significantly increased in the 2-3-fold range (Fig. 3D).

3.4. Increased CD38 expression in SLE T cells correlates with the plasma levels of several cytokines but not with the presence of anti-CD38 IgG autoantibodies

PBMCs from patients with SLE and Normal controls were analyzed for expression of CD38 in CD3^{+} T cells. The proportion of T cells expressing CD38 varied from 4% to 78%, and a threshold was set at 21% by ROC analysis of these data (AUC = 0.79, P = 0.0008501), above which samples were classified as CD38^{+} T cells. On the basis of this cut-off value, 65% of the SLE patients analyzed were considered CD38^{+} versus 6% of Normal controls (P < 0.0001, Fisher's exact test) (Fig. 4A). The differences with Normal controls were higher in clinically active SLE patients (72%, P <0.0001) than in quiescent SLE patients (54%, P = 0.0094), although the differences between both SLE subgroups were not statistically significant (P = 0.4491).

In a group of 51 SLE patients the proportion of T cells expressing CD38 was compared with the anti-CD38 IgG levels in plasma (Fig. 4B). There was no correlation between both parameters (r = 0.1148, P = 0.4226, Spearman's correlation). Rather, they seemed mutually

exclusive. Thus, there were not double positives (Fig. 4B, upper right quadrant). In contrast, CD38 expression in T cells showed a positive correlation with plasma levels of a number of cytokines, including both Type-2 cytokines such as IL-4, IL-10, and IL-13 and Type-1 cytokines such as IL-1β, IL-12, IFN-γ, and TNF-α (Table 3).

In B cells (CD19$^+$) and NK cells (CD56$^+$) the differences in CD38 expression between SLE and Normal controls were statistically significant (Fig. 4C and 4D). The ROC curve for CD38 expression in each cell type showed significant ability to differentiate SLE patients from Normal controls, as AUCs were all significantly above 0.5. Similar percentages of individuals were CD38$^+$ in B, or NK cells from clinically active and quiescent SLE patients. The data also showed that CD38 expression in B cells correlated exclusively with the plasma levels of IL-10 (r = 0.3481, P = 0.0471, Spearman's correlation), whereas CD38 expression in NK cells did not correlate with the plasma levels of any of the cytokines tested (data not shown).

3.5. Altered phenotype of SLE T cells

In a previous paper, we had some evidence that SLE T cells show an altered phenotype similar to that of activated/effector T cells [17]. To further confirm this, CD38 expression in CD4$^+$, CD8$^+$, and CD25$^+$ T cells was analyzed by flow cytometry. Indeed, the proportion of CD38$^+$ cells in the three T cell subsets studied was significantly increased in SLE patients as compared with that in Normal controls (Fig. 5A, 5B, and 5C, respectively). Moreover, most SLE patients had a significantly reduced CD4:CD8 ratio compared with that of healthy controls (Fig. 5D, P = 0.0119, t-test), which is likely caused by increased number of CD8$^+$ T cells. The CD8 data are in agreement with previous work showing significantly increased percentage of CD38$^+$CD8$^+$ T cells (activated CD8 subset) in patients with SLE in comparison to normal controls [32]. However, a small number of SLE patients had a significant increased CD4:CD8

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ratio (Fig. 5D, $P < 0.0001$, t-test), due to a drastic reduction in the percentage of CD8$^+$ subset.

We also tested for the expression of the early activation marker CD69 in the lymphocyte cell

fraction of PBMCs. As shown in Fig. 5E, 56% of the SLE lymphocytes were CD69$^+$ versus only

12% of the Normal controls ($P = 0.0461$, Fisher's exact test). The increased CD69 expression

may correspond mainly to T cells, since about 70% of the lymphocytes were T cells, and similar

results were obtained in highly purified untouched SLE T cells isolated by negative selection

from eight different SLE PBMCs (data not shown). Overall, these data further confirm the

altered phenotype of SLE T cells, which resembles to that of activated/effector T cells.

4. Discussion

SLE patients show increased levels of anti-CD38 IgG autoantibodies and higher

percentages of CD38$^+$ lymphoid cells (T, B and NK cells) as compared with Normal controls. In

contrast, in SLE patients increased levels of anti-CD38 IgM autoantibodies are not detected,

suggesting that the anti-CD38 IgG autoantibodies are not related with low avidity auto- and

polyreactive antibodies (natural antibodies), which usually are of the IgM class, and are

produced by CD5$^+$ B cells [33].

The antibody response against CD38 is likely to be diverse, involving different epitopes

within the moiety of the protein. In this study the recombinant GST-CD38 protein used in the

ELISA setting is not glycosylated and is not recognized by anti-CD38 mAbs HB136, or OKT10,

which are specific to conformational epitopes, whereas this protein is strongly reactive with a

polyclonal anti-CD38 antiserum raised against a synthetic peptide mapping the N-terminal

portion of human CD38 (H-170), or with an antibody (#77) raised against a recombinant protein

mapping the extracellular domain of human CD38 lacking the 4 N-glycosylation sites. Therefore,

GST-CD38 is recognized by anti-CD38 antibodies that react with non-glycosylated forms of

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CD38, and/or with non-conformational epitopes. In this sense, most reports on anti-CD38 autoantibodies have used non-glycosylated CD38 recombinant CD38 as antigen, either in Western blot assays [18, 34], or in ELISA [27].

Elevated anti-CD38 autoantibodies may modulate T cell and APC specific responses. Thus, in influenza hemagglutinin-specific CD38^{+} T cells CD38 blockade with specific anti-CD38 mAbs inhibits IL-2 and IFN-γ production induced by antigen-pulsed B cells [35], and in human dendritic cells blocking of CD38 signaling with anti-CD38 mAbs inhibits LPS-induced IL-12 secretion, while IL-10 is unaffected [36]. Moreover, this treatment impairs the dendritic cell-driven Th1 polarization of naive T cells [37]. In this report increased anti-CD38 IgG autoantibodies plasma levels in SLE pacients correlate with moderate increased plasma levels of IL-10, and IFN-γ, suggesting that these patients have a relatively well controlled disease, which is corroborated by their relatively low SLEDAI score. IL-10 is produced at a high level by B lymphocytes and monocytes of SLE patients, and it contributes to the abnormal production of immunoglobulins and of autoantibodies [38, 39]. On the other hand, regulatory B cells that preferentially produce IL-10 have a role in controlling autoimmune responses. Thus, it has recently shown that in human healthy controls a CD19^{+}CD24^{high}CD38^{high} B cell subset inhibits proinflammatory IFN-γ and TNF-α production by CD4^{+} T cells, which is mediated mostly by IL-10 [40]. In contrast, this subset is not functional in SLE patients, at least upon CD40 engagement [40]. In this paper CD38 expression in SLE B cells correlates exclusively with IL-10 plasma levels, which may also be linked to the maintenance of high levels of anti-CD38 autoantibodies.

In many cell types CD38 expression is regulated by a variety of cytokines including IL-1β, TNF-α, IL-13, IFN-γ, and IFN-α [41-44]. Interestingly, TNF-α and IFNs (β or γ) synergistically regulate CD38 expression in human airway smooth muscle cells, making these

cells refractory to the anti-inflammatory action of steroids [45]. In this study, increased CD38 expression in T cells strongly correlates with increased plasma levels of several cytokines, including TNF-α, IFN-γ and IL-13, which may contribute to an autocrine or paracrine induction and stabilization of CD38 expression, in particular in those SLE patients under treatment with glucocorticoids. CD38 overexpression in SLE T cells may also contribute to perpetuate the altered cytokine profile observed in SLE plasmas. Thus, up-regulation of CD38 expression in normal T cells enhances TCR-mediated calcium mobilization, and IL-2 production in an antigen-dependent manner [35], while opposite results are obtained by down-regulating surface CD38 expression by means of CD38 siRNA [35]. Increased expression of CD38 in SLE T cells, and its altered presence in membrane lipid rafts [17], may contribute, in addition with other signaling molecules that show altered T cell-surface distribution, to the observed T cell signaling abnormalities in SLE patients [46].

A close examination of SLE patients clearly shows that CD38 expression and anti-CD38 IgG levels are not related. Thus, the SLE patients that show the highest levels of anti-CD38 IgG antibodies have the lowest proportion of CD38$^+$ T cells. In the other way around, those patients with the highest proportion of CD38$^+$ T cells show the lowest anti-CD38 IgG levels. One possible explanation is that the antibodies against CD38 in SLE plasma may bind to surface CD38 on circulating lymphoid cells and saturate the binding sites of CD38, which will not be any longer available to the monoclonal anti-CD38 antibodies used in the flow-cytometry experiments. Alternatively, CD38 could be down-modulated or shed from the cell surface by high levels of circulating anti-CD38 IgG antibodies, and, therefore, not detected in T cells from SLE patients with high anti-CD38 IgG levels. Down-modulation of CD38 has been observed in CD38$^+$ Jurkat T cells upon stimulation with agonistic anti-CD38 mAbs [47]. Likewise, increased

expression of CD38 on the cell surface would facilitate the elimination of an excess of CD38 autoantibodies.

The SLE cytokine plasma levels clearly show an imbalance in the Type 2/Type 1 cytokine ratio, when IL-6, IL-5, IL-10, or IL-13 plasma levels are compared with IFN-γ in each individual sample tested (Fig. 3A). Similar results were obtained by using the IL-10/IL-12 ratio (data not shown). These findings support and extend the concept that in active SLE patients there is a shift in cytokine expression, with a predominance of Type 2-cytokine-producing T cells [48, 49]. Note that these cytokines are not exclusively synthesized by T cells. Thus, monocytes secrete IL-6, which, together with transforming growth factor β, promotes the differentiation of Th17 cells [50]. Lupus patients have elevated plasma levels of IL-6 (this paper, and [51], for a review), and high IL-6 levels may induce growth of T cells and differentiation of T cells by augmenting the production of IL-2 [52]. IL-6 plays a critical role in the B cell hyperactivity and immunopathology of human SLE, and may have a direct role in mediating tissue damage [53]. In this sense, most SLE patients have elevated plasma levels of IL-2 (Table 1), and increased percentages of $CD38^+CD8^+$ T cells (Fig. 5B, and [32]).

Other authors do not find a significant difference in the Th1/Th2 ratio in SLE patients as compared with Normal controls when analyzing the expression of the Type 1 cytokine IFN-γ and the Type 2 cytokine IL-4 in $CD4^+$ T cells [54]. Likewise, we have also not found a clear shift in the IL-4/IFN-γ ratio in SLE plasma, while the IL-13/IFN-γ ratio is clearly increased, which is also in full agreement with the data reported by others showing increased serum levels of IL-13 but not of IL-4 in SLE patients [55]. In addition, up-regulation of IL-13 mRNA transcripts, and a significant decrease in IL-4 mRNA expression have been shown in unstimulated PBMCs of SLE patients [56, 57]. IL-13 has similar biological activities with IL-4 although the production of

these cytokines by T cells is differentially regulated [58]. In this sense, in SLE T cells there is an imbalance between an overactive Ca^{2+}-mediated signaling leading to activation of the nuclear factor of activation in T cells (NFAT), and impaired activation of the activator protein 1 (AP-1) [46]. This imbalance may help to explain the skewed cytokine production towards Type 2 cytokines such as IL-10, and IL-13, but not IL-4 in SLE patients [59]. Since cytokines are key mediators of lymphocyte growth and function, a defect in the cytokine network has the potential to disrupt the normal regulation of self-reactivity, leading to the initiation of systemic autoimmunity as in lupus.

Conclusions: Prospective studies are needed to evaluate whether changes in CD38 expression in lymphoid cells, or anti-CD38 levels in plasma or serum can be useful to predict disease flares and/or organ damage. However, the results are consistent with the hypothesis that they are associated with specific immunologic abnormalities and inflammatory activity. Thus, increased CD38 expression in SLE T cells could be the consequence of the action of proinflammatory cytokines such as TNF-α and IFN-γ, and is indicative of SLE patients with a more active disease, and with an overt abnormal Type 2 and Type 1 cytokine profile. In contrast, the presence of anti-CD38 autoantibodies could be indicative of SLE patients with a relatively well-controlled disease, which is corroborated by their relatively low or negative SLEDAI score, and by the more restricted increased concentration of proinflammatory and anti-inflammatory cytokines in plasma.

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AUTHORS' CONTRIBUTIONS

The first two authors, Esther J. Pavón and Esther Zumaquero equally contributed to this work. All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Sancho had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Figure 1. Distinct immunoreactivity of SLE plasmas to GST-CD38 recombinant protein. **A**, Purified GST (lane 1), or GST-CD38 Fr #19 of the S200 chromatography column (lanes 2 and 3) were run on SDS-PAGE under reducing conditions (lane 1: 2.7 μg; lanes 2 and 3: 1.1 μg) and stained with Sypro Ruby. Bands were excised and processed as described in Material and Methods. The identification of the three GST-CD38 bands at 56 kDa, 85 kDa, and 110 kDa was done with a pool of their respective bands from lanes 2 and 3. The MS/MS data are shown in Supplemental Table 1. **B,** GST-CD38 Fr #19 of an S200 chromatography column (lanes 1, 3, 5 and 7), or purified rCD38 (lanes 2, 4, 6 and 8) were run on SDS-PAGE under non-reducing conditions (0.5 μg/lane) and transferred to PVDF membrane for Western-blot analysis. Lanes 1 and 2 were probed with P23.2 SLE plasma (1: 100 dilution), lanes 3 and 4 with the polyclonal anti-CD38 peptide antibody H-170, lanes 5 and 6 with the polyclonal anti-rCD38 antiserum #77, and lanes 7 and 8 were stained with Ponceau S before probing with P23.2 SLE plasma. Positions of 110 kDa, 85 kDa and 56 kDa GST-CD38 proteins are indicated on the left and on the right sides of the panels. **C,** Lane 1 shows the immunoreactivity of P23.2 SLE plasma (1:100 dilution) to GST-CD38 Fr #19 (0.5 μg/lane) run on SDS-PAGE under reducing conditions and transferred to PVDF membranes for Western blot analysis. Lane 2 is a SYPRO Ruby staining of GST-CD38 Fr #19 protein (0.5 μg/lane) run in parallel with lane 1. Molecular weight markers positions are indicated on the left. Positions of 85 kDa GST-CD38 protein is indicated on the right side. **D,** Immunoreactivity of C25 Normal plasma (lanes 1 and 2), or C26 Normal plasma (lanes 3 and 4), or P43 SLE (lanes 5 and 6) to GST-CD38 (lanes 1, 3, and 5), or GST (lanes 2, 4, and 6). Equal amounts of GST-CD38 and GST were loaded per lane (0.5 μg/lane). The plasmas dilution was

1:1,000. Position of the 85 kDa GST-CD38 protein is indicated on the right. Molecular weight

markers positions are indicated on the left. **E,** Binding of the polyclonal anti-CD38 Ab H-170,

anti-CD38 mAbs HB136 and OKT10, anti-CD3 mAb OKT3, SLE and Normal Control plasmas

to GST-CD38 assessed by ELISA. Antibodies were at 2 µg/ml. Plasmas were diluted at 1:4.

Results represent mean values ± SEM from triplicates from each sample.

Figure 2. Increased levels of anti-CD38 autoantibodies of IgG class (anti-CD38 IgG) in SLE

patients. **A,** Dot diagram of anti-CD38 IgG autoantibody levels in SLE patients (closed black

circles), Normal controls (open circles), SLE patients with SLEDAI = 0 (clinically quiescent)

(black squares), or with SLEDAI from 2 to 20 (clinically active) (open squares). The horizontal

line in each dot diagram denotes the mean value. Dashed line represents the cut-off value above

of which samples were considered positive for anti-CD38 IgG autoantibodies (ROC analysis). **B,**

Dot diagram of anti-CD38 IgM autoantibody levels in SLE patients (closed circles), Normal

controls (open circles), SLE patients with SLEDAI = 0 (black squares), or with SLEDAI from 2

to 20 (open squares). The horizontal line in each dot diagram denotes the mean value.

Figure 3. Distinct blood plasma cytokine profile in SLE patients. **A**, [Cytokine]/ [IFN-γ] Ratios

in SLE plasmas. Data represent the mean + SE of individual ratios obtained in each SLE patient

(n = 63). **B**, Cytokine profile in anti-CD38$^+$ SLE patients (■, n = 10) and Normal controls (□, n =

66). **C**, Cytokine profile in anti-CD38$^-$ SLE patients with circulating IL-10 concentration above

21 pg/ml (■, n = 9) and Normal controls (□, n = 66). **D**, Cytokine profile in anti-CD38$^-$ SLE

patients with circulating IL-10 concentration below 21 pg/ml (■, n = 47) and Normal controls (□,

n = 66). Column and error bars in B, C, and D panels represent the mean + SEM of the plasma

concentration (pg/ml) for each cytokine tested.

Figure 4. Increased CD38 expression in lymphoid cells from SLE patients. **A,** Dot diagram of

CD38 expression in CD3$^+$ T cells from SLE patients (closed circles), Normal controls (open circles), clinically quiescent SLE patients (black squares), and clinically active SLE patients (open squares). The dashed line represents the cut-off value above which T cells were considered CD38$^+$ (ROC analysis). Numbers on top of each dot diagram represent the number of individuals (%) that showed CD38$^+$ T cells. **B,** Dot plot of CD38 expression in CD3$^+$ T cells (X axis) versus anti-CD38 IgG levels (Y axis). The dashed lines represent the cut-off values above of which either CD38 expression in T cells (vertical), or anti-CD38 IgG autoantibodies in plasma (horizontal) were considered positive. **C,** Dot diagram of CD38 expression in CD19$^+$ B cells from the same groups as in **A**. The dashed line represents the cut-off value above of which B cells were considered CD38$^+$ (ROC analysis). **D,** Dot diagram of CD38 expression in CD56$^+$ NK cells from the same groups as in A. The dashed line represents the cut-off value above of which NK cells were considered CD38$^+$ (ROC analysis).

Figure 5. Altered phenotype of SLE T cells. **A**, Dot diagram of CD38 expression in CD4$^+$ T cells from SLE patients (closed circles), and Normal controls (open circles). **B**, Dot diagram of CD38 expression in CD8$^+$ T cells from SLE patients (closed circles), and Normal controls (open circles). **C**, Dot diagram of CD38 expression in CD25$^+$ T cells from SLE patients (closed circles), and Normal controls (open circles). **D**, Dot diagram of CD4:CD8 ratio from SLE^{low} patients (closed circles), Normal controls (open circles), and SLE^{high} patients (closed squares). **E**, Dot diagram of CD69 expression in lymphoid cells from PBMCs of SLE patients (closed circles), and Normal controls (open circles). The horizontal line in each dot diagram denotes the median. The *P* values were obtained using the Mann-Whitney *U* test.

Table 1. Increased cytokine plasma levels in SLE patients

| Cytokine | SLE (n = 63) | Controls (n = 67) | |
	Median[a] (IQR)[b]	Median[a] (IQR)[b]	P value[c]
IL-1β	0.25 (0.2-0.89)	0.21 (0.21-0.21)	0.3694
IL-2	**16.98 (1.86-35.04)**	1.05 (1.05-7.56)	<0.0001
IL-4	**0.59 (0.3-1.57)**	0.12 (0.12-0.25)	<0.0001
IL-5	1.42 (0.51-4.25)	2.02 (1.42-2.02)	0.8492
IL-6	**9.1 (1.5-33.79)**	0.82 (0.82-1.5)	<0.0001
IL-10	**5.35 (1.33-11.21)**	0.9 (0.9-4.29)	<0.0001
IL-12 (p70)	**0.93 (0.46-2.92)**	0.18 (0.16-1.1)	0.0004
IL-13	**2.2 (0.55-9.16)**	0.28 (0.28-2-2)	0.0005
IFN-γ	**1.51 (0.4-4.07)**	0.33 (0.33-0.78)	0.0010
TNF-α	**0.82 (0.31-2.37)**	0.14 (0.14-0.63)	<0.0001

[a] pg/ml.

[b] IQR, interquartile range.

[c] Mann-Whitney test.

Table 2. Analysis of the correlation of SLE anti-CD38 IgG autoantibodies with cytokine plasma levels

	Anti-CD38 IgG$^{+ a}$		Anti-CD38 IgG$^{- b}$	
Cytokine	Spearman r	P value^{c}	Spearman r	P value^{d}
IL-1β	0.3647	ns^{e}	0.07237	0.6066
IL-2	0.5897	ns	-0.03416	0.8081
IL-4	0.4377	ns	0.01900	0.8926
IL-5	0.5144	ns	-0.1321	0.3457
IL-6	0.6121	ns	-0.1832	0.1892
IL-10	**0.7939**	** ^{f}	-0.2074	0.1362
IL-12 (p70)	0.4255	ns	-0.04257	0.7621
IL-13	0.4012	ns	0.1654	0.2365
IFN-γ	**0.6505**	* ^{g}	-0.04066	0.7725
TNF-α	0.5758	ns	-0.01894	0.8929

^{a} n = 10 pairs

^{b} n = 53 pairs

^{c} P values were obtained using Spearman's correlation test. P value summary: Exact.

^{d} P values were obtained using Spearman's correlation test. P value summary: Gaussian approximation. P values > 0.05 were considered not significant.

^{e} ns: not significant. P values > 0.05.

^{f} * P values from 0.01 to 0.05 were considered significant.

^{f} ** P values from 0.001 to 0.01 were considered very significant.

Table 3. Analysis of the correlation of the percentage of SLE CD38$^+$CD3$^+$ T cells
with cytokine plasma levels

Cytokine[a]	CD38$^+$CD3$^+$ T cells[a]		
	Spearman r	*P* value[b]	*P* value summary[c]
IL-1β	**0.5012**	**0.0030**	∗∗[d]
IL-2	0.2717	0.1262	ns[e]
IL-4	**0.4889**	**0.0039**	∗∗
IL-5	0.2928	0.0982	ns
IL-6	0.3339	0.0575	ns
IL-10	**0.5173**	**0.0021**	∗∗
IL-12 (p70)	**0.5029**	**0.0029**	∗∗
IL-13	**0.4675**	**0.0061**	∗∗
IFN-γ	**0.4967**	**0.0033**	∗∗
TNF-α	**0.4908**	**0.0037**	∗∗

[a] n = 33 pairs

[b] *P* values were obtained using Spearman's correlation test.

[c] Gaussian approximation.

[d] ∗∗ *P* values from 0.001 to 0.01 were considered very significant.

[e] ns: not significant. *P* values > 0.05.

A

SYPRO Ruby

kDa | GST-CD38
188
— 110 kDa
— p85
62
— 56 kDa
49
38
GST
18

1 2 3

B Non-reducing conditions

WB: P23.2   H-170   # 77   Ponceau S

kDa
p85 —
62
56 kDa
49
38
28
18

— 110 kDa
— p85
— 56 kDa
— rCD38

1 2   3 4   5 6   7 8

C Reducing Conditions

WB: P23.2 Sypro

kDa
— 110 kDa
— p85
62 —
— 56 kDa
49 —

1   2

D Reducing conditions

WB: C25 C26 P43

kDa
62
49
38
28
18

— p85

1 2   3 4   5 6

E

ELISA

H-170, HB136, OKT10, OKT3, P43 SLE, P23 SLE, P13 SLE, C25 Normal, C26 Normal

Binding to GST-CD38 (O.D.U.)
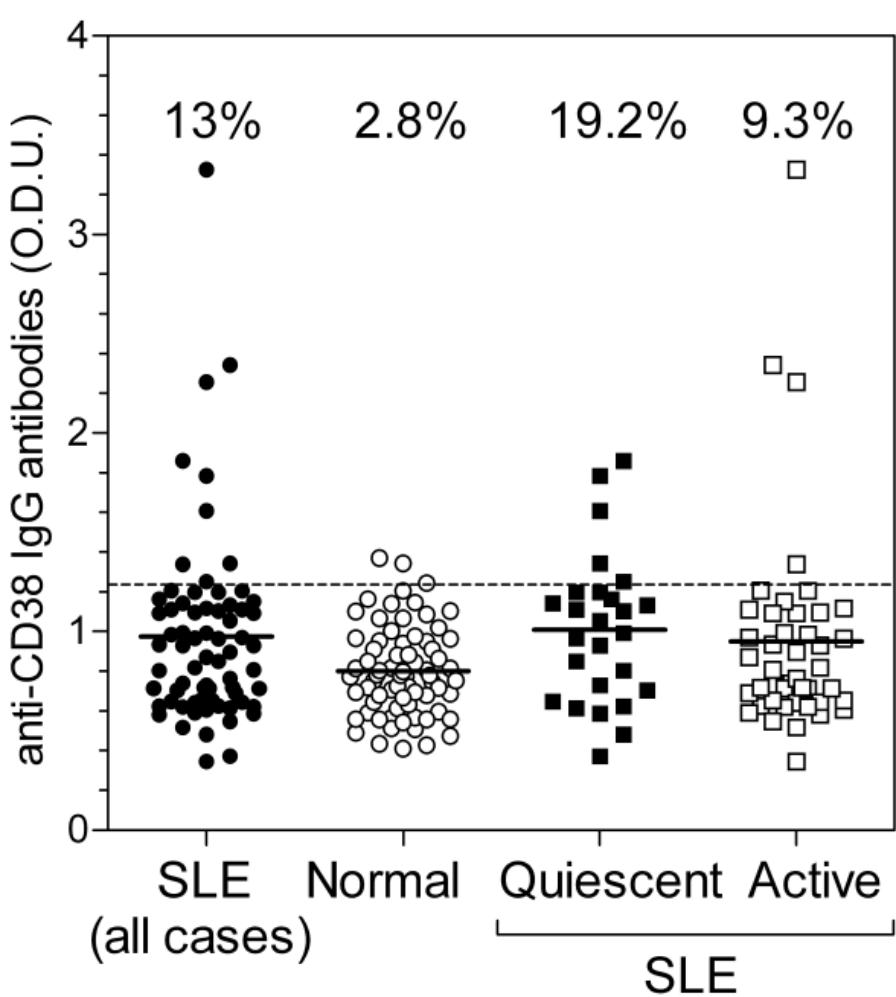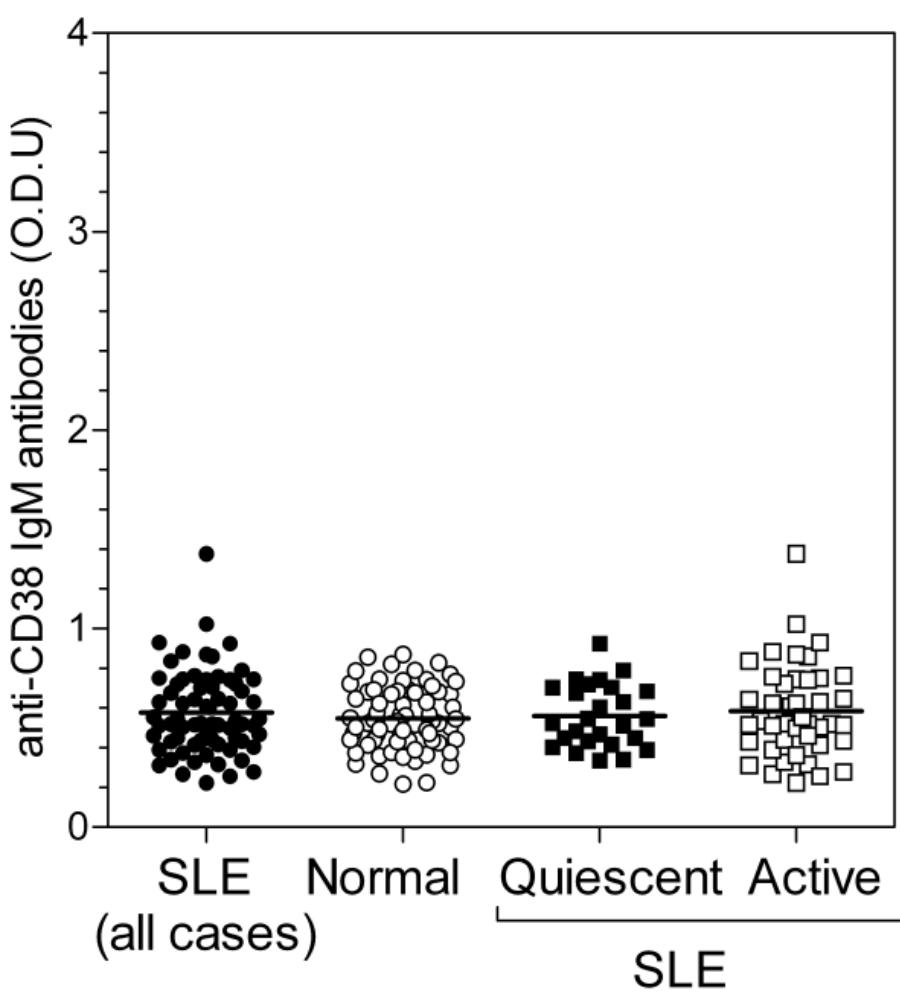
Figure 1., Pavón et al.,
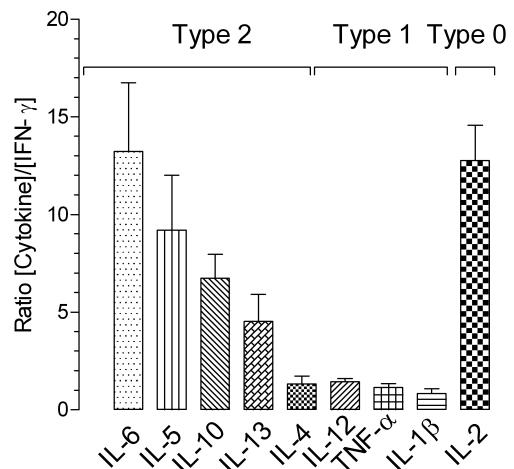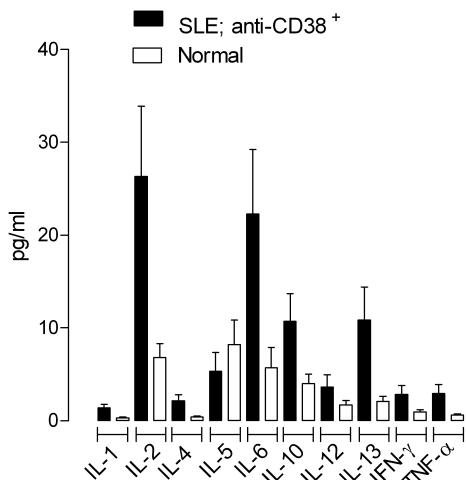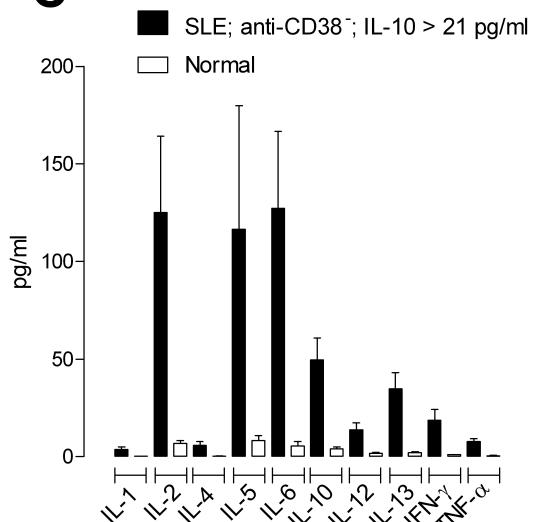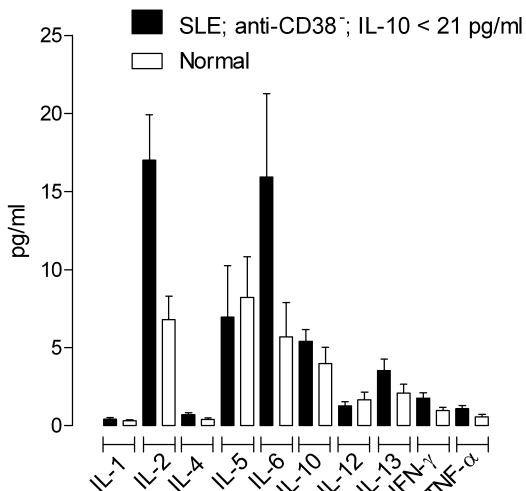
Figure 2. Pavon et al.,
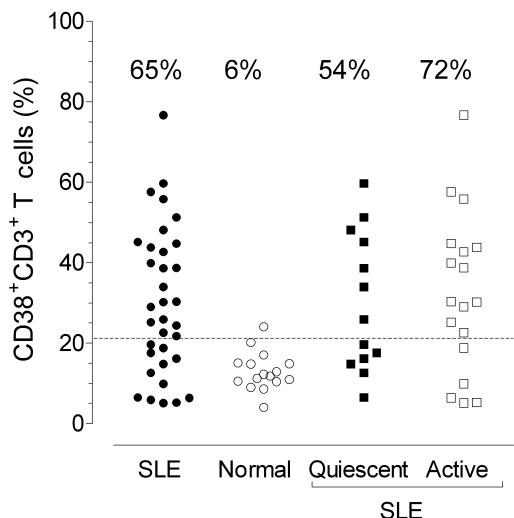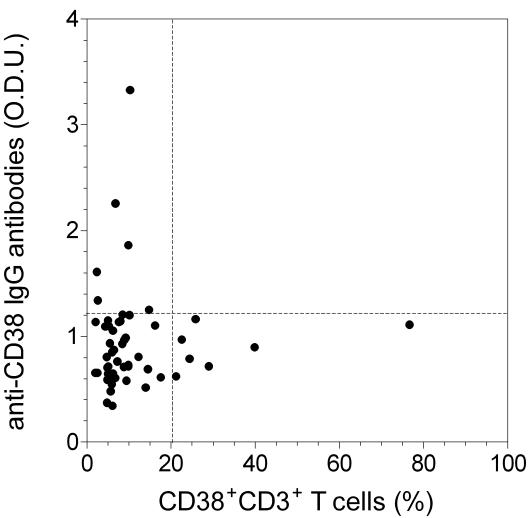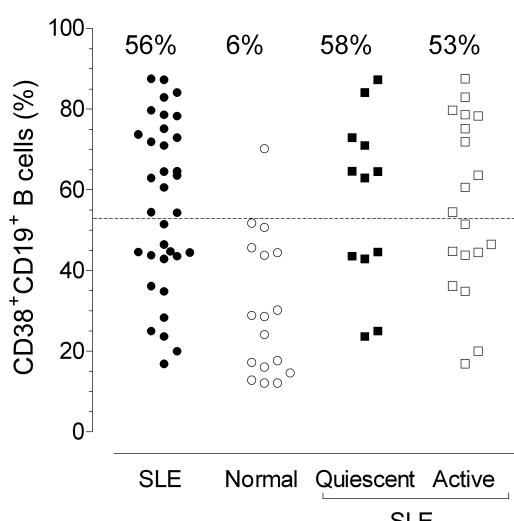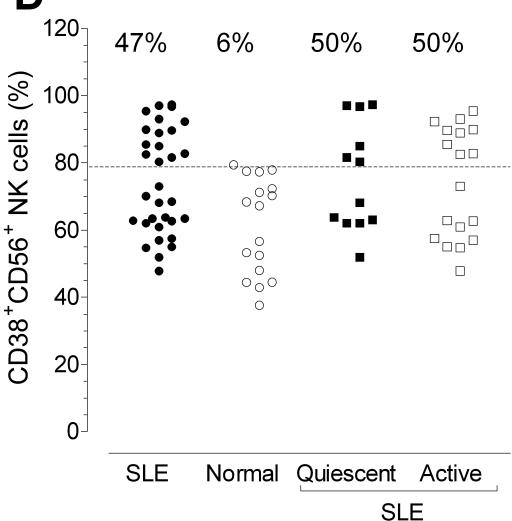
Figure 3. Pavon et al.
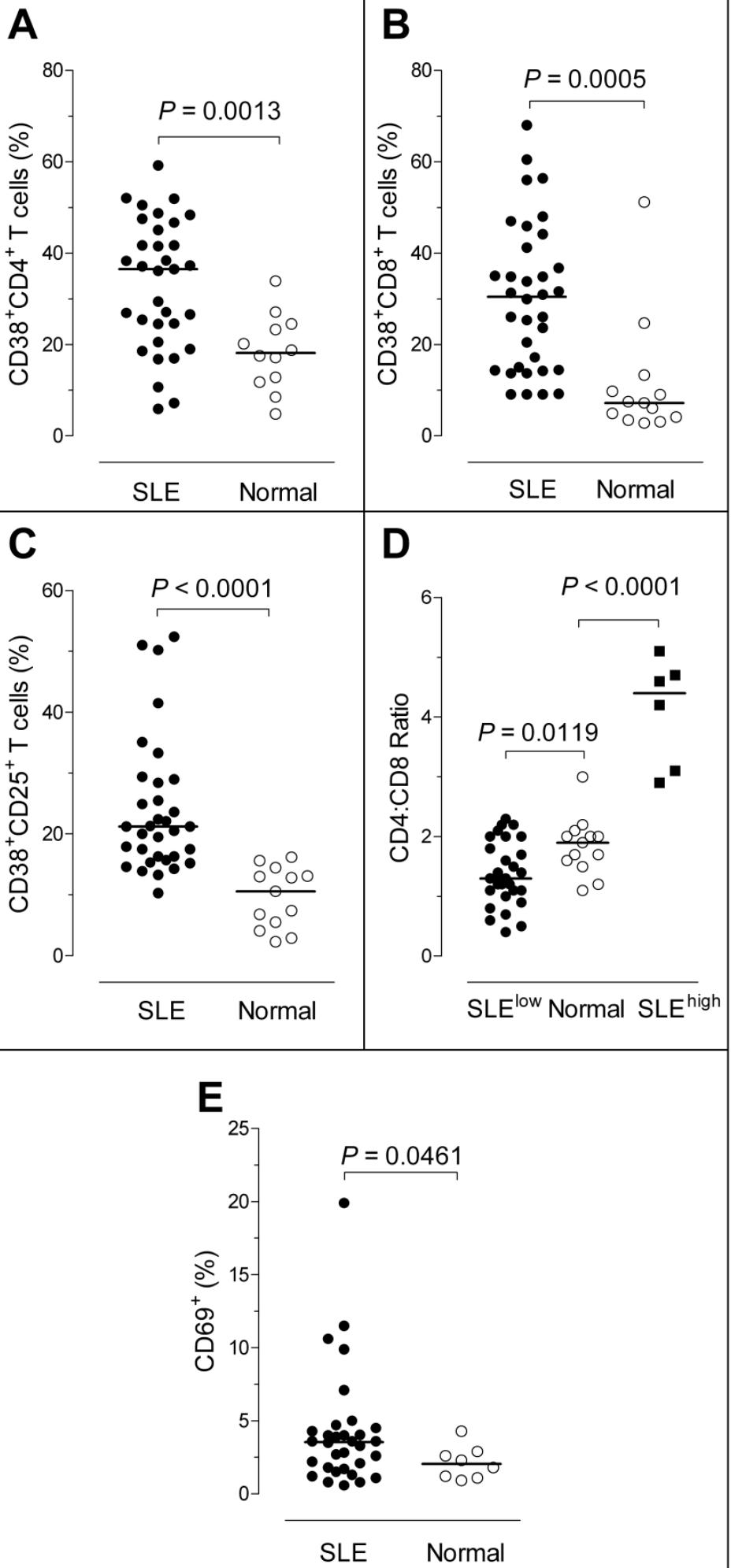
Figure 4. Pavon et al.,

Figure 5. Pavon et al.,

Clinical and immunological evaluation of a patient affected with a hemophagocytic lymphohistiocytosis associated with Leishmania infection.

1. Introduction

Hemophagocytic lymphohistiocytosis (HLH) is an unusual life-threatening immune disorder characterized by fever, splenomegaly, jaundice and constitutes a severe sepsis like illness with massive hypercytokinemia and with proliferating and organ-infiltrating phagocytes, causing cytopenia of different hematopoietic lineages [1, 2]. Historically HLH has been categorized clinically as primary or secondary. The primary form, known as familial hemophagocytic lymphohistiocytosis (FHLH), typically shows symptomatic presentation in infancy and has an autosomal-recessive inheritance. The secondary form is usually associated with a variety of infectious agents, neoplastic and autoimmune diseases and may become apparent at an older age. It has been frecuently, associated with viruses of the herpes group as Epstein-Barr virus (EBV) infection, however, others patogens has been related as CMV, parvovirus B19, *Mycobacterium tuberculosis*, *Salmonella* Typhi or *Leishmania* sp. Impaired function of natural killer (NK) cells and cytotoxic T-cells (CTL) is characteristic for both genetic and acquired forms of HLH [3]. In patients with FHLH, NK cell numbers are normal and the defect is usually persistent. Patients with acquired HLH may have low NK cell numbers and usually have decreased NK cell function with active disease. NK cell function usually reverts to normal after treatment. Malignant lymphomas, especially in adults, may be associated with HLH. A special form of HLH in rheumatic diseases is called macrophage-activation syndrome (MAS).

A young woman was admitted to the Rheumatology Department of Hospital Clínico (Granada) affected by HLA which was subsequently associated with *Leishmania* infection. Leishmaniasis is a vector-bone parasitic disease caused by protozoan of the genus *Leishmania*. *Leishmania* parasites exist as extracellular flagellated promastigotes within sandfly vectors and as intracellular amastigotes in infected mammals. Disease

manifestation depends on the infecting species and ranges from subclinical infections, to self-healing cutaneus lesions, to life-threating infections of visceral organs. Visceral (VL), which is caused by *L. donovani* and/or *L. chagasi/infantum*, is characterized by chronic parasitisation of the spleen, liver and bone marrow.

The aim of this study was to examinate the immunophenotype of lymphocytes, monocytes, and low density granulocytes (LDGs) subsets of a patient affected with hemophagocytic lymphohistiocytosis caused by *Leishmania* infection. Cytokines plasma levels were also measured using a multiparametric approach to test 10 different cytokines simultaneously.

2. Materials and Methods

Plasma and Cell Samples Collection

Blood was collected by the BD Vacutainer system into K2-EDTA tubes (BD Diagnostics, NJ) and plasma was separated from cells by density gradient centrifugation over HISTOPAQUE®-1077 (Sigma-Aldrich Química, Spain). Plasma was fractionated in aliquots and stored at -80ºC. Peripheral blood mononuclear cells (PBMCs) were isolated as described [4].

Plasma Cytokine Concentration

Cytokine plasma levels were tested with the Bio-Plex Precision Pro Human Cytokine 10-plex Panel (Bio-Rad Laboratories). The plasma factors included are pro -inflammatory cytokines: tumor necrosis factor α (TNFα), interleukin (IL) - IL-1β, IL-6; T_H1 cytokines: interferon γ (IFNγ), IL-12p70, IL-2; T_H2: IL-4, IL-5 and anti-inflammatory cytokines IL-10, IL-13. The samples were measured at a low PMT setting using directions provided in the instruction manual. The assays were performed using the Bio-Plex Pro II wash station with a magnetic plate carrier to minimize operator-related variations.

Cell Surface Marker Studies

PBMCs were examined for the expression of cell surface antigens using monoclonal antibodies for subpopulations of T cells, B cells, natural killer (NK) cells, monocytes and low density granulocytes: HIT3a (anti-CD3) and Tu 39 (anti-HLA-DR,Dp,DQ) were obtained from BD Pharmigen, 10.1 (anti-CD64) was obtained from BD Biosciences, HI 10a (anti-CD10), MCS-1 (anti-CD15), A3-B1 (anti-CD19), TP1/6 (anti-CD25), HP-4B3 (anti-CD69) were obtained from Immunostep, UCHM-1 (anti-CD14), UCHT-4 (anti-CD8) and Q4120 were obtained from Sigma and LT20 (anti-CD20) and IB6 (anti-CD38) were obtained from Miltenyi. Propidium iodide was used to stain death cells. Compensations settings were adjusted using single stained PBMCs. Isotype-matching labelled antibodies were used to calculate the non-specific staining. PMBCs were gated according with their forward and scatter characteristics. Immunofluorescence analysis was performed on a FACsCalibur flow cytometer (BD Biosciences, San Jose, CA), using the FlowJo (Tree Star, Inc. San Carlos, CA) software.

3. Results

Six consecutive blood samples from a patient diagnosed with hemophagocytic lymphohistiocytosis (HLH) were collected up to 20-months period. A healthy control sample was evaluated in parallel along the study. To avoid inter-control variability the same individual was evaluated except the first time. **Table 1** shows the treatment of the HLH patient over the 20 months.

TABLE 1.

Blood samples and treatment of a patient with hemophagocytic lymphohistiocytosis over the time

Number of blood sample	Date of blood sample (months after study opened)	Treatment	Healthy Control
1	09/02/2009 - study opened	Cyclosporine, prednisone, iv immunoglobulins	A
2	08/03/2010 (13m)	Cyclosporine, prednisone, etopoxide	B
3	01/06/2010 (16m)	Cyclosporine, prednisone, adalimumab	B
4	17/06/2010 (16.5m)	Cyclosporine, prednisone	B
5	12/07/2010 (17.5m)	Amphoterine B, prednisone	B
6	05/10/2010 (20m)	Tocilizumab	B

Table 1. Six consecutive blood samples from a patient diagnosed with a hemophagocytic syndrome were collected up to 20–months period. A healthy control was evaluated at the same time. The table shows the day of blood extraction as well as treatment at this point. Control A was tested at the beginning of the study and control B thereafter.

Plasma and peripheral blood mononuclear cells (PBMCs) were obtained after blood centrifugation over Histopaque®. Plasma samples were used to measure levels of cytokines and PBMCs were analyzed for antigen surface and mRNA was extracted to study the expression of some genes.

3.1. Increased IL-6 and IL-10 plasma levels in the HLH patient

Multiplex technology permits the determination of cytokines for a large panel of cytokines simultaneously with high sensitivity and using a small amount of sample. Cytokines were measured in plasma from a HLH patient and 2 healthy controls in six consecutive blood samples. Ten different cytokines were tested, including the pro-inflammatory cytokines; Type 1 and Type 2 cytokines and anti-inflammatory cytokines. Cytokine profiles and plasma concentration of all these cytokine are presented in **Table 2**. **Table 2.A** shows the data for the HLH patient and **Table 2.B** shows the data for the healthy controls. Plasma IL-6 and IL-10 concentration in the HLH patient is higher in all blood samples when compared with the controls.

	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IL-13	IFN-γ	TNF-α
HLH1	0.79	**5.31**	0	0	**181.7**	**112.49**	0	0	**1.07**	0.39
HLH2	1.72	**41.69**	2.84	6.19	**114.94**	**72.66**	3.47	5.2	**12.71**	2.29
HLH3	0.6	**0**	0.09	6.19	**151.38**	**143.51**	0.93	0	**4.49**	1.75
HLH4	0.6	**10.22**	0.35	0	**54**	**19.09**	1.81	1.62	**4.49**	0.39
HLH5	0.42	**0**	0	9.18	**169.57**	**21.28**	0	0	**6.16**	0.19
HLH6	0.97	**22.99**	0.88	0	**84.51**	**19.09**	2.65	3.86	**6.99**	0.8

Table 2.A. Levels of plasma citokines from a HLH patient in 6 consecutives blood samples.

	IL-1β	IL-2	IL-4	IL-5	IL-6	IL-10	IL-12	IL-13	IFN-γ	TNF-α
HC-A	OOR<	**3.76**	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<
HC-B.1	OOR<	OOR<	OOR<	OOR<	OOR<	1.82	OOR<	OOR<	OOR<	OOR<
HC-B.2	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<
HC-B.3	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<
HC-B.4	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<	OOR<
HC-B.5	0.07	OOR<	OOR<	OOR<	OOR<	0.42	OOR<	OOR<	OOR<	0

Table 2.B. Levels of plasma citokines from 2 healthy controls in consecutives blood samples.

3.2. Activated immunophenotype of low density granulocytes, monocytes, NKT and CD8+ T cells. Relative low number of NK and B cells.

PBMCs were isolated and immunophenotyped as described in material and methods. The most relevant and reliable results are the following:

a) The HLH patient shows a large population of circulating low density granulocytes with a subset of double positive for CD64 and CD10 expression.

In healthy controls peripheral blood mononuclear cells form a distinct layer at the plasma-Histopaque® interface upon centrifugation over Histopaque®, while erytrocytes and granulocytes go to the pellet. In contrast, low density granulocytes (LDG) were readily identified in the PBMC fraction from the HLH patient **(Figure 1.A, middle and right panel)**. The proportion of LDGs varied along the study with a maximum at the fourth extraction and decreasing thereafter.

To analyze the phenotype of the LDGs, CD15, CD10, CD64 and CD38 antigens were labelled with specific antibodies in the appropriate combination. CD10 expression appears late in granulocyte maturation, and CD64 is a marker of either immature (CD10neg) or activated mature granulocytes (CD10+), and it has been used as a surrogate marker of acute infection. The flow cytometry analysis of the HLH LDGs reveals several features: first, there were two LDG subpopulations according with their CD15 expression: CD15^{high} and CD15^{low} **(Figure 1.B, right panel)**; second, a subset of LDGs were double positive for CD10 and CD64, with a relatively stable expression along the study, although with a peak at the forth blood sample **(Figure 1.C, middle and left panels)**, suggesting the presence of a fraction of activated mature granulocytes; and third, LDGs were highly positive for CD38, with the highest expression upon the third blood sample **(Figure 1.D, left and right panels)**.
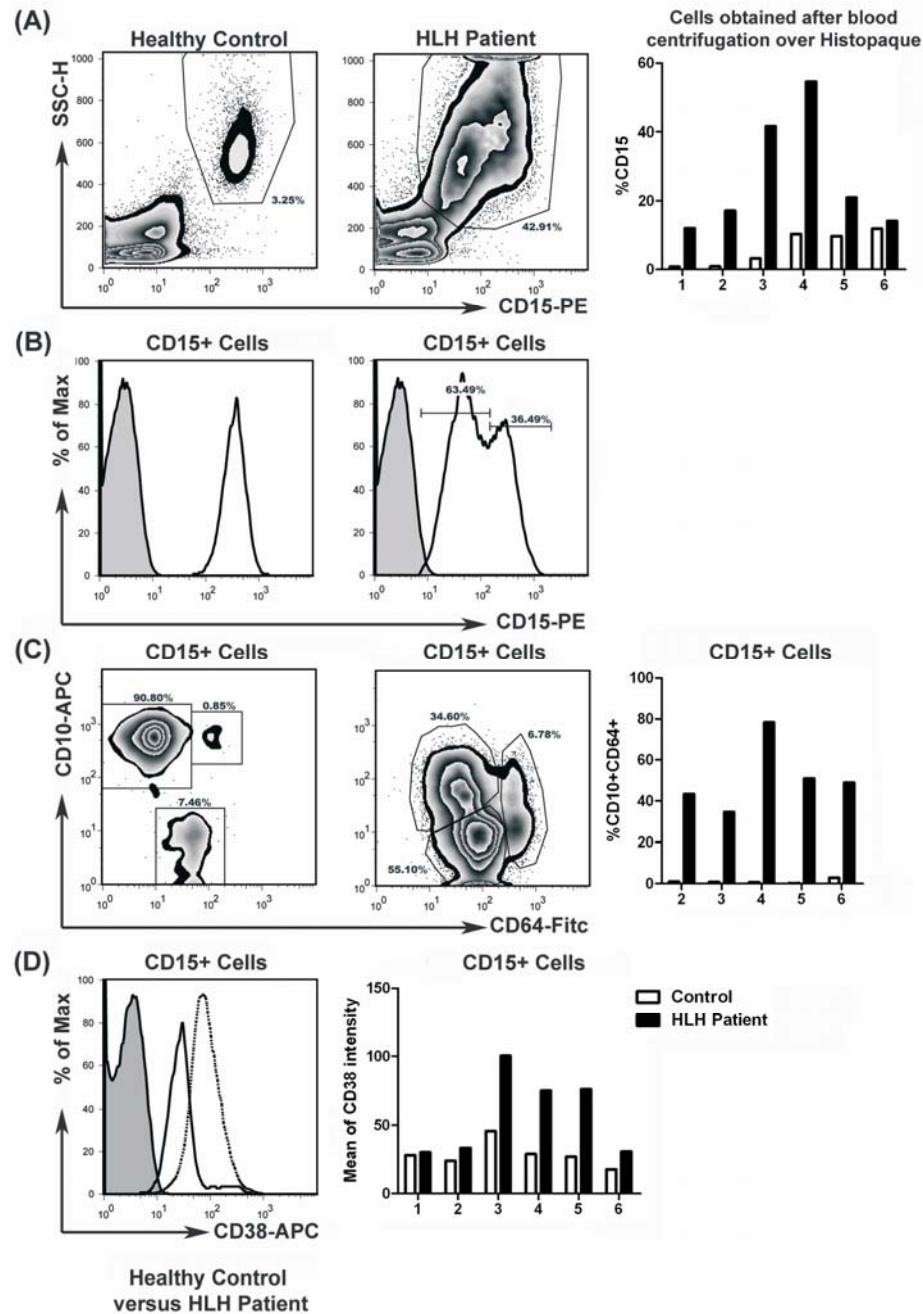
Figure 1. *Flow cytometry analysis of LDGs in PBMCs from the HLH patient.* **(A) Increased percentages of low density granulocytes:** Left and middle panels are representative SSC/CD15-PE dot plots of PBMCs obtained by density gradient centrifugation over HISTOPAQUE-1007 from a healthy control and HLH patient, respectively. The region where the low-density granulocytes are located (CD15$^+$ cells with increased side-scatter characteristics) and the percentages respect to the total number of PBMCs are shown. Right panel represents the percentages of low-density granulocytes in PBMCs obtained in six consecutive blood samples from the HLH patient (black histograms) and from the healthy control (white histograms) obtained in parallel. A higher percentage of low-density granulocytes was observed in most HLH patient samples than in healthy controls (right panel), with a maximum at the fourth blood sample and decreasing thereafter and coincidentally with the initiation of a treatment specific for *Leishmaniasis* **(B) Two subsets of low density granulocytes:** CD15 expression in LDGs from the LHL patient (right panel) and healthy control (left panel) is shown by means of histogram overlays: gray histogram for isotype control and white histogram for healthy control (left) and HLH patient (right). The HLH patient shows two subsets of granulocytes according to CD15 expression, CD15^{low} and CD15^{high}. **(C) High percentage of activated mature granulocytes:** Left and middle panels are representative two-color dot-plots for CD64 (x-axis) and CD10 (y-axis) double staining of gated low-density CD15$^+$ cells in PBMCs from a healthy control (left), and the HLH patient (middle). Regions and percentages of single (CD10$^+$, mature; or CD64$^+$, immature) and double-positive (CD10+CD64+, activated mature) cells are shown. Right panel are the data of the percentages of double-positive CD10$^+$CD64$^+$ cells on gated CD15$^+$ from five consecutive PBMCs samples of the HLH patient (solid histograms) and the healthy control (open histograms). **(D) Higher CD38 expression in low density granulocytes:** Left panel shows a representative histogram overlay for CD38 expression in the low-density CD15+ cell subpopulation of the HLH patient (dotted line), a healthy control (solid line), and an isotype control (gray histogram). Right panel shows the CD38 mean fluorescence intensity data in low-density granulocytes from six consecutive samples of the HLH patient (solid histograms), and the healthy controls (open histograms). A relatively higher CD38 expression was observed in the HLH low-density granulocytes than that in the healthy controls, in particular in the third, fifth and sixth blood samples. The dot plots and histograms correspond to the third blood sample.

b) The HLH patient shows a high percentage of circulating monocytes with increased CD64, CD38 and HLA-DR expression.

It has been suggested that the pathogenesis of lymphohistiocytosis is related with uncontrolled T-cell activation with secretion of large amounts of Th1 cytokines as IFN-γ and IL-2, which may further activate T-cells and monocytes/macrophages by increasing tumoricidal and microbicidal activities and other functions related to antigen presentation [5, 6].

PBMCs monocytes from a healthy control and a HLH patient were analyzed for the CD38, HLA-DR and CD64 expression. Monocytes are defined as CD14$^+$ population. In the HLH patient a higher percentage of monocytes were detected compared with the normal control **(Figure 2.A)**, and these cells presented a highly activated phenotype with increased CD38, HLA-DR and CD64 expression **(Figure 2.B(a)(b) and (c), third panes, respectively).**
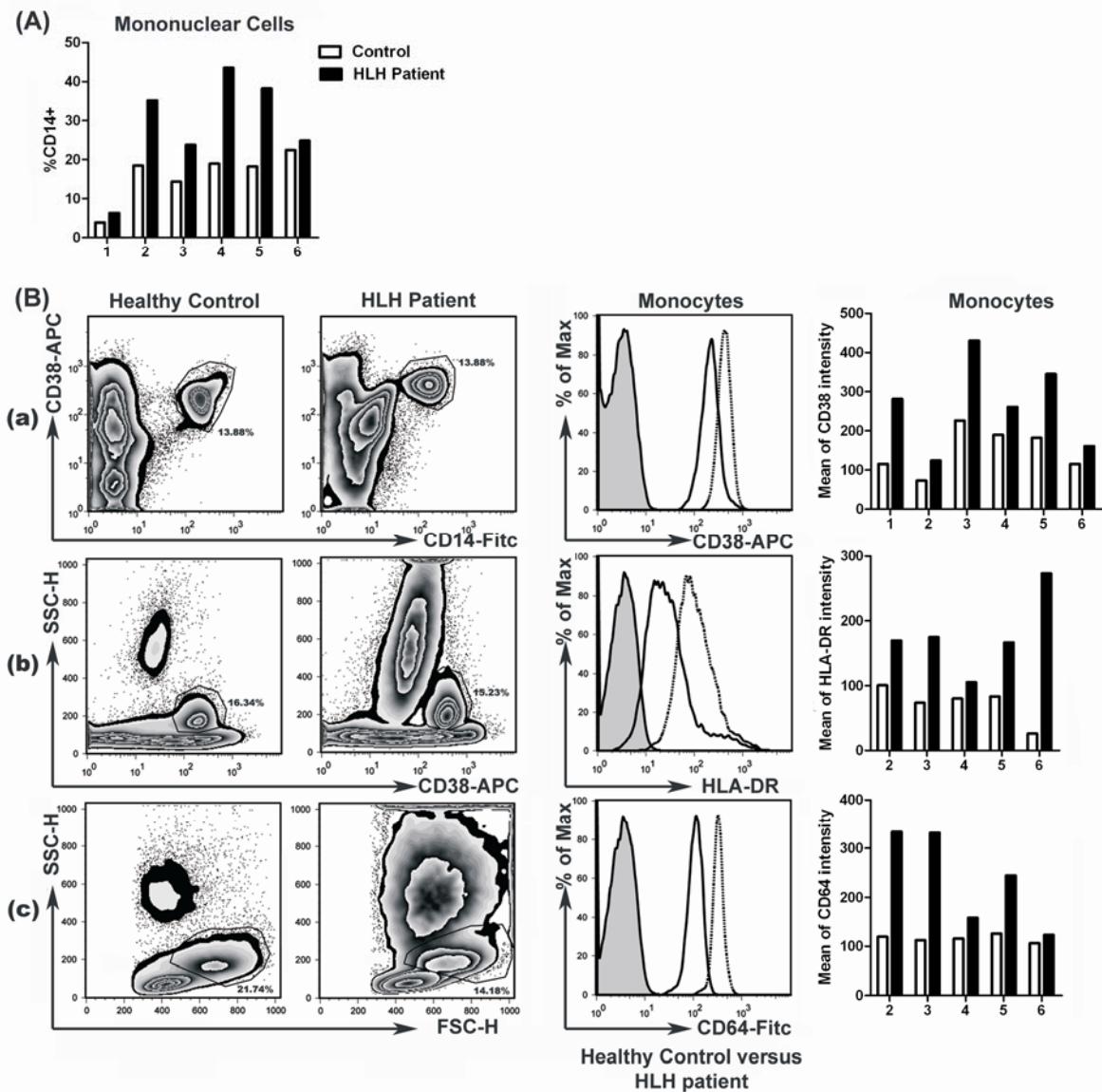
Figure 2. <u>*Flow cytometry analysis of monocytes from the HLH patient*</u>. **(A) Increased percentage of monocytes (CD14^{+}): :** Percentage of monocytes in gated genuine mononuclear cells (granulocytes were excluded by gating on forward and side scatter properties) in six blood extractions of a HLH patient (black histograms) compared with healthy controls (white histograms). An increased percentage of monocytes is observed in all the successive samples from the HLH patient, with the exception of the last one. **(B) Increased CD38, HLA-DR and CD64 expression in monocytes:** Immunophenotype of monocytes from the HLH patient was examinated and data is showed in **B(a)(b)** and **(c)** respectively. Monocytes were gated according forward and side scatter properties, CD14 and CD38 expression. First and second panel show a representative healthy control and a representative blood extraction of the HLH patient. CD38, HLA and CD64 expression in monocytes is shown in the third panel by means of histogram overlays labelled as follows: tinted line is used for isotype control, solid line is used for healthy control and dotted line is used for HLH patient. The last panel shows the mean intensity of CD38, HLA and CD64 in every blood extraction. A higher expression of CD38, HLA and CD64 is observed in the HLH patient, however, the CD38 and CD64 expression decreases in the last blood sample. The dot plots and histograms correspond to the third blood sample.

c) The HLH patient shows a low percentage of circulating NK cells, and increased CD38 expression in NKT cells.

The composition of peripheral CD56^{+} according to their expression of CD56, CD3 and CD38 antigens was investigated. Human CD56^{+} cells have been subdivided into CD3^{-}CD56^{+} (NKs) and CD3^{+}CD56^{+} (NKTs). The most intriguing defect is a decrease in the percentage of CD56^{+} cells in PBMCs **(Figure 3.A, third panel)** corresponding with a decrease in the NK cells percentage **(Figure 3.B, third panel)**. However, the percentage of NKTs is slightly lower **(Figure 3.B, fourth panel)**. CD38 expression was examined in NKs and NKTs, unlike NK cells, NKT cells are weakly positive for CD38. However, NKT cells from the HLH patient show a strong expression for CD38 (**Figure 3.B, second panel**).
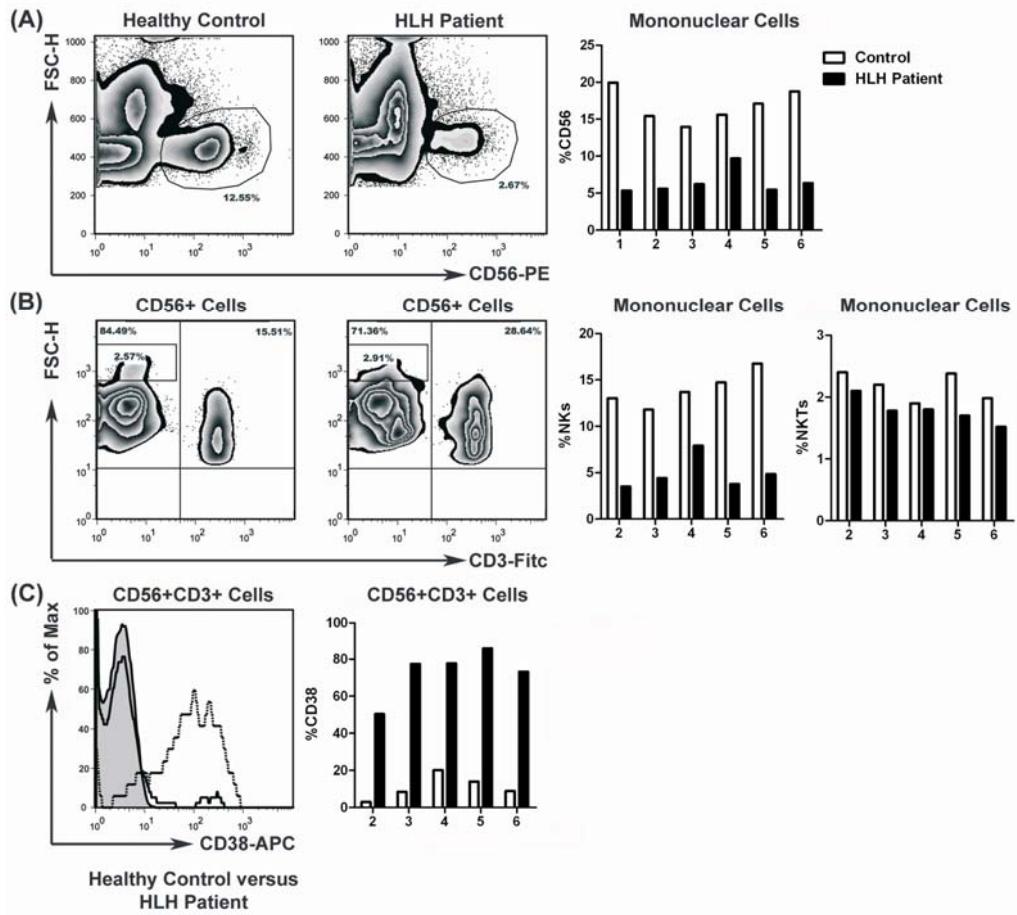
Figure 3. *Flow cytometry analysis of CD56+ cells from the HLH patient*. (A) Decreased percentages of CD56$^+$ cells: Left and middle panels are representative FSC/CD56 density-plots for CD56 expression in cells obtained after blood centrifugation over the Histopaque from a healthy control (left), and the HLH patient (right). The selected region and the percentages of CD56+ cells are shown. Right panel are the percentages of CD56$^+$ cells in gated mononuclear cells (granulocytes were previously excluded by gating on forward and side scatter characteristics) from six consecutive blood samples of the HLH patient (black histograms) and from healthy controls obtained in parallel (white histograms). **(B) Decreased percentages of NK cells (CD56$^+$):** First and second panels are representative two-color density-plots for CD3 (x-axis) and CD56 (y-axis) double-staining of gated CD3$^+$ cells from the HLH patient and a healthy control analyzed in parallel. The upper left quadrant corresponds to NK cells (CD56$^+$CD3$^-$), and the upper right quadrant corresponds to NKT cells (double-positive CD56$^+$CD3$^+$). The inset in the upper left quadrant corresponds to the CD56^{high} subpopulation of NK cells, which are likely cytokine-

producing regulatory NK cells. The percentages of the respective subsets are shown. Third and fourth panels are the percentages of NK cells (third panel), and NKT cells (fourth panel) in the six consecutive mononuclear fractions obtained from the HLH patient (solid histograms), or from the healthy controls (open histograms). **(C) Increased CD38 expression in NKT cells:** Left panel is the histogram overlay for CD38 expression in NKT cells. Gray histogram is for isotype control, solid line for healthy control and dotted line for the HLH patient. The panel on the right shows the percentage of CD38$^+$ cells in NKT cells subsets. The examples shown are from the third blood sample.

d) The HLH patient shows a low percentage of B cells with an increased subpopulation of plasmatic cells.

Freshly isolated PBMCs from a HLH patient and a healthy control were assessed for CD19, CD20 and CD38 expression by simultaneous staining with anti-CD19, anti-20 and anti-CD38 specific mAbs labelled with -PE, -Fitc and -APC, respectively. The percentage of B cells population in mononuclear cells is lower than healthy control **(Figure 4.A)**. Three distinct subsets of CD19$^+$ cells could be distinguish according with their CD38 expression levels: CD38-negative, CD38-low and, and CD38-high. The percentage of CD19$^+$CD38^{high} cell subset are shown in **Figure 4.B, left and middle panel**. CD19$^+$CD38^{high} subset is CD20-negative, corresponding to plasma cells. The percentage of plasma cells in CD19$^+$ gated cells is higher in the HLH patient than in the healthy control.
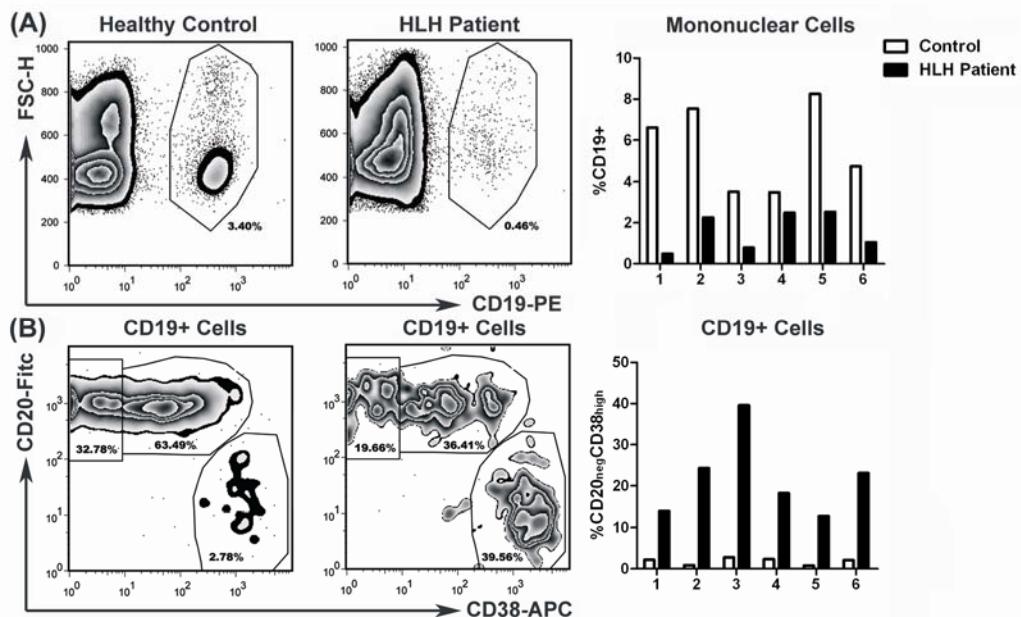
Figure 4. *Flow cytometry analysis of B cells from the HLH patient*. **(A) Decreased percentage of CD19+ B cells:** Left and middle panels are representative density plots for FSC/CD19 analysis in the cells obtained after blood centrifugation over Histopaque of a healthy control (left), and the HLH patient (right). Percentages of gated B cells (CD19^{+}) are shown. Right panel are the percentage of CD19^{+} in gated mononuclear cells (granulocytes were previously excluded by gating on forward and side scatter characteristics) from six consecutive blood samples from an HLH patient (black histograms) and from healthy controls obtained in parallel (white histograms). **(B) Increased percentage of CD20^{neg}CD38^{high} plasma cells:** Left and middle panels are representative two-color density-plots for CD38 (x-axis) and CD20 (y-axis) double staining of PBMCs (gated on CD19^{+} cells). Right panel represents the data on the percentages of the CD20^{neg}CD38^{high} cell subset, corresponding to plasma cells, along the six consecutive samples analyzed (solid histograms, HLH patient; open histograms, healthy controls analyzed in parallel). The examples shown are from the third blood sample.

e) The patient has a high CD38 expression in CD3+CD8+ population

The percentage of CD3$^+$ in gated mononuclear cells was very similar in the HLH patient compared to the healthy control **(Figure 5.A)**. CD38 expression was analyzed in CD3$^+$, CD3$^+$CD4$^+$, CD3$^+$CD8$^+$ and CD3$^+$CD25$^+$ subsets in the six consecutive blood samples **(Figure 5.B)**. Interestingly, there was a higher CD38 expression in CD3$^+$ cells as judged by the mean fluorescence intensity (MFI) values, which is likely due to an increased CD38 expression in the CD3$^+$CD8$^+$ subset and not in the CD3+CD4+ subset. Note, however, that CD38 expression normalized upon the initiation of the Tocilizumab treatment **(Figure 5.B(c), right panel)**.
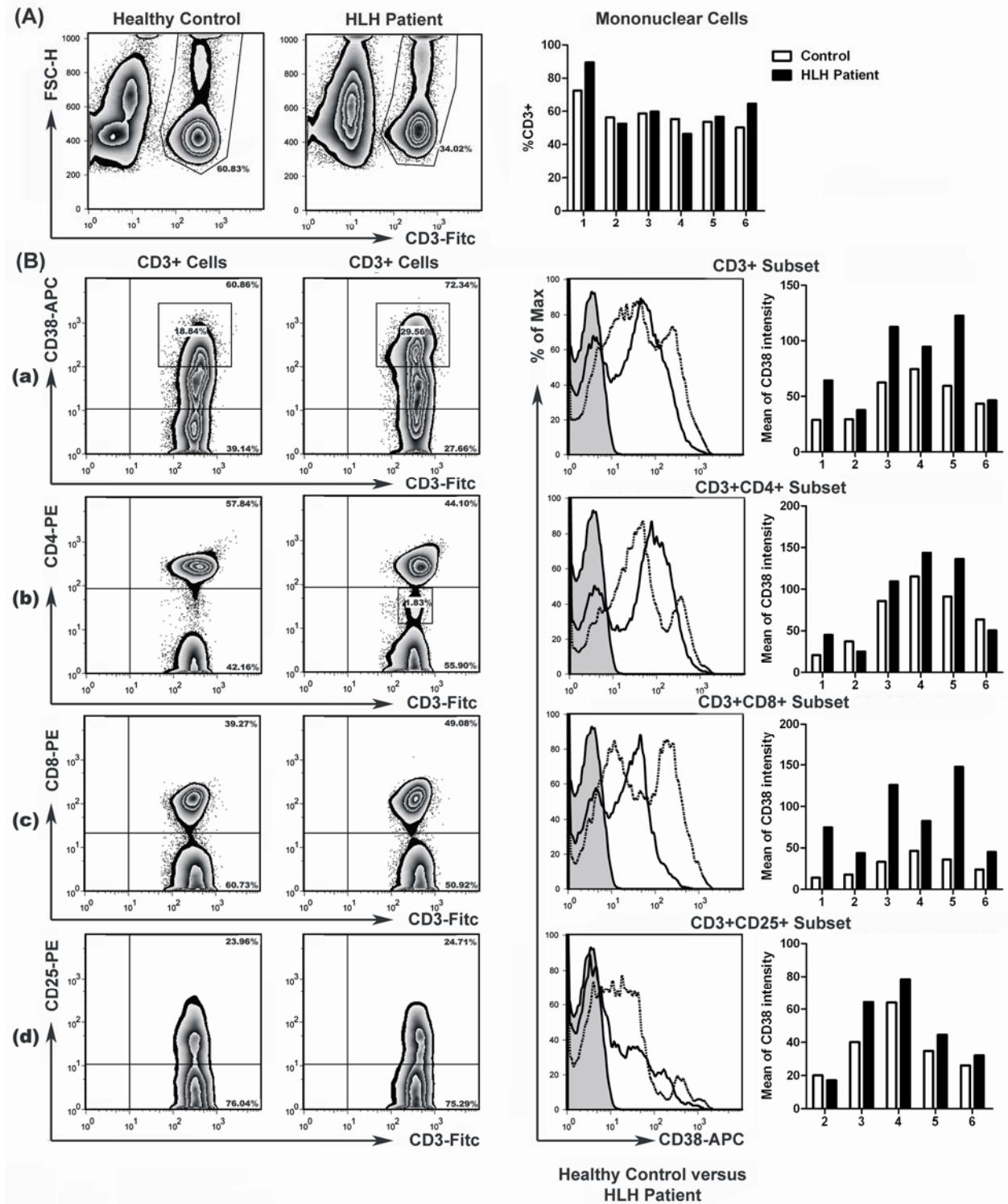
Figure 5. <u>*Flow cytometry analysis of T cells from the HLH patient.*</u> **(A) Same percentage of CD3+ T cells:** Left and middle panels are representative FSC/CD3 density plots for CD3 expression in cells obtained after blood centrifugation over Histopaque from a healthy control (left), and the HLH patient (right). The selected region and the percentage of CD3$^+$ cells are shown. Right panel are the percentages of CD3$^+$ in gated mononuclear cells (granulocytes were previously excluded by gating on forward and side scatter characteristics) from six consecutive blood samples of the HLH patient (black histograms) and from healthy controls obtained in parallel (white histograms). **(B) Increased percentage of CD38 in CD3$^+$ T cells and in CD3$^+$CD8$^+$ cells:** Cells were labelled with CD3-FITC, CD4-PE, CD8-PE or CD25-PE and CD38-APC and analyzed by flow cytometry. First and second panels are representative two-colour density plots for CD3 (x-axis) and CD38 **(a)**, CD4 **(b)**, CD8 **(c)** and CD25 **(d)** (y-axis) of gated CD3$^+$ population. Third panels are the histograms overlay for CD38 expression in CD3$^+$ **(a)**, CD3$^+$CD4$^+$ **(b)**, CD3$^+$CD8$^+$ **(c),** and CD3$^+$CD25$^+$ **(d)** subsets. Gray histogram is for isotype control, solid line for healthy control and dotted line for the HLH patient. The panels on the right show the mean of CD38 intensity in these cells populations in six consecutive blood samples (except for the CD3$^+$CD25$^+$ subset in the first blood sample, which was not performed).

4. Discussion

Levels of cytokines in plasma provide useful diagnostic and prognostic information about disease pathogenesis. Many studies have described cytokine abnormalities in hemophagocytic syndrome. In addition to high levels of interleukin-6 (IL-6), IL-8, interferon-γ (IFN-γ), IL-10, macrophage inflammatory protein-1α (MIP-1α), macrophage-colony-stimulating factor (M-CSF), and tumor necrosis factor-α (TNF-α), very high plasma concentrations of sCD25, sCD95-ligand [7, 8], and IL-18 [9] are characteristic of lymphohistiocytosis. The alpha chain of the soluble IL-2 receptor (sCD25) is a valuable disease marker because of constantly increased levels during active HLH. By contrast, the inflammatory marker IL-1β is not elevated [10], however it is controversial. The HLH patient shows increased IL-6 and IL-10 plasma levels in all blood samples compared to the control. Moreover, IL-2 and IFNγ are weakly higher in the HLH patients in some blood extraction.

The observation of high levels of IL-10 in plasma of the HLH patient leaded us to think the HLH was associated to a chronic infection, then it was confirmed. IL-10 has been implicated as a key regulator during infection with a variety of parasitic, bacterial, viral a fungal pathogens [11-13]. In experimental visceral leishmaniasis (VL) one of the strategies adopted by *Leishmania* to evade protective immune responses is the induction of immune suppressive IL-10 responses [14-17]. Although IL-10 responses are typically generated to balance excessive Th1 and CD8+T-cell responses and prevent immunopathology, overproduction of IL-10 has been shown to inhibit proinflammatory responses leading to susceptibility to infectious pathogens such as *Malaria* [18, 19], *Leishmania* [20, 21], lymphocytic chroriomeningitis virus (LCMV) [22, 23] and *Mycobacteria* spp [24].

Various studies have now shown that one of the major contributing factors to disease progression in leishmaniasis is indeed IL-10. IL-10 receptor blockade in mice infected with *L. donavani* nearly abrogates infection [25]; and *Il10*^{-/-} are highly resistant to VL [26]. Several cell populations have been shown to express IL-10 during Leishmania infections including, natural regulatory T cells (Tregs) [14], Th1 cells [15, 27], NK cells [28], macrophages [29], B cells [28] and DCs [30].

In humans, plasma levels of IL-10 are strongly linked to susceptibility to a variety of infectious diseases, such as malaria [31], leprosy [32] and HIV [33]. Persistent *L.donovani* infectious are also associated with increased levels of IL-10 in the serum of VL patients [34], however the cellular sources of IL-10 have been elusive. Recently, a study demostrated that individuals with VL display an accumulation of IL-10 mRNA in CD25 Foxp3 T cells within the spleen [35].

Another mechanism of evasion of host-protective immunity is the capacity of *L. donovani* to escape CD8+T-cell responses [16]. CD8+T cells are essential for the control of primary infections and [36-40] to the main mediators of resistance to rechallenge in the mouse model of VL [36]. Recently, it has been characterized the antigen-specific CD8+T-cell responses in *L. donovani*-infected mice using transgenic parasites expressing the model antigen ovalbumin [16]. This study shows that *L. donovani* evades CD8+T-cell responses by limiting clonal expansion and by inducing functional exhaustion during chronic disease. Reasons for limited expansion may be multifactorial, either resulting from a reduced capacity of dendritic cells to process and present antigen and/or due to a

suppressive environment. Recent studies have indicated that Leishmania may interfere with the antigen cross-presenting machinery of dendritic cells [41] and actively downregulate various signalling events in DCs [42], suggesting that infected DCs may not be able to prime effectively CD8+T cells and this limited expansion of antigen-specific CD8+ T cells could result from antigen paucity and from poor activation of antigen-presenting DCs. Following infection, IL-10 can act on antigen-bearing APC (antigen presenting cell) and naïve T cells to alter APC stimulatory activity and the activation and differentiation of T cells to alter APC stimulatory activity and the activation and differentiation of T cells. Following T cell priming, IL-10 might influence the properties and differentiation of developing effector and memory T cells, attenuating effector function and altering T cell memory formation. IL-10 may be produced directly by infected DCs, and/or by CD4+T cells (Tr1) as a consequence of their activation by DCs bearing the ability to drive IL-10 responses. IL10 maintains its own production by the establishment of a feedback loop. During virulent *L. major* infections, which are characterized by particularly strong inflammatory responses and high parasite burdens [15, 43], suggesting that development of IL-10-producing Th1 cells may be correlated with the extent of Th1 cell activation and/or the concentration of proinflammatory cytokines.

The recent observation that persistent viral infection can be resolved by neutralizing the effect of IL-10 *in vivo* suggest that successful treatment of other chronic infections also associated with high IL-10 production may be achieved using the same approach. Interestintly, IL-10 production was found to correlate with the pathology of the parasitic infection leishmaniasis in humans [34], and the associated loss of function of anti-parasitic T cells was restored *in vitro* by anti-IL-10 blocking mABs [44]. *In vivo*, a comparable therapeutic approach consisting of neutralizing IL-10 signalling has provided a sterile cure from leishmaniasis [45], as well as a number of intracelular bacterial infections [46].

One of the most intringuing data showed in the present study is the large population of low density granulocytes on PBMCs, characterized by 2 subpopulations of CD15^{hight} and CD15^{low} expression.

When the hematopoietic system is activated following infection, an inflammatory process or stress, immature leukocytes appear in the peripheral blood. The presence of

increased numbers of immature neutrophils (bands, metamyelocytes, and occasionally promyelocytes) in the circulation is called a "left shift" [47]. This will be represented by an increased number of non-segmented or band neutrophils in the bloodstream, and if the "left shift" is more severe, there can be matamyelocytes as well as myelocytes present. These immature granulocytes stay at the plasma-Histopaque® interface.

Surface neutrophils antigens undergo several changes during neutrophilic maturation to accommodate the cell´s function. Surface antigens may appear with neutrophilic maturation, such as CD16b, CD35, and CD10; disappear with maturation, such as CD49d and CD64 or be maintained during maturation, such as CD32, CD59, and CD82. In order to analyzed the phenotype of these low density granulocytes we label CD10 (a marker for mature neutrophils) and CD64 (a marker for immature neutrophils) antigens. However, CD64 is also up-regulated in activated neutrophils (see next paragraph). Unlike the healthy control, in which most of the LDGs are CD10+CD64-, and therefore considered mature neutrophils, the HLH LDGs show two distinct phenotypes, one subpopulation is CD10-CD64+ and may correspond to immature neutrophils, and the other one is CD10+CD64+ and may correspond to mature activated neutrophils. Moreover, these low density granulocytes are highly positive for CD38.

High affinity receptor for IgG (FcγRI/CD64) stands out as a particularly important molecule [48, 49], in a view of its role in mediating Ab-dependent cellular cytotoxicity and the release of several inflammatory mediators [50]. While FcRI/CD64 is expressed constitutively in mononuclear phagocytes, band and segmented neutrophils express only very low levels of CD69 antigen in the resting, non-activated state [51, 52]; upon neutrophils activation it is strongly upregulated by the proinflammatory cytokines IFNγ and granulocyte colony stimulating factor (G-CSF) which are produced during infections or exposure to endotoxin [53]. The Fc receptors on white blood cells are very important for effective phagocytosis and are up regulated during infection. *In vitro* stimulation with IL-15 [54], IFNγ [48, 49, 55-57] or G-CSF [58] produce upregulation of CD64 on the surface polimorphonuclear neutrophils [54]. Centenarians have increased CD64+ neutrophils, which may be related to higher level of interferon (INF)γ [59]. Some reports found that during pregnancy, neutrophil CD64 expression is higher compared with non-pregnant women [60-62], mimicking activation of the innate immune system. CD64 expression has been investigated to be used as a diagnostic marker of bacterial and viral infection, sepsis

and inflammation [63-65]. Quantitative measurement of CD64 can distinguish between systemic infection and the flare of autoimmune diseases [66]. Indeed, simultaneous quantitative analysis of CD64 and CD35 on neutrophils could potentially distinguish between inflammatory and infectious diseases [67]. It has been suggested that changes of neutrophils by circulating factors may facilitate their attachment to endothelium, which may be an important factor in the induction of organ dysfunction.

A remarkable increase in neutrophil CD64 expression in patients would be induce unusually high concentrations of circulating cytokines and can be used as a diagnostic indicator of neutrophil activation.

It has been suggested that the pathogenesis of lymphohistiocytosis is uncontrolled T-cell activation with secrete large amounts of Th1 cytokines as IFN-γ and IL-2, further activating T-cells and monocytes/macrophages by increasing tumoricidal and microbicidal activities and other functions related to antigen presentation [5, 6]. Given the state of activation of neutrophils in the HLH patient, the expression of activation markers CD64 and CD38, as well as HLA-DR was analyzed.

As granulocytes, monocytes also express CD64 and upregulate this receptor during activation. Monocytes from HLH patient shows higher CD64 expression. The longitudinal patterns of monocyte and neutrophil CD64 upregulation are strikingly similar, although the change in CD64 expression is considerably higher for neutrophils [68], as in our HLH patient. Some investigations have demonstrated that in human monocytes and murine macrophages, CD64 gene and surface expression can also be upregulated by IL-10, both *in vitro* and *in vivo* [69-71], surprisingly IL-10 fails to stimulate either the mRNA accumulation or the surface expression of CD64 in human neutrophils [72].

As CD64, the HLH patient shows a higher CD38 expression on monocytes. IFN-γ up-regulates constitutive CD38 expression on peripheral blood monocytes and monocytic cell lines. In addition, IL-2 treatment of monocytes increases CD38 expression although to a lesser extent than IFN-γ [73]. The enhancement of CD38 expression induced by IFN-γ to stimulate monocytes may affect other functional activities involving CD38, such as antigen presentation or T-cell activation. In addition, the effect of IFN-γ in the upregulation of CD38-CD31 interaction [73] may play a critical role during extravasation of monocytes from vessels in conditions such as bacterial infections or autoimmune diseases

characterized by the prevalence of Th1 cells [74, 75]. CD38 signals by using other molecules specialized in signalling. One candidate molecule associated with CD38 in monocytes is HLA class II, it has been shown that human CD38 may cooperate with MHC class II by acting as co-receptor in superantigen-induced activation [76]. A higher HLA-DR expression is observed in the HLH patient. Interestingly, CD38, human leukocyte antigen-DR (HLA-DR) and the tetraspanin CD9 are functionally and physically associated in lipid rafts microdomains and share a common pathway of tyrosin kinase activation in human monocytes [77]. In addition, the MHC classII/CD38/CD9 complex is implicated in antigen-presentation to T cells [77].

It has been observed a strong increase in the percentage of CD14+/CD16^{bright}/DR^{++} monocytes in hemophagocytic syndrome [78]. These proinflammatory monocytes show high expression of the CD16 and HLA-DR antigen, act as differentiated monocytes or tissue macrophages, showing increased migration into tissues [79]. These cells produce large amounts of the proinflammatory cytokines TNF-α and IL-1 [80] but little or no IL-10 [81]. Elevated serum cytokines together with increase CD14+/CD16^{bright} monocytes suggest that either CD14+/CD16^{bright}/DR^{++} monocytes produce cytokines or cytokines induce CD14+/CD16^{bright}/DR^{++} monocytes. Blumenstein et al. [82] reported TNF-α production before CD14+/CD16^{bright} monocyte population expansion in a sepsis patient, concluding that cytokines induced expansion of the CD14+/CD16^{bright}/DR^{++} monocyte subset in human septicemia. Glucocorticoid-treated multiple sclerosis patients showed depletion of CD14^{dim}/CD16^{bright}/DR^{++} monocytes after suppression of proinflammatory cytokine production, again suggesting that cytokines participate importantly in upregulating CD16 production on monocytes [83]. Feature of CD14+/CD16^{bright}/DR^{++} monocytes suggest important participation in the proinflammatory state, expanded numbers of CD14+/CD16^{bright}/DR^{++} monocytes in the blood can be considered a valuable excessive inflammation indicator that could help to evaluate the inflammatory state.

Human CD56^{+} cells, have been subdivided in 2 subsets, NK cells (CD56^{+}CD3^{-}) and NKT cells (CD56^{+}CD3^{+}). NK cells play a vital role in early pathogens containment and they are widely regarded as beneficial for host protection against intracellular pathogens. An examination of NKs from the HLH patient show a very low percentage in mononuclear cells gate. On the basis of the intensity of CD56 stainig, human NK cells have been

subdividided into two distinct subsets with distinct functional characteristics. CD56^{bright} NK cells have the ability to produce high levels of immunoregulatory cytokines, in particular interferon (IFN)-γ, but are in general poorly cytotoxic [84]. By contrast, CD56^{dim} NK cells produce relatively low levels of cytokines and are potent cytotoxic effector cells expressing high levels of perforin. An observation in detail of NKs from the HLH patient indicates that CD56^{bright} subset is not as well defined as in healthy controls. It has been previously described a lack of the circulating CD56^{bright} NK cells in patients with macrophage activation syndrome and juvenile rheumatoid arthritis [85]. The disappearance of CD56^{bright} NK cells from peripheral circulation is therefore unlikely to account for the defects in cytolytic activity of NK cells. In contrast, CD56^{bright} NK cells might have a function in regulating the CD56^{dim} perforin-^{bright} cells, and in this case their disappearance might have an effect on cytolytic activity. Alternatively, the apparent absence of immunoregulatory NK cells in peripheral circulation might reflect their active recruitment to sites of inflammation. The appearance of two subpopulations with CD56^{low} and CD56^{brigh} is another interesting feature of NK cells from the HLH patient. Moreover, NKT cells from the HLH patient are not diminished, however, unlike the healthy control, NK cells from the HLH patient are highly positive for CD38. NKT cells are unique extrathymically differentiated lymphocytes. They express both an NK receptor and TCR. CD56+ T cells mediate non-major histocompatibility complex-restricted cytotoxicity and are mostly located in the bone marrow and liver, where pathological changes frequently occur in HLH patients. Interestingly, it has been reported that invariant NK cells from HIV-1 or Mycobacterium tuberculosis infected patients express an activated phenotype with a significantly increased expression of activation markers as CD38.

The HLH patient shows a low percentage of B cells on gated mononuclear cells. Interestingly, low numbers of B cells have been reported previously [86]. However, the percentage of plasmatic cells in gated B cells is significantly higher in the HLH patient.

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Deficient activation of peripheral blood T Cells from Pemphigus patients upon stimulation with a mixture of superantigens

1. Introduction

Pemphigus is a life-threatening autoimmune blistering disease affecting the skin and mucous membranes. It is mediated by the production of pathogenic autoantibodies directed againt desmogleins (Dsg), adhesion molecules of the epidermis that are responsible for the cohesion between keratinocytes in skin and mucosa [1-3].

Two main classical subtypes of pemphigus have been described, pemphigus vulgaris (PV) and pemphigus foliaceus (PF), in which pathogenic autoantibodies are directed against desmosomal transmembrane glycoproteins belonging to the cadherin family, desmoglein 3 (Dsg3), and desmoglein 1 (Dsg1), respectively. Although the autoimmune response in the mucosal form of PV is directed against Dsg3, in the mucocutaneous form of the disease, both Dsg1 and Dsg3 are targeted by autoantibodies [4, 5]. The autoantibody responses are directed against conformational calcium-dependent epitopes of the Dsg extracellular domain, particularly the N-terminal adhesive region (extracellular 1 and 2 domains) [6]. Anti-Dsg autoantibodies, mainly IgG4 and IgG1, are pathogenic. Their in vivo binding to their targets after injection into normal mice leads to the loss of adhesion between keratinocytes and the formation of intraepidermal blisters [7-10].

Patients with severe pemphigus require long-term treatment with corticosteroids and other immunosuppresive drugs, which can lead to serious adverse events [11, 12].

T cells are clearly required for the production of autoantibodies in PV. However, the precise role of T cells in disease pathogenesis and evolution remains poorly understood. The purpose of this study was to determine whether T cells from PV patients were able to respond to superantigen stimulation in vitro. Superantigens are microbial or viral toxins that comprise a class of disease-associated, immunostimulatory molecules and act as Vβ-restricted extremely potent polyclonal T cell mitogens and induce massive cytokine production. They bind major histocompatibility complex (MHC) class-II molecules without

any prior processing and stimulate large number of T cells (up to 20% of all T cells) on the basis of epitope specified by this receptor. These properties are attributable to their unique ability to cross-link MHC class II and the T cell receptor (TCR), forming a trimolecular complex.

Superantigen-induced Tcell:Bcell conjugation was assessed in peripheral blood mononuclear cells (PBMCs) from pemphigus and healthy controls, moreover the capacity of lymphocyte activation and cytokines production were also evaluated upon stimulation with superantigen. Cytokine plasma levels were measured in patients with pemphigus and a healthy control group using a multiparametric approach to test 10 different cytokines simultaneously. Immunophenotyping of lymphocyte and monocyte subsets was performed by flow cytometry.

2. Materials and Methods

Healthy controls, patients and treatment

Plasma and cell samples collection

Blood was collected by the BD Vacutainer system into K2-EDTA tubes (BD Diagnostics, NJ) and plasma was separated from cells by density gradient centrifugation over HISTOPAQUE®-1077 (Sigma-Aldrich Química, Spain). Plasma was fractionated in aliquots and stored at -80ºC. Informed consent was obtained from each participant, and the study was approved by the Hospital Review Board and Ethics Committee of the Hospital San Cecilio (Granada, Spain).

FACS analysis

PBMCs were analyzed for surface expression of CD3, CD4, CD8, CD25, CD69, CD19, CD56, CD14, CD15 and CD38 using FITC-, PE- or APC-labeled specific monoclonal antibodies (mAbs) in the relevant combinations. Propidium iodide was used to stain death cells. Compensations settings were adjusted using single stained PBMCs. Isotype-matching labelled antibodies were used to calculate the non-specific staining. PBMCs were gated according with their forward and scatter characteristics.

Immunofluorescence analysis was performed on a FACsCalibur flow cytometer (BD Biosciences, San Jose, CA), using the FlowJo (Tree Star, Inc. San Carlos, CA) software.

Conjugate formation

PBMCs were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics at a concentration of 1×10^6 cells/0.1ml. Cells were stimulated by the addition of superantigens SEE (0.5 µg/ml) and SEB (0.5 µg/ml) obtained from Toxin Technology (Sarasota, FL), and incubated for 25 min at 37ºC and then centrifugated for 1 min. The cell pellet was incubated at 37ºC for further 5 minutes before resuspending (by manual shaking), and analyzing them immediately by flow cytometry.

Cell stimulation

PBMCs were resuspended in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics at a concentration of 1×10^6 cells/ml. Cells were then stimulated by the addition of SEE (0.5 µg/ml) and SEB (0.5 µg/ml) at 37ºC in a humidified atmosphere of 5% CO_2 for 18h. Then cells were centrifuged and washed a couple of times with PBS and analyzed by flow cytometry. The supernatant was centrifuged for 10min at high speed and fractionated in aliquots and stored at -20ºC for determination of cytokines.

Determination of cytokines production

Cytokines production was measured on plasma samples and cell supernatants after stimulation of PBMCs with superantigens using multiplex assay (Bio-Plex™, Bio-Rad Laboratories).

Statistical analysis

To compare sample groups statistical analysis were performed using the Mann-Whitney U test. *P* values less than 0.05 were considered significant. The tests were performed using the GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA).

3. Results

1. Pemhigus patients show a defective T cell:B cell conjugate formation upon superantigen stimulation.

T cell interacting with antigen-presenting cells (APCs) form a specialized contact interface called immunologic synapse (IS) [15]. This cell-cell contact area has 2 spatially segregated regions: the central supramolecular activation complex (c-AMAC; containing the T-cell receptor (TCR), costimulatory molecules, and signalling molecules), surrounded by the peripheral SMAC (p-SMAC; enriched in adhesion molecules). Previous to IS, the formation of a conjugate between the T cell and an APC takes place. The conjugate formation requires the activation of integrins on the T cell surface and remodeling of cytoskeletal elements at the cell-cell contact site via inside-out signaling. However, the early events in this signaling pathway are not well understood.

Using three colors flow cytometry analysis, we evaluated conjugate formation in PBMCs from **6** pemphigus patients and in **7** healthy controls. After PBMCs incubation with a mixture of the two superantigens, SEB and SEE, for 30 minutes at 37ºC, PBMCs were analyzed for surface expression of CD3, CD19 and CD38 by three-staining using FITC-, PE- and APC-labeled anti-CD3, anti-CD19, and anti-CD38 mAbs, respectively. Positive double staining for CD3 and CD19 indicated the percentage of conjugates in total PBMCs **(Figure 1.A)**.

The results are expressed as the fold increase in the number of conjugates in response to SEE/SEB over the spontaneous conjugates formation for total B cells subset. The data show that pemphigus patients generate a lower number of conjugates compared with healthy controls **(Figure 1.B)**.

FIGURE 3.A
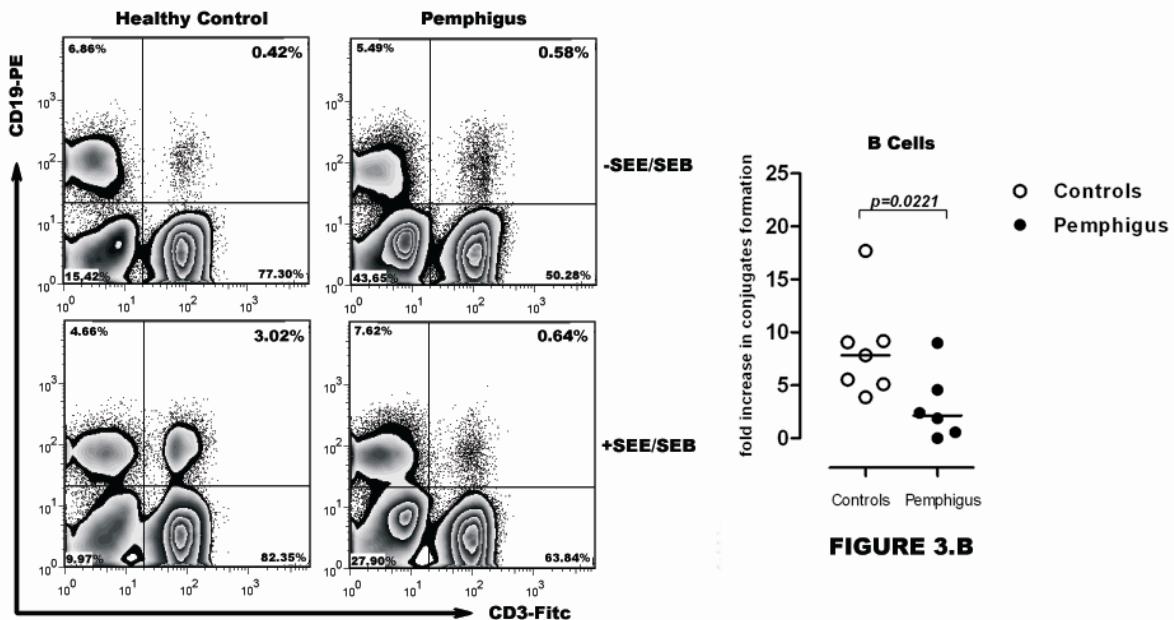
FIGURE 3.B

Figure 1. *Quantification of T:B conjugates by flow cytometry*. After pulsing or not PBMCs with a mixture of SEE and SEB, cells were pelleted and allowed to interact at 37ºC. Cells were then resuspended, labeled with CD3-FITC, CD19-PE, and CD38-APC and analyzed by flow cytometry. T cells were detected in the FL1 channel, B cells in the FL2 channel, and T:B cell conjugates were detected as dual color events in the upper right quadrant. A representative experiment of a pemphigus patient and a healthy control is shown **(Figure 1.A)**. The results are expressed as the fold increase in the number of conjugates in response to SEE/SEB over the spontaneous T:B conjugate formation in the absence of these superantigens **(Figure 1.B)** . Data from six experiments were collected and analyzed for statistical significance by Mann-Whitney U test. *P<0.05* was considered statistically significant.

2. PBMCs from pemphigus patients show a relatively low production of IL-2, IL-5, IL-10, IL-12 and IFNγ upon in vitro stimulation with a mixture of superantigens.

PBMCs from pemphigus patients and healthy controls were cultured in the presence and absence of a mixture of SEB and SEE. After 18h of incubation at 37ºC the supernatant from **5** pemphigus and **4** healthy controls were analyzed for cytokines production using a multiplex assay. The results in **Figure 2** show that after SEE/SEB stimulation, the IL-2. IL-5, IL-10, IL-12 and IFNγ level was significantly lower in the supernatant from pemphigus patients than in those from the healthy controls.
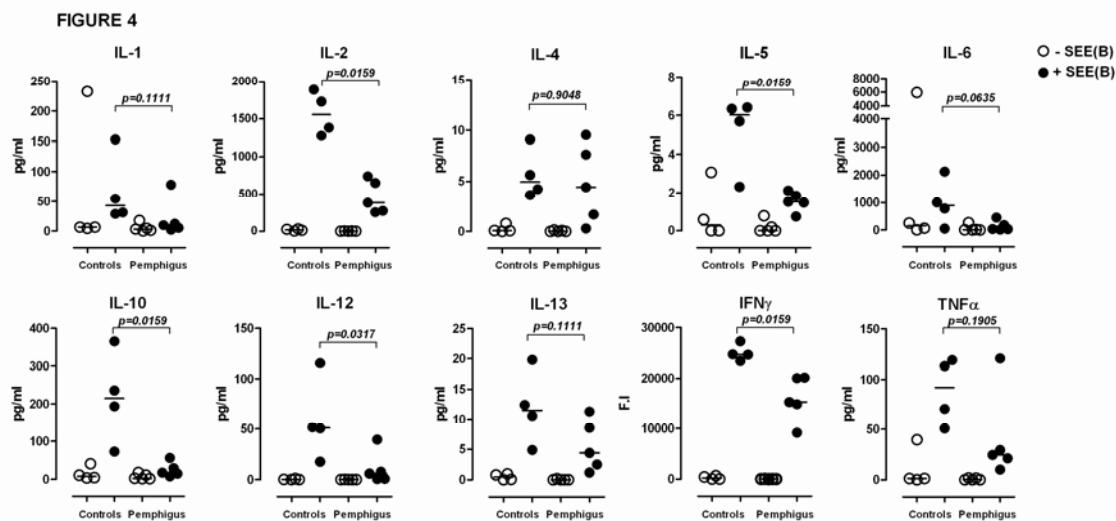

Figure 2. *Production of cytokines after stimulation with a mixture of SEE and SEB*. PBMCs from pemphigus patients and control subjects were cultured in the presence (black balls), or in the absence (white balls) of a mixture of the superantigens SEE and SEB for 18h. The cytokine levels were determined in the supernatants by a multiplex assay. The cytokine concentration (Y-axis) was expressed in picograms per milliliter with the exception of IFNγ that was expressed in fluorescence intensity units. Horizontal lines represent median values. The statistical significance between groups is indicated (*P<0.05*, Mann-Whitney U test).

3. In vitro stimulation of PBMCs with a mixture of superantigens from pemphigus patients shows a defective increase in CD25 expression in lymphocytes.

PBMCs from pemphigus patients and healthy controls were cultured in the presence and absence of a mixture of SEB and SEE. After 18h of incubation at 37ºC the cells from 5 pemphigus patients and 4 controls were analyzed for surface expression of the activation markers CD25, CD64 and CD38 in the lymphocyte cell subpopulation. The results show that lymphocytes from pemphigus patients have a significant lower increase in CD25. Increase CD64 expression after SEB/SEE was also lower in the pemphigus group, however, this did not reach stadistical significance. CD38 expression did not differ between pemphigus and healthy controls **(Figure 3)**.


7
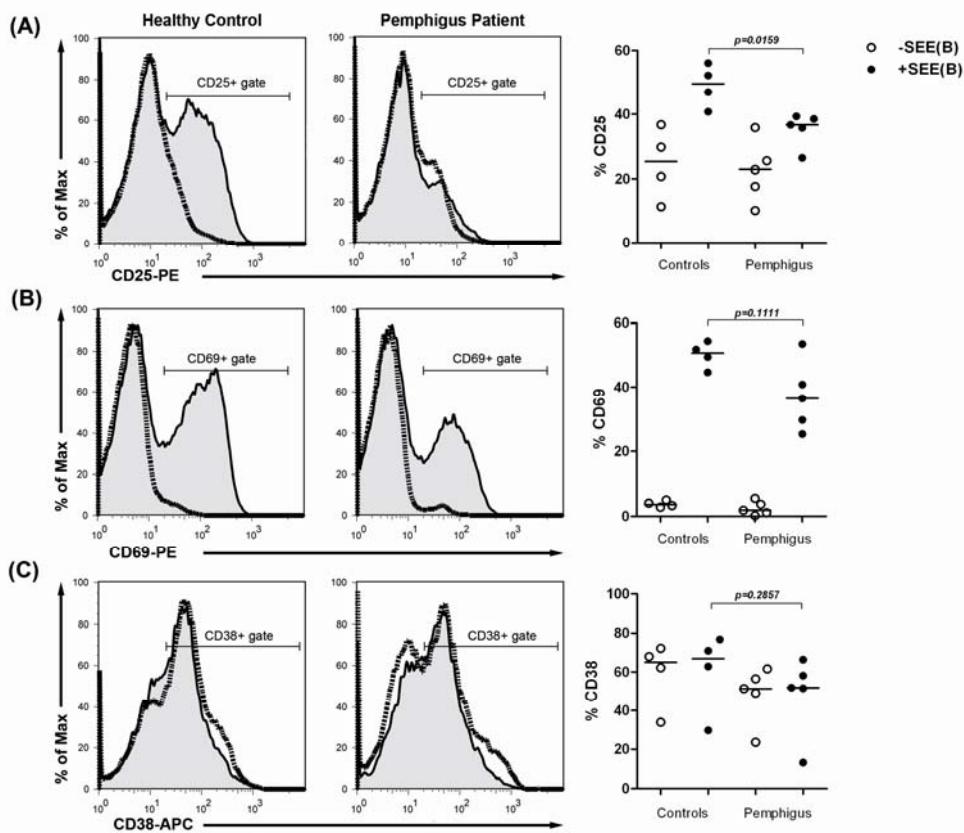
Figure 3. <u>Flow cytometry analysis of activation markers in the lymphocyte subset upon in vitro stimulation of PBMCs from pemphigus patients and healthy controls using a mixture of superantigens.</u> PBMCs derived from patients and healthy controls were *in vitro* stimulated with a mixture of two superantigens (SEE + SEB) for 18h, and then stained with fluorochrome-labeled mAbs specific for the activation markers CD25, CD69 and CD38. Surface expression of these markers was tested by FACS gating the lymphocyte cell subpopulation. For every activation marker analyzed, left and middle panels show a representative expression profile of a healthy control and a pemphigus patient, respectively. The histogram overlays are labeled as follows: tinted line is for lymphocytes stimulated with superantigen and dotted line is for lymphocytes without stimulation. Right panel show the activation markers analysis from 5 pemphigus patients and 4 controls. Black histograms are for SEE/SEB-stimulated cells and white histograms are used for non stimulated cells. The mean value for CD25 expression was significantly lower in pemphigus lymphoid cells than in healthy controls, whereas the differences were not statistically significant for the other two markers CD69 and CD38. Mann-Whitney U test was performed. $P < 0.05$ was considered significant.

4. Pemphigus patients show increased circulating IL-6 levels compared to healthy controls.

Levels of cytokines in plasma provide useful diagnostic and prognostic information about disease pathogenesis. Few studies have described cytokine abnormalities in pemphigus and they do not have measured more than 3 or 4 cytokines. Multiplex technology permits the determination of cytokines for a large panel of cytokines simultaneously with high sensitivity and using a small amount of sample. Cytokines were measured in plasma from **8** pemphigus patients and **16** healthy controls. A group of **8** patients diagnosticated with pemphigoid, another bullous skin disorder, was also analyzed. <u>In the pemphigoids, IgG autoantibodies against components of the dermoepidermal basement membrane such as bullous pemphigoid (BP) antigen 180 (BP180), BP antigen 230 (BP230) and laminin 5 interfere with the adhesion of basal epidermal keratinocytes to the dermoepidermal basement membrane zone.</u> Ten different cytokines were tested, including the pro -inflammatory cytokines: tumor necrosis factor α (TNFα), interleukin (IL)-1β, IL-6; Type 1 cytokines: interferon γ (IFNγ), IL-12p70, IL-2; Type 2: IL-4, IL-5 and anti-inflammatory cytokines IL-10, IL-13. Cytokine profiles and the median plasma concentration of all these cytokines are presented in **Figure 4**. Plasma IL-6 concentration in pemphigus and pemphigoid group was significantly higher when compared to the controls. Interestingly the pemphigus patient with very high level of IL-6

(shown in the *Figure 4* as an asterisk) also presents higher level of others cytokines as IL-1β, IL-10, IL-12 or IFN-γ. Moreover, such pemphigus patient died.


FIGURE 1
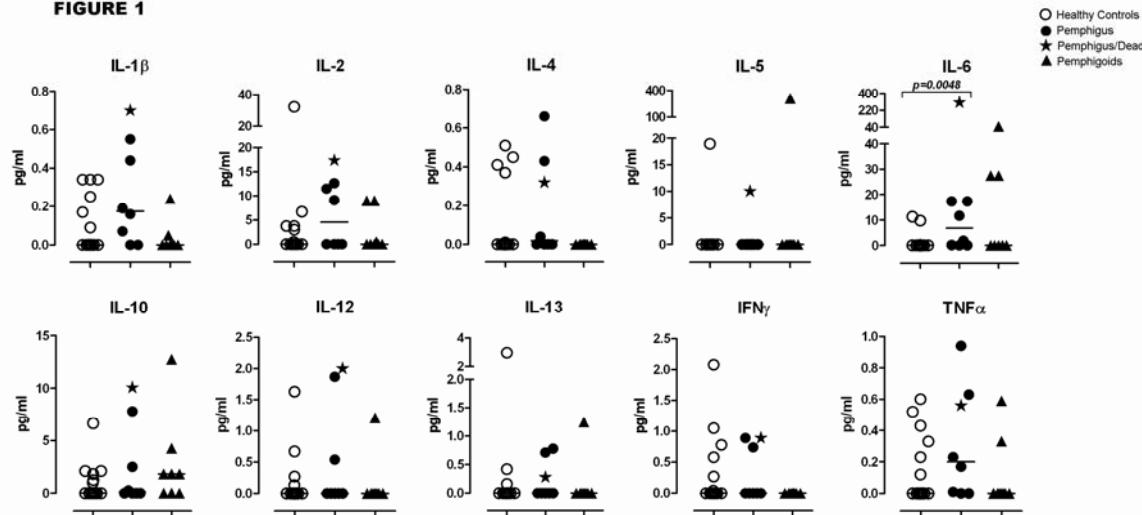
Figure 4. **_Analysis of cytokine plasma levels from pemphigus and pemphigoid patients and healthy controls._** Plasma concentration of 10 cytokines was measured in **8** patients with pemphigus (black balls), **8** patients with pemphigoids (black triangles) and **16** healthy controls (white balls) by using multiplex cytokine assay. A pemphigus patient with very high levels of IL-6 is shown with an asterisk. Horizontal bars denote the median values. Median plasma level of IL-6 was significantly higher in pemphigus patients *versus* healthy controls, Mann Whitney U test was used to compare healthy controls with patients. *P<0.05* was considered statistically significant.

5. Pemphigus patients show a significant increase in a CD19+ B cell subset with high CD38 expression, a lower percentage of CD56^{bright} on natural killer (NK) cells and a large proportion of low density granulocytes (LDG) in PBMCs.

The immunophenotypic characteristics of PBMCs from **8** pemphigus patients and **9** control subjects were determined by flow cytometry using appropriate lymphocyte-specific labeled monoclonal antibodies.

The most relevant and reliable results that we determined by flow cytometry are the following:

a) A low density granulocyte population in PBMCs.

Centrifugation over Histopaque® causes the mononuclear cells to form a distinct layer at the plasma-Histopaque interface, this means erytrocytes and granulocytes go to the pellet. When the hematopoietic system is activated following infection, an inflammatory process or stress, immature leukocytes appear in the peripheral blood. The presence of increased numbers of immature neutrophils (bands, metamyelocytes, and occasionally promyelocytes) in the circulation is called "left shift" [13]. This will be represented by an increased number of non-segmented or band neutrophils in the bloodstream, and if the "left shift" is more severe, there can be metamyelocytes as well as myelocytes present. Interestingly, a relatively large population of low density granulocytes was readily identified in the PBMCs fraction from pemphigus patients **(Figure 5.A)**.

b) A B cell subset with high CD38 expression.

Freshly isolated PBMCs from pemphigus patients and healthy controls were assessed for CD19 and CD38 expression by simultaneous staining with anti-CD19 and anti-CD38 specific mAbs labeled with -PE, and with -APC, respectively. Three distinct subsets of CD19$^+$ cells could be distinguished according with their CD38 expression levels: CD38-negative, CD38-low, and CD38-high**.** The percentages of CD19$^+$CD38^{high} cell subset are shown graphically in **Figure 5.B**. The data show that pemphigus patients had a higher percentage of CD19$^+$CD38^{high} than normal controls. This subset may correspond to plasma cells **(Figure 2.B)**.

c) A lower percentage of CD56^{bright} on NK cells.

FACs analysis of peripheral CD56$^+$ cells showed a lower frequency of the CD56^{bright} in pemphigus patients than in healthy controls **(Figure 2.C).** On basis of the intensity of CD56 staining, human NK cells have been subdivided into two distinct subsets with distinct functional characteristics. CD56^{bright} NK cells have the ability to produce high levels of immunoregulatory cytokines, in particular IFN-γ, but are in general poorly cytotoxic [14]. By contrast, CD56^{dim} NK cells produce relatively low levels of cytokines and are potent cytotoxic effector cells expressing high levels of perforin.
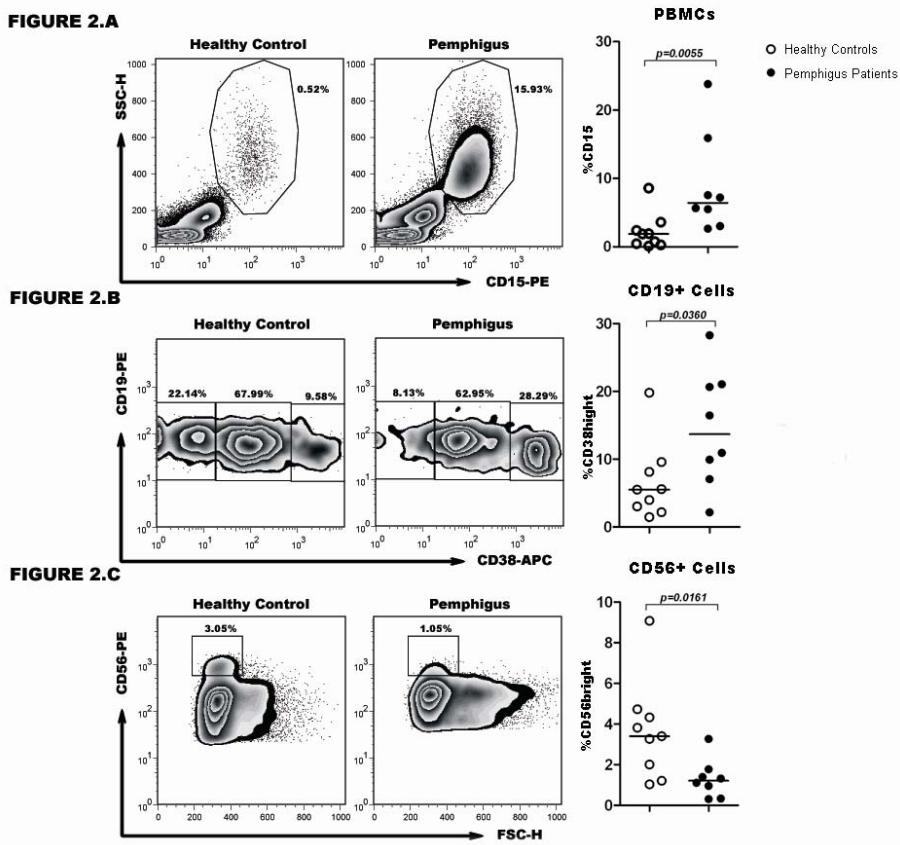
Figure 5. *Analysis of PBMCs from pemphigus patients and healthy controls by flow cytometry.* **(A)** Flow cytometry analysis of the mononuclear cell fraction from healthy controls and pemphigus patients shows a CD15^{+} subpopulation with increased side-scatter characteristics (SSC^{high}) (left and middle panels, respectively). The proportion of these low-density CD15+ cells was significantly higher in pemphigus patients than in healthy controls (right panel). **(B)** CD19^{+} gated peripheral blood B cells were examined by flow cytometry for CD38 expression. The dot-plot representation (left and middle panels) illustrates CD19^{+} versus CD38 expression (gated on CD19^{+} cells). The percentage of CD19^{+}CD38^{high} cell subset was significantly higher in pemphigus patients than in healthy controls (right panel). **(C)** Flow cytometry analysis of the CD56^{+} gated cells allowed the identification of two subpopulations: CD56^{bright} and CD56^{dim} (left and middle panels). A significant decreased proportion of circulating CD56^{bright} cells was shown in pemphigus patients as compared with that in normal healthy controls (right panel). In A, B and C, the left and middle panels show a representative healthy control (left panel) and pemphigus patient (middle panel), whereas the right panel shows the median of 8 pemphigus patients (black histograms) and 9 healthy controls (white histograms). Mann-Whitney U test was performed. *P<0.05* was considered statistically significant.

4. Discussion

An original observation from this work is a decrease in T:B cell conjugate formation in PBMCs from pemphigus patients pulsed with a mixture of superantigens compared with that in PBMCs from a healthy control group. Formation of stable adhesive interactions between B cells and T cells is an essential step that allows for bidirectional signal transfer (references), and LFA-1–ICAM interactions are known to be critical for B cell-T cell interaction (references). Another molecule that could mediate these T:B cell contacts is CD28. Thus, CD28 may function as an adhesion molecule, since anti-CD28 mAb affected T:B cell conjugate formation, and the impairment of T:B cell conjugates formation correlates with T cell surface expression of CD28 [27]. Moreover, T cells from CD28 knockout had a marked reduction in the number of stable conjugates [28]. Interestingly, it has been reported that the proportion of $CD8^+CD28^-$ T cells is significantly enhanced in pemphigus patients [26], and many of individuals who have elevated number of $CD28^-$ T cells have an autoimmune disease. Another possibility to take into account is the potential effect of immunosuppressive treatments, some drugs impairs integrin avidity and integrin-mediated signals leading to the abrogation of successful Tcell/APC interaction [29]. CD28 ligation in the presence of limited concentrations of anti-CD3 or antigen promotes T cell proliferation and IL-2 production by regulating IL-2 mRNA at the level of both transcription and translation [30, 31]. The data of this study show a significant low production of IL-2 by PBMCs from pemphigus after 18h of incubation with a mixture of two superantigens. Presumably, a lower number of conjugates in pemphigus patients is related with the unpaired production of IL-2 and other cytokines as IFN-γ, IL-5, IL-10 or IL-12 [32]. Co-stimulation via CD28 also mediates strong up-regulation of the IL-2 receptor α (CD25) [33] and β chains [34], interestingly a lower increase in CD25 expression after stimulation with a mixture of superantigens is shown in pemphigus patient.

In this study it is also observed the alteration in the plasma levels of IL-6 in patients affected with pemphigus as compared with healthy subjects. TNF-α, IL-1, and IL-6 sera concentration in PV patients has been analyzed in the active stage of the disease or in clinical remission by *J. Narbutt et al.* [16]. In the patients presenting the active stage of the disease or in clinical remission, only IL-6 serum concentration was significantly higher when compared to the control group, however, TNFα and IL-1 sera concentration were higher in the active stage of the disease. Likewise, in this study, a pemphigus patient showing a very active stage also show high levels of IL-6 and the other cytokines as IL-1β,

IL-10, IL-12 or TNFα. An elevated serum IL-6 levels has been observed in the majority of cases of a rare type of pemphigus, paraneoplastic pemphigus (PNP) [17]. In animal models of pemphigus vulgaris, deficiency of IL-1 or TNF-α partially protects from pemphigus IgG-induced blister formation [18]. Local production of proinflammatory cytokines are associated with endemic pemphigus foliaceus lesions [19] and overexpression of IL-1α and TNFα in lesional and perilesional areas have been confirmed by reverse transcriptase-polymerase chain reaction in pemphigus vulgaris. Also the role of cytokines IL-1 and TNFα have been confirmed *in vivo* using passive transfer studies [18].

Flow cytometry analysis shows a population of low density granulocytes (LDGs). Infection, an inflammatory process or stress can produce that immature leukocytes appear in the peripheral blood, this phenomenon is called "left shift" [13]. LDGs have been previously detected in patients with systemic lupus erythematosus (SLE) by *M.F. Denny et al*, however the phenotype and functional characteristics of these LDGs correspond to an activated phenotype. The low density of these neutrophils is probably caused by an extensive degranulation and release of proinflammatory cytokines. Thus, SLE LDGs have the capacity to synthesize and secrete high levels of type I IFNs, TNFα, and IFNγ. Moreover, they induce significant endothelial cell cytotoxicity but show impaired phagocytic potencial [20]. Further studies are needed to know whether the LDGs detected in PBMCs from pemphigus patients are either immature or activated mature granulocytes. FACs analysis of pemphigus PBMCs also showed a higher percentage of a B cell subset with high CD38 expression, which is like to correspond to antibody-producing plasma cells. Pemphigus pathogenesis is associated with autoantibodies against Dsg3 and Dsg1 and serum levels of anti-Dsg1 and anti-Dsg3 antibodies generally correlate with disease activity. In view of the key role played by autoAb in pemphigus, over the past few years rituximab has been used in cases of severe pemphigus with impressive results. The studies by *Mouquet et al.* and *Eming et al.* demostrate significantly decreased levels of anti-Dsg autoAb in most rituximab treated patients undergoing clinical improvement and increasing titers of anti-Dsg antibodies in relapsing patients [21, 22]. It is clear a correlation between clinical response and decreasing autoAb titers. A higher percentage of plasma cells could be related with antibodies production. Another interesting result from the flow cytometry analysis is the lower percentage of CD56^{bright} in CD56 gated cells. CD56^{bright} NK cells express the IL-2-receptor and have the ability to produce high levels of immunoregulatory cytokines, in particular interferon (IFN)-γ, but are in general poorly cytotoxic [14]. It has been previously described a lack of the circulating CD56^{bright} NK cells

in patients with macrophage activation syndrome and juvenile rheumatoid arthritis [23]. A decrease of immunoregulatory NK cells in peripheral circulation might reflect their active recruitment to sites of inflammation. The observation that CD56^{bright} NK cells and CD56^{dim} NK cells differ in their pattern of expression of chemokine receptors and adhesion molecules is consistent with the idea that the two subsets of cells home to different sites [24]. Moreover, it has been published that CD56^{bright} subset of NK cells is expanded at site of inflammation as synovial fluid of patients with rheumatoid arthritis and express more IFN-γ that peripheral NK cells [25].

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Anexo III: *Colaboraciones*

Role of murine CD38, TRPM2 and purinergic receptors in migration of immune cells

Role of murine CD38, TRPM2 and Purinergic Receptors in Migration of Immune Cells

1. Introduction

1.1 The ectoenzyme CD38 catalyzes the production of NAADP, cADPR and ADPR, calcium-mobilizing second messengers

1.2 CD38 acts at the intersection of innate and adaptative immunity

1.2.1 <u>CD38 as a regulator of innate immune responses</u>

a) CD38 regulates neutrophils migration and lung inflammatory responses
b) CD38 regulates dendritic cell trafficking *in vitro* and *in vivo*
c) CD38 regulates leukocyte trafficking by producing calcium mobilizing second messengers

      i.   CD38 regulate leukocyte trafficking by producing cADPR
      ii.   CD38 regulate leukocyte trafficking by producing ADPR

d) CD38 regulates leukocyte trafficking by an alterantive pathway of chemokine recptor signalling

e) CD38 regulates leukocyte trafficking by activating calcium influx through a plasma membrane channel

      i.   TRPM2
      ii.   Purinergic receptors

1.2.2 <u>CD38 regulates T cell- dependent immune responses</u>

2. Materials and Methods

Mice
Reagents and antibodies
Cell purification
In vitro generation of bone marrow-derived dentritic cells
Chemotaxis assays
RNA isolation and gene expression analyses
Stadistical analysis

3. Results

3.1 *In vitro* chemotaxis of mouse cells to chemokines that signal in a CD38-dependent manner is not TRPM2 dependent

a) *In vitro* chemotaxis of neutrophils to fMLP and IL-8 is not TRPM2 dependent.
b) *In vitro* chemotaxis of mature dendritic cells to ELC and immature DCs to SDF-1 is not TRPM2 dependent
c) *In vitro* chemotaxis of CD4$^+$ cells to SDF-1, ELC and SLC is not TRPM2 dependent
d) *In vitro* chemotaxis of B cells to SDF-1 is not TRPM2 dependent
e) *In vitro* chemotaxis of monocytes to MCP-1 is not CD38 and TRPM2 dependent

3.2 Bone marrow neutrophils show a large expression of P2Y$_{13}$ and P2X$_7$ receptors

3.3 Suramin inhibits *in vitro* chemotaxis of mouse bone marrow neutrophils to fMLP but not to IL-8

a) *In vitro* chemotaxis of mouse bone marrow neutrophils to fMLP is inhibited by suramin and CGS-15943
b) In *vitro* chemotaxis of bone marrow neutrophils to IL-8 is inhibited by CGS-15943 but not by suramin

4. Discussion

5. References

Role of murine CD38, TRPM2 and Purinergic Receptors in Migration of Immune Cells

Kinases, phosphatases and others intracellular enzymes play critical roles in regulating the development, activation, differentiation and survival of lymphocytes [1]. However, cells of the immune system also express many membrane-associated ecto-enzymes with important immune cell functions. Ecto-enzymes have their catalytic sites directed towards the extracellular space, so these enzymes utilize substrates that are found in the extracellular milieu. Some of these enzymes, such as CD73, CD38, CD39, ART2, and PC-1, utilize nucleotides as substrates. These nucleotide-utilizing enzymes might be envolved in salvaging purines [2] or in generating products such ATP, ADP, and adenosine that function as signalling molecules for purinergic receptors [3]. Many of these enzymes play very important roles in regulating the survival, activation, and effector function of leukocytes [4].

This work I explain below has been developed during my visit to Frances E. Lund´s lab. Dr. Lund is an expert on signals that determine how and when inflammatory cells go to sites of infection and tissue damage. She is interested in the function of the ectoenzyme CD38 and the metabolites produced by its enzymatic activities because this molecule regulates immune responses by controlling the trafficking of neutrophils, monocytes, dendritic cells and T cells to sites of inflamation and infection. Dr. Lund´s lab is focused on learning the molecular mechanism by which CD38 regulates chemokine receptor signalling and finally controls cell migration. Dr. Lund is looking at the possibility of targeting CD38 to control several chemokines simultaneously. The work is relevant to broad array of infections and autoimmune diseases.

1.Introduction

1.1 The ectoenzyme CD38 catalyzes the production of NAADP, cADPR and ADPR, calcium-mobilizing second messengers.

CD38 is a multifunctional enzyme widely expressed in hematopoietic and non-hematopoietic tissues [5, 6]. CD38 uses β-NAD(P)$^+$ as a substrate to catalyze the production of a number of different products including nicotinamide, nicotinic acid-adenine dinucleotide phosphate (NAADP), ADP-ribose (ADPR) and cyclic ADP-ribose (cADPR) [7-9] (**Figure 1**). All three products of the CD38 enzyme reaction function as signalling molecules either by mobilizing calcium from intracellular stores or by activating calcium entry from the extracellular space [10-14].
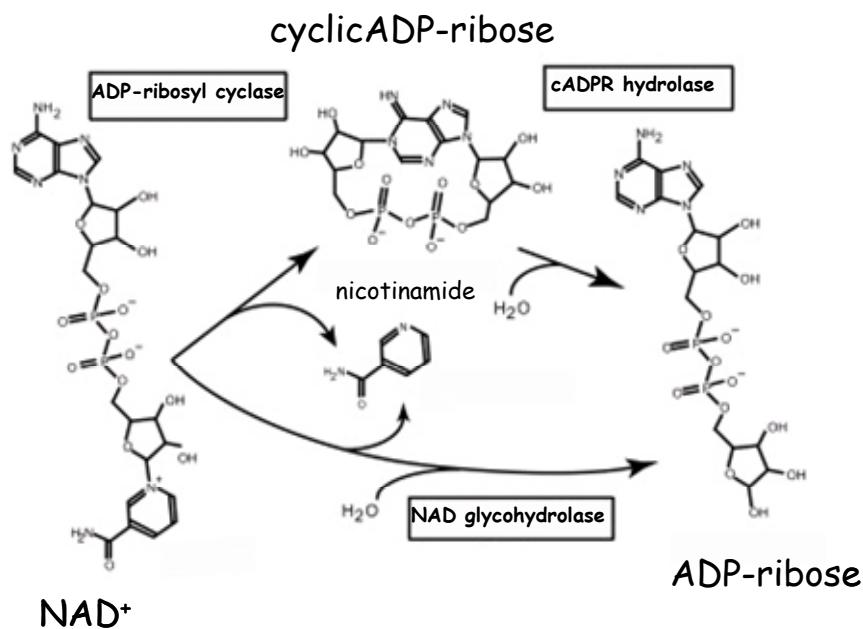

Figure 1: The three ectoenzyme activities, NAD glycohydrolase (NADase), ADP-ribosyl cyclase (ADPRC) and cADPR hydrolase.

- **cADPR**: Initially, most research focused on the production of cADPR, given its central role in cell physiology. cADPR induces intracellular Ca^{2+} release from ryanodine receptor (RyR)-dependent Ca^{2+} stores [15-17] (**Figure 2**). Interestingly, the cADPR-triggered, RyR-gated stores are found in a number of different mammalian cell types including smooth muscle and neuronal cells. RyR-gated stores are spatially, functionally and pharmacologically distinct from the calcium stores controlled by inositol triphosphate (IP3), indicating that cADPR mobilizes intracellular calcium in an IP3-independent way [18].
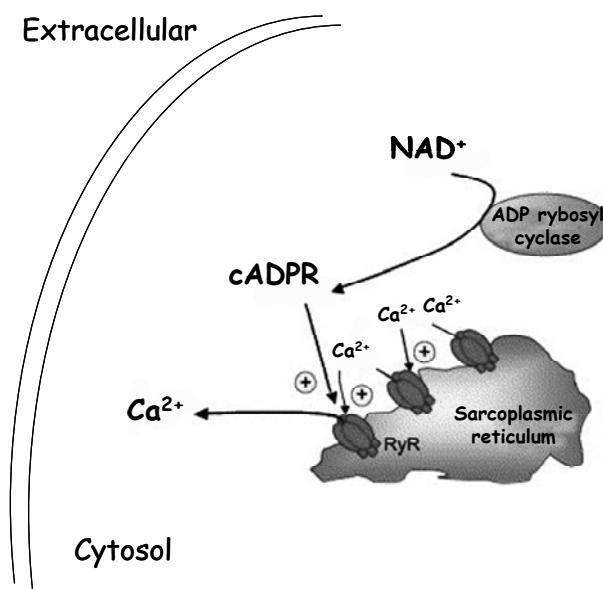

Figure 2: cADPR induces intrcellular Ca^{2+} release from ryanodine receptor (RyR)-dependent Ca^{2+} stores.

cADPR is known to induce intracellular Ca^{2+} release in a variety of cells types isolated from plants, animals and protists [19]. cADPR also has the ability to induce Ca^{2+} entry, as it was shown that microinjection with cADPR induces Ca^{2+} influx in Jurkat T cells [20]. CD38 is a very inefficient cyclase, with cADPR representing only 1-3% of the final product and ADPR accounting for the rest [7], but studies from CD38 deficient mice *(CD38$^{-/-}$)* have suggested that CD38 and this small amount of cADPR is biologically relevant [7] and may play an important role in regulating insulin secretion [21], muscarinic receptor-mediated Ca^{2+} signalling in pancreatic acinar cells [22], and in chemotaxis in response to N-formyl methioyl leucyl (fMLP) in neutrophils [23].

- **NAADP$^+$:** NAADP is a potent Ca^{2+}-mobilizing metabolite [8, 24]. This activity occurs selectively at an acidic pH; the acidic dependence of NAADP metabolism, coupled with it is biological function in targeting the acidic Ca^{2+} stores in cells, suggest that NAADP serves as a specific Ca^{2+} messenger for the acidic organelles of the endocytic pathway in cells [25] (**Figure 3**). There is support for NAADP-elicited Ca^{2+} release from lysosomes and endosomes [26, 27]. However, it was also shown that NAADP can release Ca^{2+} from the endoplasmic reticulum [28, 29].
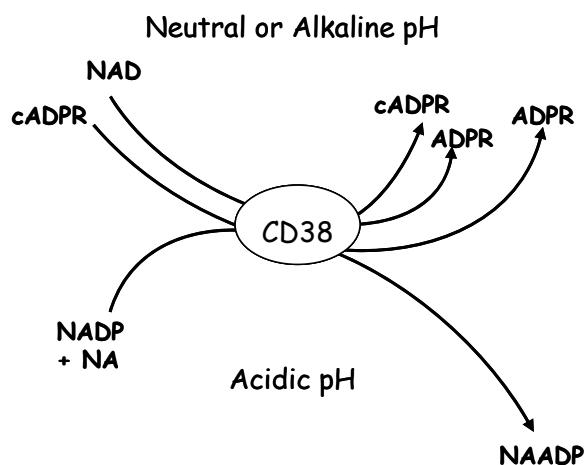

Figure 3: The multiple enzymatic reactions catalyzed by CD38 according to environmental pH.

- **ADPR**: Specific attention has recently turned to ADPR. Although it is the main product of CD38, ADPR initially lacked a clear role as an intracellular signalling molecule in vertebrate systems [13]. It was later shown that ADPR activates the melastatin-related transient receptor potential cation channel TRPM2 after binding to the Nudix domain (**Figure 4**). These data revealed that ADPR acts as intracellular messenger and may play an important role in Ca^{2+} influx by activating TRPM2 in immunocytes [13, 14, 30].
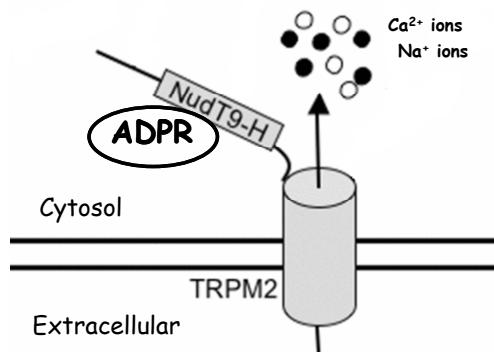
Figure 4: ADPR activates the melastatin-related transient receptor potential cation channel TRPM2 after binding to the Nudix domain.

Taken together, in addition to IP_3, internal Ca^{2+} stores can be mobilized by at least two other molecules, cADPR and NAADP, which target separate Ca^{2+} stores and are bound by distinct receptors [31, 32] (**Figure 5**). Some studies have revealed a functionally important interplay between IP_3, cADPR and NAADP in the transformation of local cytosolic Ca^{2+} spikes (via IP3) to global Ca^{2+} transients (via cADPR and NAADP) [33]. Moreover, ADPR may be involved in Ca^{2+} influx by activating TRPM2 [14].

The physiological relevance of NAADP, cADPR and ADPR as novel Ca^{2+} second messengers is just beginning to be explored.
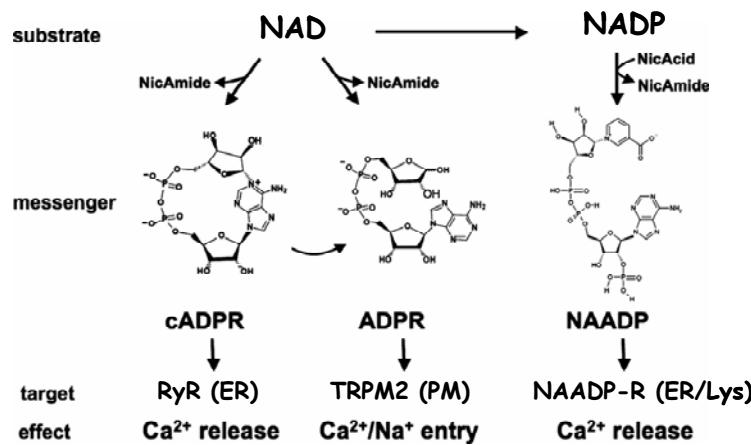

Figure 5: Metabolic relationships between the Ca^{2+} messenger cADPR, ADPR and NAADP, their target and effect.

1.2 CD38 acts at the intersection of innate and adaptative immunity

1.2.1. <u>CD38 as a regulator of innate immune responses</u>

a) CD38 regulates neutrophil migration and lung inflammatory responses

$CD38^{-/-}$ mice infected with the gram$^+$ organism *S. pneumoniae* show a reduced inflammatory cell infiltrate in the lungs of these mice and the bacteria rapidly disseminate from the lungs to the blood [23], showing that these animals have profound defects in their ability to generate an innate inflammatory response after infection with *S. pneumoniae.*

Neutrophils do not accumulate in the lung airways of S. pneumonia-infected $CD38^{-/-}$ mice [23]. This effect appears to be due, at least in part, to the defective chemotactic response of $CD38^{-/-}$ neutrophils to the bacterial-derived chemoattractant N-formyl methioyl leucyl phenylalanine (fMLP) [23]. Interestingly, $CD38^{-/-}$ neutrophils are unable to migrate in response to several endogenous chemoattractants and chemokines but are able to respond to the CXCR1/2 ligands, IL-8 and MIP-2 [23].

b) CD38 regulates dendritic cell trafficking *in vitro* and *in vivo*

Dendritic cells (DCs) migrate from the blood to the peripheral tissues and from the peripheral sites to the draining lymph nodes in response to inflammation [34]. T cell-dependent immune responses in the lymph nodes are significantly impaired in animals that lack the chemokines or chemokine receptors that induce DCs migration [35, 36]. Sort-purified immature and mature DCs from cultures of bone marrow-derived $CD38^{-/-}$ and normal wild-type cells show an intrinsic defect in their chemotactic response to an array of chemokines in *in vitro* chemotaxis assays [37]. The immature DCs from the $CD38^{-/-}$ bone marrow cultures were unable to migrate in response to the SDF-1 and MCP-1. $CD38^{-/-}$ DCs matured with

TNFα did not migrate effectively in response to ELC, SLC and SDF-1. This is not due to an inability of the *CD38$^{-/-}$* DCs to mature in response to TNFα.

In support of the *in vitro* results, *in vivo* migration assays demonstrate that the trafficking of DCs precursors from the blood to the skin and DCs trafficking from the skin to lymph nodes is significantly impaired in *CD38$^{-/-}$* mice [37].

c) CD38 regulates leukocyte trafficking by producing calcium-mobilizing second messengers

i) CD38 regulate leukocyte trafficking by producing cADPR

Frances E. Lund lab has demonstrated that cADPR induces intracellular and extracellular Ca^{2+} mobilization and regulates phagocyte chemotaxis and cell migration of immune cells [23, 37-39]. The treatment of DCs or neutrophils with the cADPR antagonist 8Br-cADPR or the CD38 substrate analog 8Br-NAD blocks the calcium response and chemotactic potential of mouse leukocytes [23, 37, 39]. Although cADPR signalling is an obligate component of the process by which CD38 modulates chemotaxis, the molecular mechanism of how this metabolite exerts its activity is not fully understood. A number of groups have demonstrated that cADPR activate calcium release from Ryanodine Receptor-gated stores in the endoplasmic reticulum (ER) [18]. Moreover, Guse lab indicates that intracellular calcium released by cADPR control extracellular calcium influx through an unknown plasma membrane calcium channel [40].

ii) CD38 regulate leukocyte trafficking by producing ADPR

The most striking defect in the chemokine/chemoattractant-treated *CD38$^{-/-}$* cells is a reduction in Ca^{2+} influx from the extracellular space [23, 37, 41].

ADPR, the main metabolite produced by CD38, directly activates Ca^{2+} influx through TRPM2. This indicated that ADPR alone, or in combination with cADPR, may play a role in CD38-dependent Ca^{2+} influx and cell migration. Importantly, TRPM2 activity is highly facilitated by synergism between ADPR and cADPR [42]. Although high concentrations of

cADPR by itself were reported to gate the channel, experiments from Fleig and Penner´s group show that cADPR at lower concentrations can significantly potentiate the effects of ADPR [42]. Similar synergism between cADPR and ADPR was reported to induce TRPM2 activation and favors insulin secretion in pancreatic β-cells [43]. Furthermore, it is known that intracellular free Ca^{2+} also enhances ADPR-gating of TRPM2 [44]. NAADP appears to regulate TRPM2 activity in synergy with ADPR [45]. cADPR and NAADP, in combination with ADPR, are physiological co-activators of TRPM2 contributing to Ca^{2+} influx signalling immune cells.

Because of all these characteristics, Lund´s group decided to test the analog 8Br-ADPR that can specifically block ADPR-gated cation entry without inhibiting Ca^{2+} influx through store-operated channel (SOCs) or hindering intracellular Ca^{2+} release mediated by IP3, cADPR, or NAADP. They show the ADPR antagonist blocks chemokine/chemoattractant receptor signal transduction and chemotaxis in primary mouse leukocyte populations. This is due to the fact that 8Br-ADPR blocks Ca^{2+} influx in leukocytes activated through chemokine receptors such as the formyl peptide receptor (FPR). They also demonstrated that PARP-1 is not required for chemoattractant-induce Ca^{2+} signalling or chemotaxis in these cells [41].

We conclude that CD38, through its ability to produce cADPR and ADPR, regulates cell trafficking and plays an important role in inflammatory responses. Moreover, compounds that block the activity of either cADPR or ADPR, or NAD analogues that can be catabolised to ADPR or cADPR antagonists inhibit leukocyte trafficking, at least *in vitro*. The data also strongly suggest that inhibitors of CD38, RyR, or the ADPR-gated cation channel could also be used to inhibit DCs and neutrophil migration *in vivo.*

d) CD38 regulates leukocyte trafficking by an alternative pathway of chemokine receptor signalling.

As mentionated above, there are many examples of chemokine receptors that signal in either a CD38/cADPR/ADPR-dependent or –independent manner [23, 37, 39]. For example, the *in vitro* chemotaxis of mouse formyl peptide receptor (mFPR)1-expressing mouse bone marrow neutrophils to the chemoattractant fMLP is dependent on CD38 and its products, in contrast, chemotaxis to the CXC chemokine receptor CXCR1/CXCR2 ligand IL-8 is independent on CD38 or its products. It is clear that there must be at least two subclasses

of chemokine receptors that can be distinguished from one another based on their requirement for CD38.

G proteins are of central importance in leukocyte trafficking, since one of the components of the chemokine receptor signalling is the trimeric G protein complex. Unlike Gαi-containing G proteins, which are required for the chemotaxis of virtually all hematopoietic cells to all chemoattractants [46], CD38 is not universally required for cell migration. *Shi et al.* discovered a novel "alternative" chemokine receptor signalling pathway that is required for cell chemotaxis [47]. Similar to the "classical" chemokine receptor signalling pathway, the alternative chemokine receptor signalling pathway is dependent on Gαi. However, unlike the classical chemokine receptor signalling pathway, the alternative pathway is also dependent on Gαq-containing G proteins. Interestingly, Gαq, like CD38, is not obligatorily required for the chemokine-induced early IP3-dependent calcium response of neutrophils activated with either IL-8 (a ligand of the classical pathway) or fMLP (a ligand of the alternative pathway). Gaq, like CD38, regulates the extracellular calcium entry in chemokine-stimulated cells through a plasma membrane channel, and evidence from Shi et al suggests that Gαq and CD38 are coregulators (**Figure 6**) of the same cation/calcium channel. Gαq-deficient (*Gnaq*$^{-/-}$) DCs and monocytes are unable to migrate to inflammatory sites and LNs *in vivo,* demonstrating that this alternative Gαq-coupled chemokine receptor signalling pathway is critically important for the initiation of immune response [47].
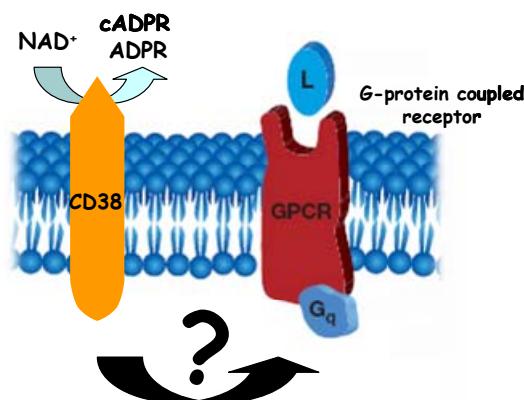

Figure 6: The chemokine receptors that signal in a CD38-dependent use a novel "alternative" chemokine receptor pathway dependent on the Gq class of G proteins.

e) CD38 regulates leukocyte trafficking by activating calcium influx through a plasma membrane.

We previously showed that extracellular calcium influx is required for the chemotaxis of cells in response to ligands of the alternative pathway and the data strongly suggest that a major mechanism by which CD38/Gαq regulates chemotaxis is by controlling the sustained calcium entry response (**Figure 7**). Although, we do not know yet the identity of the CD38/Gαq-activated calcium/cation we know that this channel is sensitive to the presence of cADPR antagonists, is dependent on intracellular Ca^{2+} release, can be blocked with an ADPR analog, and is not a classical SOC like Orail/CRACM.
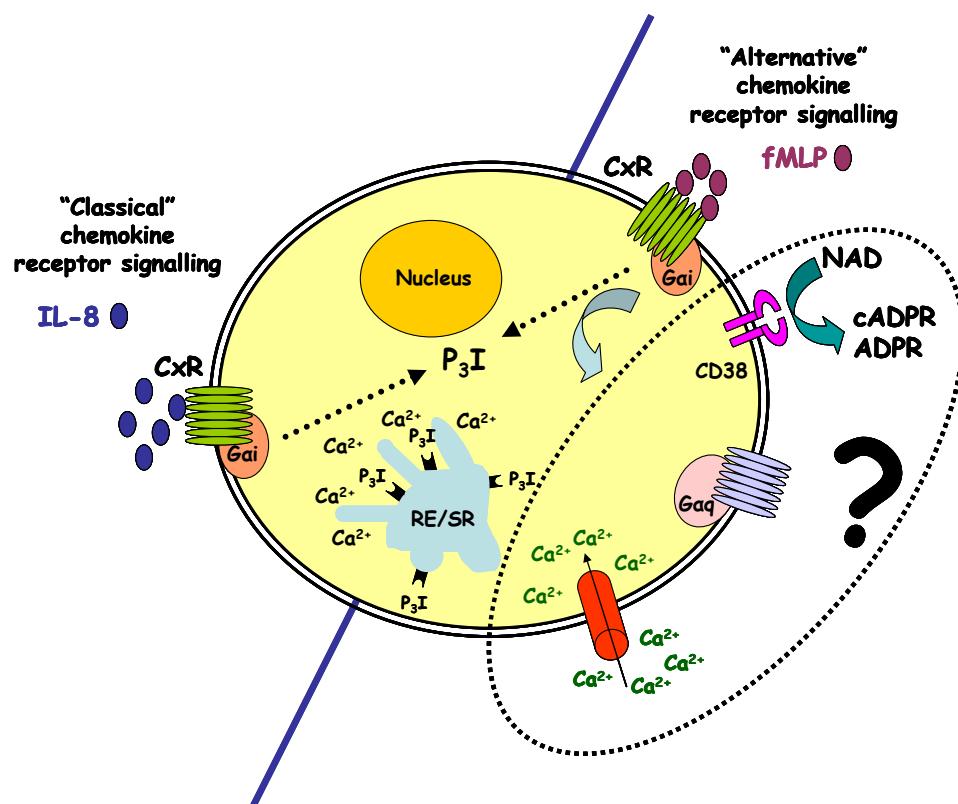

Figure 7: "Classical" and "alternative" chemokine receptor signalling. Both chemokine receptor signalling are dependent on Gαi, however, the alternative pathway is also dependent on Gαq-containing G proteins and CD38. Moreover, Gαq and CD38 regulates the extracellular calcium entry in chemokine-stimulated cells through a plasma membrane channel.

Several possibilities have been considered. Given that TRPM2 is the only known plasma membrane channel activated by ADPR, it has been speculated that chemokine receptor-mediated Ca^{2+} signalling and chemotaxis might be dependent on TRPM2. Purinergic receptors are another interesting possibility.

i) TRPM2 channel

TRPM2 (transient receptor potential channel, melastatin subfamily type 2), formerly designated TRPC7 and LTRPC2, is a non-selective cation channel permeable to Na^+, K^+ and Ca^{2+} with unique gating properties that are conferred by a domain that is homologous with the mitochondrial ADPR-metabolizing enzyme NUDT9 [13]. Therefore, TRPM2 is a unique combination of a cation channel and enzyme. In addition to its role as a plasma membrane-resident Ca-influx channel, TRPM2 can also function as an intracellular Ca^{2+}-release channel in lysosomes of pancreatic β cells [48].

TRPM2 is synergistically activated and regulated by multiple signalling pathways through various adenine dinucleotides (ADPR, cADPR and NAADP) and intracellular calcium concentration [13, 14, 42-45, 49, 50]. Structural and mutational analysis revealed direct binding of ADPR into a cleft formed by the NUDT9 homology region, thereby inducing the channel opening of TRPM2. Since the enzymatic ADPR hydrolase activity is largely reduced in the NUDT9 homology region of the channel [13], this motif primarily serves as an interaction site for ADPR. ADPR hydrolase activity does not appear to influence ADPR gating of TRPM2, suggesting that enzymatic function is not needed for channel opening [49].

Free ADPR can be generated extracellularly and intracellularly by different cellular pathways, hydrolysis of NAD^+ through NADases [10], the action of both, poly(ADPR) polymerases (PARP enzymes) and poly(ADPR) glycohydrolases (PARG enzymes) [51] or hydrolysis of cADPR [10].

TRPM2 is highly expressed in human brain microglial cells and also in heart, liver, lung, cells of the monocytic lineage, macrophage cell lines [13, 14, 30], pancreatic islets [52, 53] and insulinoma cell lines [53]. As reported for human, murine TRPM2 expresion has been confirmed to be abundant in tissues of hematopoietic origin and non-hematopoietic tissues containing bone marrow-derived cells; including: spleen, brain lung and liver. TRPM2

transcrips are abundant in mouse primary neutrophils, peritoneal macrophages and bone marrow-derived dendritic cells (DCs) [54].

Massullo et al. proposed **a model of ADPR/TRPM2 pathway** that regulates Ca^{2+} influx and chemotaxis [54] (**Figure 8**). Under normal homeostatic conditions, very small quantities of CD38-derived metabolites are produced since NAD^+ is in limited supply [55]; under this steady-state scenario, connexin 43 (Cx43) hemichannels would pump out NAD^+ to feed CD38. However, upon inflammation and subsequent tissue damage, the concentration of inflammatory cytokines, chemokines, and extracellular NAD^+ increases. Available NAD^+ may reach concentration levels that exceed the Km of CD38 [56]. The presence of abundant free NAD^+ generate Ca^{2+} second messengers which are rapidly internalized by CD38 or nucleoside transporters [57]. Next, chemokines and/or chemotactic peptides will bind chemokine receptors on migrating cells, inducing them to follow these chemotactic trails. Chemokines induce the activation of G protein coupled receptor (GPCR) that lead signalling cascades resulting in PLC activation and IP3 production and calcium release from ER intracellular stores [58]. The Ca^{2+} mobilized by IP3, along with cADPR, facilitates the release of Ca^{2+} from RyR-gated ER Ca^{2+} stores. Furthermore, cADPR in combination with ADPR will enhace extracellular Ca^{2+} influx through TRPM2 channels. The presence of CD38-catalyzed metabolites will sustain the Ca^{2+} response for longer periods of time and facilitate phagocyte migration. When tissue damage is resolved, chemokine production and diffusion is down regulated, extracellular NAD^+ levels are lowered, cADPR production is reduced, and chemokine receptor signalling is attenuated.

Unfortunately, this hypothesis could not be experimentally validated because no specific TRPM2 antagonists have been identified and TRPM2-deficient mice were not available until now.
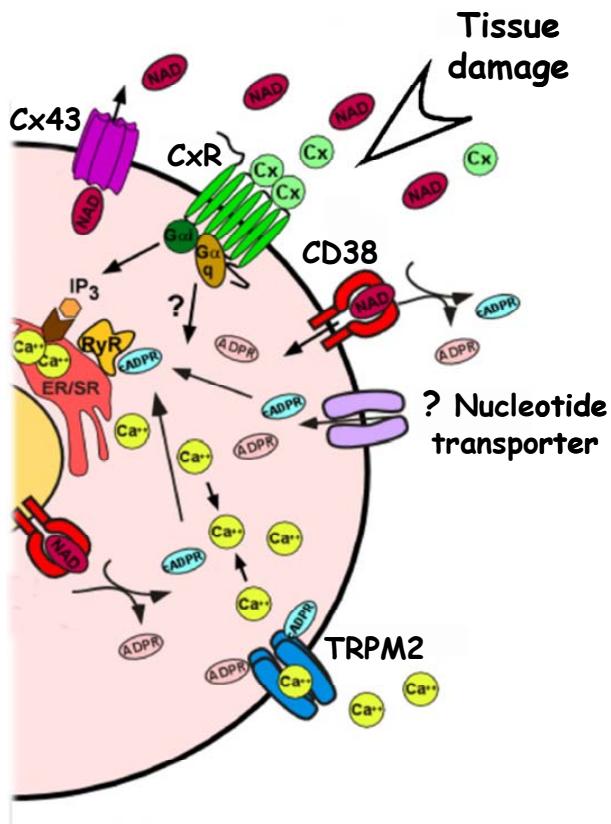
Figure 8: Model for ADPR and TRPM2 mediated regulation of chemotaxis. *Massullo et al.* proposed that CD38, through conversion of NAD into the Ca2+ second messengers cADPR and ADPR will activate extracellular Ca2+ influx cooperatively with chemokine receptor (CxR) ligation [54].

ii) Purinergic receptors

The extracellular actions of ATP, ADP, UTP, UDP, UDP-glucose and Ado are mediated through purinergic P2 and P1 receptors. Recently NAD^+ and $NAADP^+$ have been shown to act as agonists of purinergic receptors [59-61]. One of the aspects than have not been studied so far is the possibility that products of CD38 or its breakdown products ADP or adenosine can signal via purinergic receptor, however, a recent report from *Lange et al.* [48] shows that extracellular ADPR production by the ectoenzyme CD38 from its substrates NAD^+ or cADPR causes IP3-dependent Ca^{2+} release via P2Y and adenosine receptors in β cells.

Purinoreceptors comprise adenosine-sensitive P1 receptors (A_1, A_{2a}, A_{2b}, and A_3) and P2 receptors, with are activated by ATP, ADP, UTP, UDP or UDP-glucose [62, 63] and NAD^+ or $NAADP^+$ [59-61]. According to the molecular structure, P2 receptors are divided into two subfamilies: the ionotropic P2X receptors, and metabotropic P2Y receptors (**Figure 9**).
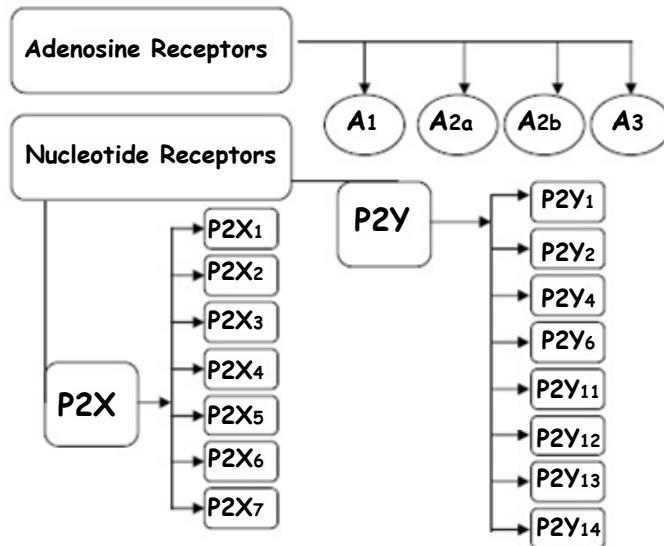
Figure 9. Classification of purinergic receptors. Adenosine P1 receptors consist of A_1, A_{2a}, A_{2b} and A_3 receptors and nucleotide P2 receptors are divided into P2X receptors ($P2X_{1-7}$) and P2Y receptors ($P2Y_{1,2,4,6,11-14}$).

- **P1 receptors:**

Four subtypes of P1 receptors have been cloned, namely A_1, A_{2A}, A_{2B} and A_3 [62, 64]. All P1 adenosine receptors are typical G protein-coupled (metabotropic) receptors. These receptor subtypes bind extracellular Ado with different affinities. It must be noted that the breakdown product of Ado (inosine) also exhibits immunomodulatory properties by agonistic action on A_1, A_{2A} and A_3 receptors [65-68] (**Table 1**). Specific agonists and antagonists are available for each subtype. P1 receptors are antagonized by methylxanthines. Adenosine receptors are expressed in most tissues in the body.

Receptor	Physiologic Ligands	Mechanism
A_1	Ado Inosine	Gi/o → decreased cAMP
A_{2A}	Ado Inosine	Gs → increased cAMP
A_{2B}	Ado	Gs → increased cAMP Gq → DAG-IP3-Ca2+
A_3	Ado Inosine	Gi/o → decreased cAMP

Table 1: P1 receptor subtypes and signalling. P1 receptors consist of four G protein-coupled receptors (GPCRs): A_1, A_{2a}, A_{2b} and A_3

- **P2X receptors:**

P2X receptors are non-selective ligand-gated ion channels. A P2X receptor monomer consists of two transmembrane domains connected by a large extracellular loop containing the putative ATP binding site (**Figure 10**). Upon binding ATP, P2X receptors permit the passage of cations along their electrochemical gradients; in cells this leads to the influx of sodium and calcium. This in turn leads to depolarization of the cell and downstream calcium signalling. P2X receptors comprise seven subtypes ($P2X_1$ throught $P2X_7$). They have widespread and overlapping distribution in excitable and nonexcitable cells of vertebrates and play key roles in inter alia afferent signalling (including pain), regulation of renal blood flow, vascular endothelium, and inflammatory responses.
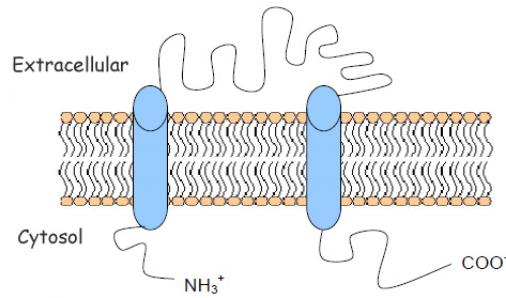
Figure 10. Structure of P2X receptor. P2X ion channel monomer consists of intracellular carboxy and amino termini, two transmembrane spanning domains, and a large extracellular ligand-binding loop.

Stoichiometry of $P2X_{1-7}$ receptors is thought to involve three subunits, which form a trimer. Heteromultimers as well as homomultimers are involved in forming the trimer ion pore. Heteromultimers are clearly established for $P2X_{2/3}$, $P2X_{4/6}$, $P2X_{1/5}$, $P2X_{2/6}$, $P2X_{1/4}$ and $P2X_{1/2}$ receptors. $P2X_7$ receptors do not form heteromultimers [69] and $P2X_6$ receptors will not form a functional homomultimer [70].

- **P2Y receptors:**

P2Y are typical G protein-couple receptors (GPCRs). Structurally, GPCRs are seven transmembrane spanning receptors (7TM) (**Figure 11**). P2Y receptor stimulation leads to activation of heterotrimetic G proteins and their dissociation into α and βγ subunits that can then interact with a variety of effector proteins and results in the release of calcium from intracellular stores.
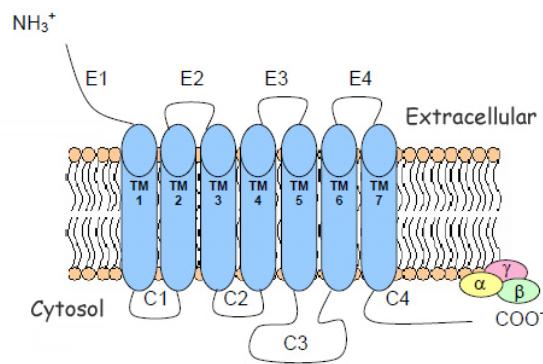

Figure 11. P2Y receptors belong to the family of G protein-coupled receptors. G-Protein coupled receptor consists of seven transmembrane spanning domains, an intracellular carboxy terminus, and extracellular amino terminus.

The P2Y receptors are subdivided into Gq-coupled subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) and Gi-coupled subtypes ($P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$). The $P2Y_{11}$ receptor additionally couples to Gs and activates adenylyl cyclase (**Table 2**).

Pharmacologically, P2Y receptors can be subdivided into the adenine-nucleotide-preferring receptors mainly responding to ATP and ADP (human and rodent $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ and human $P2Y_{11}$), the uracil-nucleotide-preferring receptors (human $P2Y_4$ and $P2Y_6$) responding to either UTP or UDP, receptors of mixed selectivity (human and rodent $P2Y_2$ and rodent $P2Y_4$), as well as nucleotide-sugar-preferring human $P2Y_{14}$ receptor responding to UDP-glucose and UDP-galactose [71, 72] (**Table2**).

Receptor	Physiologic Ligands	G Protein	Signalling
$P2Y_1$	ADP	Gq/11	PLC (+), Ca^{2+} release
$P2Y_2$	ATP, UTP	Gq/11	PLC (+), Ca^{2+} release
$P2Y_4$	UTP	Gq/11	PLC (+), Ca^{2+} release
$P2Y_6$	UDP	Gq/11	PLC (+), Ca^{2+} release
$P2Y_{11}$	ATP	Gq/11	PLC (+), Ca^{2+} release
	ATP	Gs	AC (+), increased cAMP
$P2Y_{12}$	ADP	Gi	AC (-), decreased cAMP
$P2Y_{13}$	ADP	Gi/o	AC (-), decreased cAMP PLC (+), Ca^{2+} release
$P2Y_{14}$	ADP-glucose	Gi/o	PLC (+), Ca^{2+} release

Table 2. P2Y subtypes, G-protein coupling and signalling. Modified from *Laurie Erb et al. Eur J Physiol (2006)*, a good review of intracellular signalling.

- **Purinergic receptor signalling in immunity inflammation:**

These endogenous signalling molecules and their purinergic receptors play a major role in immunity and inflammation, however their role in immunity and inflammation is extremely complex and interdependent. For the purpose of clarity, inflammatory and immune responses are divided into three stages, which partly overlap [73-75].

The **first stage** encompasses the onset of acute inflammation and the initiation of primary immune responses upon encounter with infectious or injurious agents. Extracellular ATP mainly functions as a pro-inflammatory and immunostimulatory mediator in the microenviroment of damaged cells. ATP is present in the cell cytoplasm at millimolar concentrations and is released from the intracellular compartment upon cellular stress or non-physiological necrotic cell death. Extracellular ATP concentrations in the local microenviroment contribute to the promotion of inflammation and the initiation of primary immune responses through P2 receptor-mediated purinergic signalling. When ATP levels are highest, the receptor subtype most likely involved is the $P2X_7$ receptor [76-79], this receptor subtypes appears to be up-regulated by pro-inflammatory mediators [80, 81]. Via activation of $P2X_7$ receptors, ATP induces the production of cytokines such as IL-1β, IL-2, IL-12, IL-18 and TNFα by residing immune cells, triggering the inflammatory response and inducing type 1 lymphocyte polarization (Th1). High level signalling through other P2 receptors probably also contributes to the pro-inflammatory and immunostimulatory role of ATP. Recruitment of leukocytes to damaged sites is promoted by ATP as well as other adenine and uridine nucleotides, which also induce inflammatory activation of neutrophils, classical activation of macrophages, maturation and Th1 cell-stimulatory capacity of DCs, and proliferation of lymphocytes.

Over-activation of the immune system may lead to uncontrolled or chronic inflammation resulting in collateral cell damage and destruction of healthy tissues, so the **second stage** comprises the modulation and fine-tuning of ongoing inflammatory and immune responses by endogenous immunoregulatory substances. The immunological role of ATP appears to shift gradually from being mostly immunostimulatory to being more immunomodulatory. The expression profile of purinergic receptors as well as of ecto-enzymes by immune cells changes under inflammatory conditions, allowing for the progressive acquisition of an immunomodulatory purinergic repertoire expressed by the cells

involved in inflammatory and immune responses. The ecto-enzymes CD39 and CD73 control extracellular adenine-containing nucleotide concentrations and thereby regulate the extent of purinergic signalling [82-87], these nucleotides when present in decreased concentrations induce low-level purinergic signalling by P2 receptors. Activation of P2 receptors, most probably P2Y receptor subtypes, attenuates pro-inflammatory cytokine production by monocytes and macrophages (Mo/Mø), diminishes Th_1 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions. Moreover, Ado-mediated P1 receptor signalling during the second stage down-regulates neutrophil effector functions, contributes to alternative activation of macrophages, stimulates Th2 cell-stimulatory capacity of DCs and inhibits lymphocyte effector functions.

Finally, the **third stage** encompasses the down-regulation of immune responses, the induction of inflammatory resolution and the restoration of damaged tissues to preserve cellular homeostasis. Extracellular Ado appears to be an important immunosuppressive and tissue-healing factor. Inflammatory mediators progressively up-regulate the expression of the P1 receptor subtypes through which Ado mediates its immunosuppressive effects [88-97]. The A_{2A} receptor subtype has been considered to play a non-redundant role in down-regulating cell-mediated immunity and in activating pro-reslolution pathways [92, 98-108]. A_1, A_{2B} and A_3 receptors also contribute to the Ado-mediated negative feedback signalling [67, 109-116]. Ado mediated signalling by these purinergic receptors deactivates macrophages, suppresses proliferation and effector functions of lymphocytes, and promotes angiogenesis and tissue regeneration.

In conclusion, ATP, ADP, UTP, UDP and Ado are crucial endogenous signalling molecules in immunity and inflammation. Because these molecules are unstable molecules with a short half-life, they probably operate only transiently in the local microenvironment of cells in an autocrine or paracrine manner. Interestingly, purinergic receptors have been found to localize to lipid rafts.

1.2.2. <u>CD38 regulates T cell-dependent immune responses</u>

CD38 is not obligate for the development of any of the hematopoietic lineages but was necessary for optimal T-cell dependent humoral immune responses [117]. Subsequent experiments indicate that $Cd38^{-/-}$ B cells proliferated normally in response to BCR ligation [6] and that the defective humoral immune response seen in the $Cd38^{-/-}$ mice was not due to the loss of CD38 on B lymphocytes [37].

If CD38 regulates the migration of DCs both *in vitro* and *in vivo*, it is predicted that the priming and the activation of CD4 T cells is likely to be impaired in the $Cd38^{-/-}$ mice due to an intrinsic inability of $Cd38^{-/-}$ DCs to mobilize Ca^{2+} in response to chemokine receptor ligation and to migrate in response to chemokines that normally direct their trafficking *in vivo*. To test this hypothesis, Partida-Sánchez et al. [37] transferred normal T cell receptor (TCR) transgenic ovalbumin (OVA)-specific CFSE-labeled T cells into $Cd38^{-/-}$ or normal *wild-type* hosts and assessed the activation, proliferation, and expansion of these cells after immunization with OVA peptide in alum. They found that the normal TCR transgenic T cells expanded upon immunization and underwent multiple rounds of proliferation in both the spleen and lymph node. In contrast, the T cells transferred into the $Cd38^{-/-}$ hosts did not proliferate as extensively as assessed by dilution of the CFSE dye and a larger proportion of the transferred OVA-specific T cells remained $CD44^{lo}$ in the CD38 hosts. These data indicated that $Cd38^{-/-}$ DCs were less effective at priming CD4 T cells, even when the T cells were able to express CD38.

The impairment in DC trafficking and CD4 T cell priming observed in the $Cd38^{-/-}$ mice had functional consequences for the humoral immune response. Partida-Sanchez et al. sensitized the skin of *wild-type* and $Cd38^{-/-}$ with FITC and showed a significant lower number of antigen-specific CD4 T cells and B cells present in the draining lymph node. Furthermore, they found that primary and secondary FITC-specific IgG and IgG1 responses were severely attenuated in the $Cd38^{-/-}$ mice.

The main objective if this work is to study in more detail the chemokine receptors that signal in a CD38-dependent manner and also require the Gq class of G proteins, with particular emphasis on the pursuit of the membrane calcium channel involved in Ca^{2+} influx necessary for a proper chemotaxis of neutrophils and DCs to a subsets of chemokines.

2. Materials and Methods

Mice

Wild-type C57BL/6J mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME) and then maintained in the University of Rochester Animal Breeding Facility. $Trpm2^{-/-}$ [118] and C57BL/6J.129 $Cd38^{-/-}$ mice [117] were bred and maintained at the University of Rochester Animal Breeding Facility.

Reagents and Antibodies

Recombinant mouse chemokines and chemoattractants used in this study were purchased from Peprotech (Rocky Hill, NJ) and Sigma-Aldrich (St. Louise, MO). fMLP and L-8 were obtained from Sigma-Aldrich and MCP-1, SDF-1, ELC, SLC and BLC from Peprotech. Suramin sodium salt, a non-selective antagonists for P2 receptors, CGS-15943, a non-selective antagonist for P1 receptors, and ATP disodium salt, a native agonist for P2 receptors were obtained from Sigma-Aldrich. All fluorescent mAbs were from Pharmigen (San Diego, CA) with the exception of bio-CD115 that was obtained from eBioscience (San Diego, CA). Flow cytometry was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) and a Accuri C6 flow cytometer.

Cell Purification

Neutrophils: Mouse bone marrow cells were prepared by flushing bone marrow from tibias and femurs of *wild-type* C57BL/6J, *Trpm2-/-* and *Cd38-/-* mice, then were stained with biotinylated anti-GR1 antibody (BD Pharmigen, San Diego, CA) and MACS Streptavidin Microbeads (Miltenyi Biotec, Auburn, CA) and then positively selected on a MACS midi column. Purity was ≥90% as assessed by FACs.

B and T Cells: B and T cells were purified from spleen using anti-CD19 and anti-CD4 microbeads (Miltenyi Biotec)

Monocytes: Bone marrow cells were first stained with biotinylated anti-CD115 antibody and MACS streptavidin microbeads (Miltenyi Biotec) and then positively selected on a MACS midi column.

Dendritic Cells: Dendritic cells were purified from spleen or culture by positive selection using anti-CD11c microbeads (Miltenyi Biotec)

All purified cells were washed and resuspended in HBSS (Ca^{2+}, Mg^{2+}) supplemented with 1% FBS to perform chemotaxis assays.

In vitro generation of BM-derived DCs

Bone marrow cells obtained from mice were incubated in complete RPMI medium supplemented with 10% FBS containing GM-CSF (Peprotech) at 20ng/ml. At day 6, non adherent cells were collected and stimulated for 36-48h with LPS (Sigma-Aldrich) at 1µg/ml or anti-CD40 (clone 10C8) [119] at 10µg/ml. Mature dendritic cells were purified from culture by positive selection using anti-CD11c microbeads (Miltenyi Biotec).

Chemotaxis assays

Chemotaxis assays with mouse neutrophils were performed using 24-well Transwell plates (Costar, Cambridge, MA) with a 3µm pore size polycarbonate filter. Chemotaxis assays with mouse T cells and monocytes were performed using 24-well Transwell with a 5µm pore size polycarbonate filter, Chemotaxis assays with B cells and dendritic cells were performed using 96-well Transwell plates (Costar, Cambridge, MA) with 5µm pore size polycarbonate filter. The number of cells used in each assay were adjusted relative to the dimensions of the membranes in the wells. Chemoattractants were diluted in HBSS media and placed in the lower chamber and then cells were added to the upper chamber of the Transwell. In some experiments neutrophils were pretreated for 15 minutes with Suramin (1-300µM), CGS-15943 (0.1-100µM) or ATP (10-300µM), and then added to the upper chamber of the transwell in the continued presence of the drug. For neutrophil assays, Transwell plates were incubated at 37ºC for 45min. For all other cell types, transwell plates were

incubated for 2h. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. To determine the absolute number of cells in each sample, a standard number of 20 µM size fluorescent microspheres (Polysciences, Inc., Warrington, PA) was added to each tube and counted along with the cells. The total number of transmigrated cells was counted. The total number of transmigrated cells equals the number of counted cells X the total number of beads/the number of beads counted. The results are expressed as the mean ±SD of triplicate cultures.

RNA isolation and gene expression analyses

Total RNA was isolated from wild type, *Cd38^{-/-}* and *Trpm2^{-/-}* bone marrow neutrophils using the RNeasy kit (Qiagen, La Jolla, Ca). DNase-treated RNA was reverse transcribed with random hexamers and Superscript II (Invitrogen). Quantitative PCR was performed with Taqman master mix, according to the Applied Biosystems protocol. Primers and probes for P1, P2X and P2Y receptors were obtained from Applied Biosystems. Quantitative PCR assays were performed using a Roche LighCycler 480 instrument from Applied Biosystems available through the Cancer Center at University of Rochester. Standard PCRs were performed with 50 ng of cDNA. GADPH house-keeping was used for levels normalization. The "fold increase" in signal relative to that of $P2Y_1$ receptor in *wild-type* mouse was determinated with the "$\Delta\Delta C_T$" (change in cycling threshold) calculation recommended by Applied Biosystems.

Stadistical Analysis

Data sets were analyzed using GraphPad Prism version 4.0 for Macintosh or PC (GradhPad Software, San Diego, CA). Student´s t test or Mann-Whitney analyses were applied to the data sets to determine statistically significant differences between groups. Differences were considered significant when p values were ≤0.05.

3. Results

3.1. *In vitro* chemotaxis of mouse cells to chemokines that signal in a CD38-dependent manner is not TRPM2 dependent

Shi et al. [47] showed that chemokine receptors can be divided into CD38-dependent and –independent subclasses and CD38-dependent chemokine receptor signaling is also dependent on Gαq, however, we do not know if the Gαq is physically associated with the chemokine receptor or is associated with another receptor that is also required for the chemotactic response. Furthermore, we do not know the identity of the membrane calcium/cation channel that is required for signalling through the CD38/Gaq dependent chemokine receptors. To determine whether TRPM2, a cation channel activated by ADPR, is necessary, we tested chemotaxis of different types of *wild-type* C57BL/6J and TRPM2 deficient mouse cells to CD38-dependent chemokines and CD38-independent chemokines.

a) *In vitro* chemotaxis of neutrophils to fMLP and IL-8 is not TRPM2 dependent

Neutrophils migrate to sites of infection in response to gradients of chemokines and chemoattractants that are produced by the local cells and by the invading pathogen. Chemoattractants rapidly activate neutrophils and induce random migration (chemokinesis). If a chemotactic gradient exists, the activated neutrophils polarize their leading edge toward the highest concentration of the gradient and migrate directionally (chemotaxis). Neutrophils home to sites of infection upon stimulation of their N-formylpeptide receptor by bacterially derived formylated peptides such as Formyl-Methionyl-Leucyl-Phenylalanine (fMLP). Moreover, the chemokine interleukin-8 is a potent activator of neutrophils. Interestingly, $Cd38^{-/-}$ neutrophils were defective in their ability to chemotax to fMLP, however, the IL-8-induced chemotaxis of $Cd38^{-/-}$ and wild-type neutrophils was equivalent [23]. This data indicate that $Cd38^{-/-}$ neutrophils make defective chemotactic responses to some, but not all, chemoattractants. According to the model for ADPR/TRPM2 mediated regulation of chemotaxis [54], CD38, through its production of cADPR and ADPR, controls the chemotaxis of neutrophils and DCs towards exogenosus bacterially –derived peptides and endogenous inflammatory chemokines. CD38 will convert NAD^+ into the second messengers cADPR and ADPR, which, in turn will activate extracellular Ca^{2+} influx through TRPM2. The presence of

CD38-catalyzed metabolites will sustain the Ca^{2+} response for longer periods of time and facilitate migration. It has been hypothesized that the CD38 products cADPR and ADPR are involved in the influx of Ca^{2+} through TRPM2. If this hypothesis is correct, then *Trpm2-/-* neutrophils should make a defective chemotactic response to fMLP. To test this hypothesis, bone marrow neutrophils were purified from C57BL/6J and *Trpm2-/-* mice by positive selection. Purity was ≥90% as assessed by FACs. We assessed the ability of *Trpm2-/-* and *wild-type* neutrophils to migrate to fMLP by chemotaxis in a Transwell assay. As a control we also tested the migration of mouse neutrophils to IL-8. Chemotaxis assays with mouse neutrophils were performed with 24-well Transwell plates (Costar, Cambridge, MA) with a 3-µm pore size polycarbonate filters. fMLP and IL-8 were diluted in HBSS at 1µM and 100nM respectively and placed in the lower chamber, cells were then added to the upper chamber of the Transwell. For mouse neutrophil assays, $1x10^6$ cells/Transwell were incubated at 37ºC for 45min. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. Surprisingly, as it is shown in **Figure 12** *Trpm2-/-* neutrophils did not appear to have an intrinsic defect in chemotaxis to fMLP and chemotaxis to IL-8 was normal, as expected.
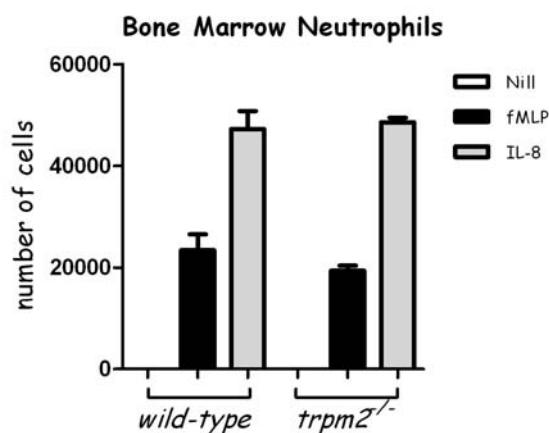

Figure 12. Chemotaxis of purified bone-marrow neutrophils from *wild-type* and *Trpm2$^{-/-}$* mice to fMLP and IL-8. We purified bone-marrow neutrophils from *wild-type* C57BL/6J and *Trpm2$^{-/-}$* mice. We tested for their ability to migrate in response to medium, fMLP or IL-8 in a conventional transwell chemotaxis assay. Mouse neutrophils were placed in the upper wells of chemotaxis chambers and the lower wells of the chamber contain HBSS plus fMLP (1µM) or IL-8 (100nM). Transmigrated cells were collected after 45min from the bottom chamber and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown are representative of three independent experiments.

b) *In vitro* **chemotaxis of mature dendritic cells to ELC and immature DCs to SDF-1 is not TRPM2 dependent**

Previous reports showed that *wild-type* C57BL/6J immature dendritic cells (DCs) migrated poorly to CCR7 ligands CCL21 (SLC or seconday lymphoid organ chemokine) and CCL19 (ELC or EB1 ligand chemokine), which are involved in regulating trafficking of dendritic cells to lymph nodes (LNs), but responded efficiently to the CCR2 ligand CCL2 (MCP-1 or monocyte chemotactic protein-1) that regulates migration of DCs precursors to inflamed tissues and the CXCR4 ligand CXCL12 (SDF-1 or stromal derived factor-1). By contrast, mature DCs migrated vigorously to SLC and ELC and also responded, albeit less well, to SDF-1. *Shi et al.* [47] demonstrated that *Cd38*$^{-/-}$ DCs were unable to migrate efficiently *in vivo* in response to inflammatory stimuli. Mature DCs were unable to migrate *in vitro* in response to SLC and ELC and immature DCs were also unable to migrate *in vitro* in response to MCP-1 and SDF-1. We hypothesized that the *Trpm2* $^{-/-}$ DCs might also be unable to migrate in response to these chemokines. We prepared *in vitro*-cultured anti-CD40- or LPS-stimulated mature DCs from *wild-type* and *Trpm2*$^{-/-}$ bone marrow cells and CD11c^{+} cells were purified from the bulk cultures. The chemotactic potential of mDCs was determined by transwell chemotaxis assays using ELC. We also examinated chemotaxis of immature DCs to SDF-1. Chemotaxis assay using purified mature dendritic cells derived from bone marrow were performed with 96-well Transwell plates (Costar, Cambridge, MA) with a 5-μm pore size polycarbonate filters, however chemotaxis assay using non-purified immature dendritic cells were performed with 24-well Transwell plates (Costar, Cambridge, MA) with a 5-μm pore size polycarbonate filters. ELC and SDF-1 were diluted in HBSS at 25ng/ml and 100ng/ml respectively and placed in the lower chamber, cells were then added to the upper chamber of the Transwell. 2.5x10^{4}cells/Transwell for mouse mature DCs assays and 5x10^{5}cells/Transwell for immature DCs assays were used and then they were incubated at 37ºC for 2h. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. Unexpectedly, *Trpm2*$^{-/-}$ mature DCs migrated efficiently in response to the CCR7 ligand ELC (**Figure 13.a**). Likewise, chemotaxis of *Trpm2*$^{-/-}$ immature DCs to SDF-1 was completely normal (**Figure 13.b**).
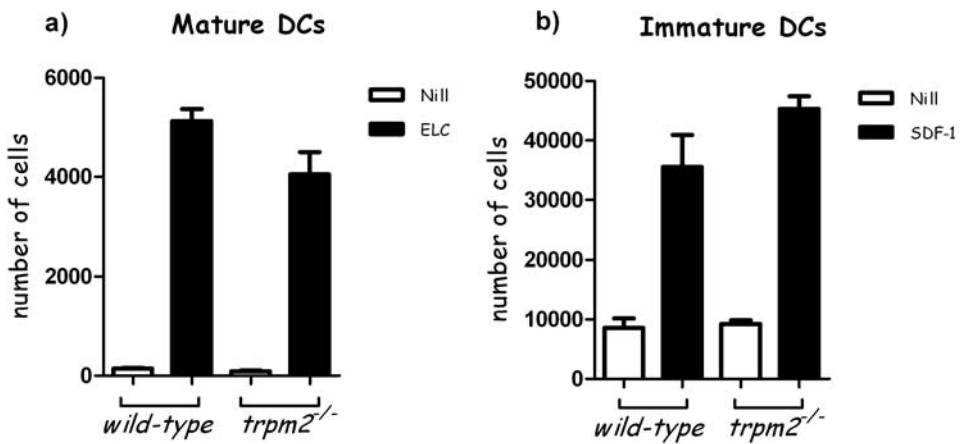
Figure 13. **Chemotaxis of purified bone-marrow derived mature DCs and non-purified bone marrow derived immature DCs from *wild-type* and *Trpm2^{-/-}* mice to ELC and SDF-1, respectively.** We prepared *in vitro*-cultured immature and α-CD40 or LPS stimulated mature DCs from *wild-type* C57BL/6J and *Trpm2^{-/-}* mice. Purified mature DCs from the bulk cultures and total non adherent cells from the unstimulated cultures were tested for their ability to migrate in response to ELC and SDF-1, respectively in a conventional transwell chemotaxis assay. Mouse DCs were placed in the upper wells of chemotaxis chambers and the lower wells of the chamber contained HBSS plus ELC (25ng/ml) or SDF-1 (100ng/ml). Transmigrated cells were collected after 2h from the bottom chamber and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown from mature DCs are representative of three independent experiments and the data shown from immature DCs are representative of two independent experiments.

c) *In vitro* chemotaxis of CD4^{+} cells to SDF-1, ELC and SLC is not TRPM2 dependent

CD4^{+} T cells, like DCs and neutrophils, are reported to express TRPM2. Signaling through CXCR4 and CCR7 in T cells is not Gαq dependent and CD38 is not required for the chemotaxis of these cells to the CXCR4 and CCR7 ligands CXCL12 (SDF-1), CCL21 (SLC) and CCL19 (ELC). Therefore, we predicted that T cell chemotaxis to these ligands would occur in a TRPM2 independent fashion. To test this prediction, we examined the *in vitro* chemotactic response of peripheral T cells from *wild-type* C57BL/6J and *Trpm2^{-/-}* mice to SDF-1, ELC and SLC. CD4^{+} T cells were isolated from spleen of *wild-type* C57BL/6J and *Trpm2^{-/-}* mice by positive selection. Purity was ≥90% as assessed by FACs. Chemotaxis assays with mouse T cells were performed with 24-well Transwell plates (Costar, Cambridge,

MA) with a 5-µm pore size polycarbonate filters. SDF-1, SLC and ELC were diluted in HBSS at 300ng/ml and placed in the lower chamber and cells were added to the upper chamber of the Transwell. For T cell assays, 1×10^6 cells/Transwell were incubated at 37°C for 2h. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. As expected the chemotaxis of T cells to these chemokines is TRPM2 independent (**Figure 14**).
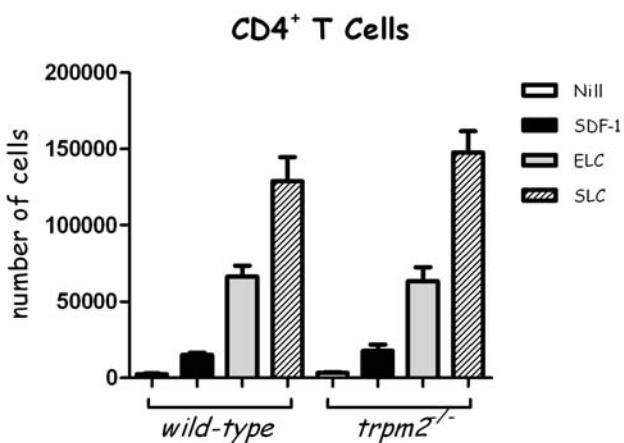

Figure 14. Chemotaxis of mouse spleen T cells to SDF-1, SLC and ELC. Splenic CD4+ T cells were purified from *wild-type* C57BL/6J and Trpm2^{-/-} mice. T cells were placed in the upper wells of chemotaxis chambers and the lower wells of the chamber contain HBSS plus SDF-1, SLC or ELC at 300ng/ml. Transmigrated cells were collected after 2h from the bottom chamber and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown are representative of four or more independent experiments.

d) Chemotaxis of B cells to SDF-1 is not TRPM2 dependent

B cells also express TRPM2. Signalling through CXCR4 and CXCR5 in B cells is not Gαq dependent [120] and CD38 is not required for the chemotaxis of these cells to the CXCR4 and CXCR5 ligands CXCL12 (SDF-1), and CXCL13 (BLC). Therefore, we predicted that B cell chemotaxis to these ligands is TRPM2 independent. To test this prediction, we measured the *in vitro* chemotaxis of peripheral B cells from *wild-type* and *Trpm2^{-/-}* mice to SDF-1 and BLC. B cells were isolated from spleen of *wild-type* C57BL/6J and *Trpm2-/-* mice by positive selection. Purity was ≥90% as assessed by FACs. Chemotaxis assays with

mouse B cells were performed with 96-well Transwell plates (Costar, Cambridge, MA) with a 5-μm pore size polycarbonate filters. SDF-1 and BLC were diluited in HBSS at 300ng/ml, and placed in the lower chamber and cells were added to the upper chamber of the Transwell. For B cell assays, 3.5×10^5 cells/Transwell were incubated at 37ºC for 2h. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. As expected chemotaxis of *Trpm2*$^{-/-}$ B cells to SDF-1 chemokine is completely normal, however, surprisingly we did not observe an efficient chemotaxis to BLC of *wild-type* or *Trpm2*$^{-/-}$ B cells (**Figure 15**). We tested chemotaxis of splenic B cells to different concentrations of BLC (10-300ng/ml) and observed the same results (data not shown).
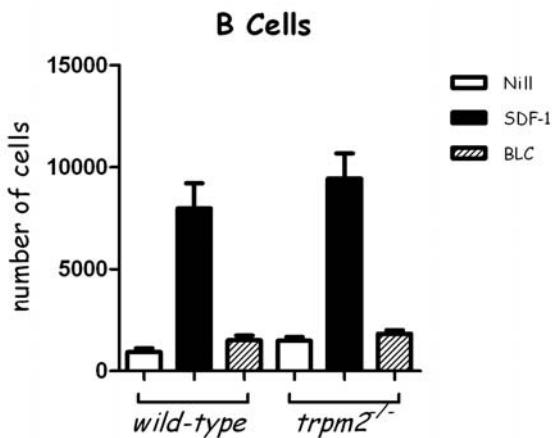

Figure 15. **Chemotaxis of mouse spleen B cells to SDF-1 and BLC**. Splenic B cells were purified from *wild-type* C57BL/6J and *Trpm2*$^{-/-}$ mice. B cells were placed in the upper wells of chemotaxis chambers and the lower wells of the chamber contain HBSS plus SDF-1 or BLC (300ng/ml). Transmigrated cells were collected after 2h from the bottom chamber and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown are representative of three independent experiments.

e) *In vitro* chemotaxis of monocytes to MCP-1 is not CD38 and TRPM2 dependent

It has been reported that cADPR regulates signalling through only a subset of the chemoattractant receptors expressed on human monocytes. Chemotaxis of human monocytes to the FPR ligand fMLP was independent of cADPR, however, chemotaxis of human monocytes to the CCR1/CCR5 ligands RANTES and MIP-1 and to the CXCR4 ligand

SDF-1 was regulated by cADPR. Unlike human monocytes, mouse monocyte chemotaxis has not been extensively studied, due to the difficulty in obtaining large numbers of highly purified monocytes. Some not yet published results in a *Listeria* infection model using *Cd38-/-* mice could be explained by an impaired migration of monocytes, so we hypothesized that the CD38/cADPR/ADPR signaling pathway would also be impaired in the TRPM2 deficient monocytes. To test this hypothesis, we examined the chemotaxis of mouse monocytes to MCP-1. Mouse monocytes were isolated from bone marrow of *wild-type* C57BL/6J, *Trpm2*$^{-/-}$ and *CD38*$^{-/-}$ mice by positive selection using anti-CD115. Purity was ≥70% as assessed by FACs. Chemotaxis assays with mouse monocytes were performed with 24-well Transwell plates (Costar, Cambridge, MA) with a 5-µm pore size polycarbonate filters. MCP-1 was diluted in HBSS, and placed in the lower chamber and cells were added to the upper chamber of the Transwell. For mouse monocyte assays, 5x10^{5}cells/Transwell were incubated at 37ºC for 2h. The transmigrated cells were collected from the lower chamber, fixed, and counted on a flow cytometer. MPC-1 was tested in *wild-type* monocytes at different concentrations from 10ng/ml to 300ng/ml. Interestingly, monocytes have a higher chemotactic response to low concentrations of MCP-1 (data not shown). Using 25ng/ml of MCP-1 as the chemoattractant, *Trpm2*$^{-/-}$ and *Cd38*$^{-/-}$ monocytes did not appear to have an intrinsic defect in chemotaxis to MCP-1 (**Figure 16**).
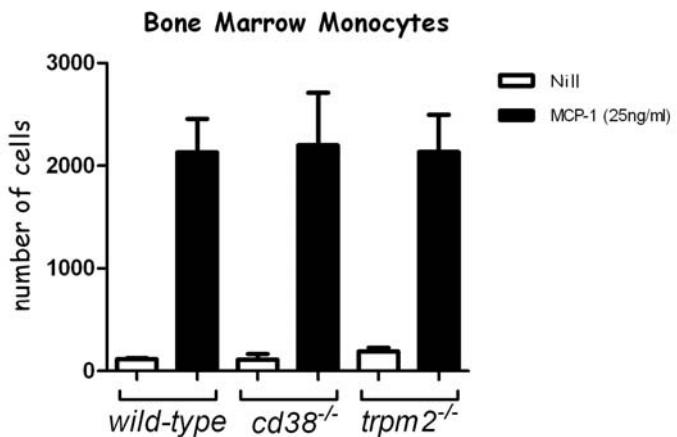
Figure 16. Chemotaxis of mouse bone marrow monocytes to MCP-1. Bone marrow monocytes were purified from *wild-type* C57BL/6J, *Cd38*$^{-/-}$ and *Trpm2*$^{-/-}$ mice. B cells were placed in the upper wells of chemotaxis chambers and the lower wells of the chamber contain HBSS plus MCP-1 (25ng/ml). Transmigrated cells were collected after 2h from the bottom chamber and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown are representative of four or more independent experiments.

3.2. Mouse neutrophils show a large expression of P_2Y_{13} and P_2X_7.

Collectively, our data using *Trpm2$^{-/-}$* cells indicated that TRPM2 channel is not required for the chemotaxis of mouse leukocytes to chemokines that signal in a CD38-dependent fashion. Therefore, our original hypothesis was incorrect and we were still left with the question of how CD38 and its metabolites, cADPR and ADPR, regulate calcium influx in chemokine stimulated cells. One possibility that we considered is that the ADPR made by CD38 might directly or indirectly activate other receptors that can mediate calcium mobilization. We therefore decided to address the hypothesis that ADPR or its breakdown products ADP or adenosine could activate P1 or P2 receptors. To test this hypothesis, we first analyzed P1 and P2X/P2Y receptor expression (mRNA) in mouse bone marrow neutrophils.

Expression of mRNA of P1 and P2 receptors in mice neutrophils was determined with Real-Time RT-PCR using neutrophils isolated from *wild-type*, *Cd38$^{-/-}$* and *Trpm2$^{-/-}$* mice. Purinergic receptors such as A_1, A_{2b}, A_3, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{10}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$, $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$ and $P2X_7$ were examined and RT-PCR analysis suggested that mouse neutrophils express predominantly $P2Y_{13}$ and $P2X_7$ receptors (**Figure 17**). However, A_3, $P2Y_6$, $P2Y_{12}$, $P2X_1$ and $P2X_4$ mRNAs are also highly expressed in mouse neutrophils. The mRNA purinergic receptor data are shown as fold change in mRNA expression compared with *wild-type* $P2Y_1$ (noted in the graph). We can not ignore the fact that low mRNA levels may still be biologically significant, moreover, high levels of mRNA do not necessarily imply that functional protein is produced. The data also show that there are no differences in the expression of receptors analyzed in *wild-type*, *Cd38$^{-/-}$* and *Trpm2$^{-/-}$* mice.
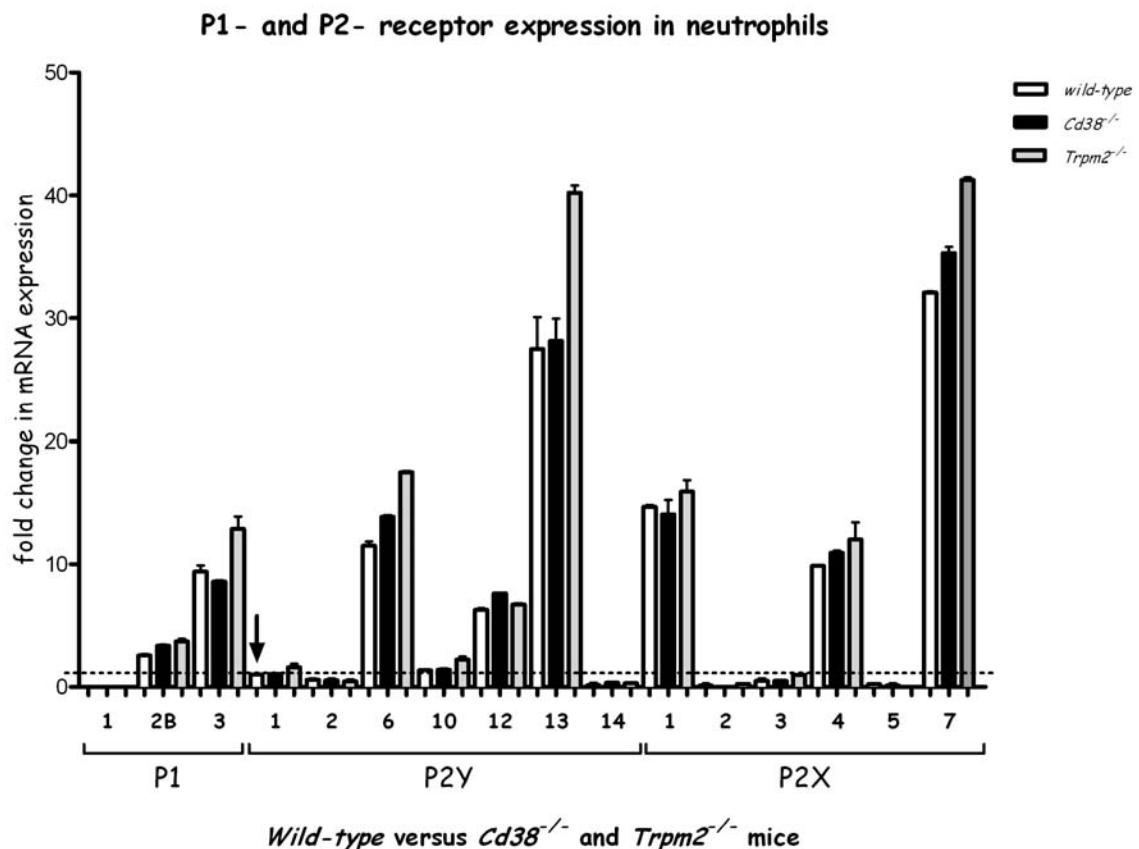
Figure 17. Purinergic receptor expression in mice bone marrow neutrophils. Basal purinergic receptor mRNA expression in mice neutrophils from *wild-type*, *Cd38^{-/-}* and *Trpm2^{-/-}* was estimated with real-time RT-PCR analysis. Data are expressed as fold increase in relation to $P2Y_1$ in *wild-type*. Error bars indicate ±SD of 2-6 determinations.

3.3. Suramin inhibits chemotaxis of mouse bone marrow neutrophils to fMLP but not to IL-8

a) *In vitro* chemotaxis of mouse bone marrow neutrophils to fMLP is inhibited by suramin and CGS-15943.

Next, to address whether inhibition of either P1 or P2 receptors blocks chemotaxis of mouse neutrophils, we treated the cells with three different antagonists, suramin, CGS-15943 and ATP. Suramin is a non-selective antagonist of P2X and P2Y receptors, it blocks homomeric $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$, heteromeric $P2X_{2/3}$ and $P2X_{1/5}$ and $P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{13}$ receptors, but is weak or inactive as an antagonist at homomeric $P2X_4$,

P2X$_6$ and P2X$_7$, heteromeric P2X$_{4/6}$ and P2Y$_4$ and P2Y$_6$ receptors. In contrast, CGS-15943 is a widely used highly potent, non-selective adenosine receptor (P1 receptor) antagonist while ATP in high concentrations can block signaling through both purinergic P2 and P1 receptors. We incubated mouse neutrophils in the presence or absence of Suramin, CGS-15943 or ATP at multiple concentrations for 15min and then measured the migration of these cells to fMLP. Suramin was tested at different concentrations from 1µM to 300µM (concentrations higher than 100µM produce complete inhibitory effect, data not shown). CGS-15943 was tested from 0.1µM to 100µM. ATP was tested from 10µM to 300µM. Suramin did inhibit the migration of mouse neutrophils, 10 µM was sufficient to inhibit strongly the migration to fMLP (**Figure 18.a**). Likewise, CGS-15943 inhibited the migration of mouse neutophils (**Figure 8.b**). However, while 10µM CGS-15943 has the same inhibitory effect as 100µM of CGS-15943, 100µM of suramin produced a total inhibition of mouse neutrophil to fMLP. Lastly, ATP produced a weak inhibition of neutrophil migration to fMLP (**Figure 8.c**), even using high concentrations as 300µM (data not shown).
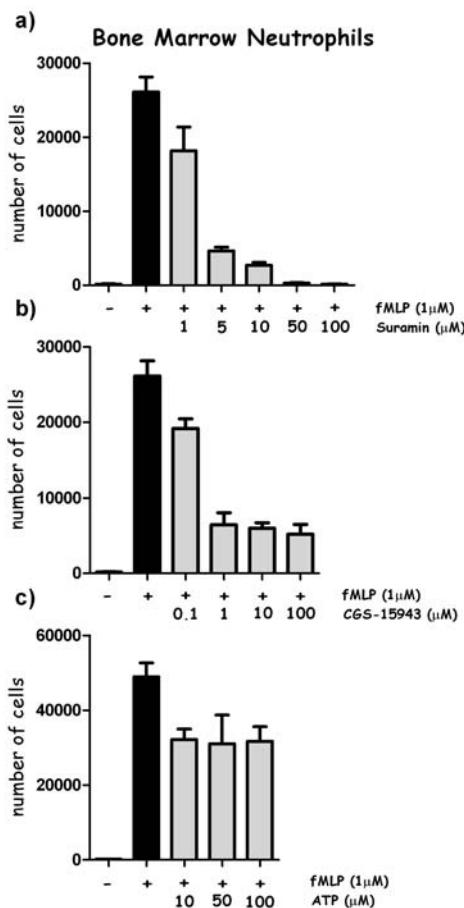
Figure 18. Chemotaxis of mouse bone marrow neutrophils to fMLP is inhibited by Suramin and CGS-15943. Bone marrow neutrophils isolated from *wild-type* C57BL/6J mice were preincubated for 15 min in medium or medium containing suramin (a), CGS-15943 (b) and ATP (c) at different concentrations. Cells were then placed in the upper wells of chemotaxis chambers that contained medium and medium plus fMLP in the lower wells of the chamber. Transmigrated cells were collected after 45 min and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown are representative of three or more independent experiments.

b) *In vitro* chemotaxis of mouse bone marrow neutrophils to IL-8 is inhibited by CGS-15943 but not by suramin.

The chemokine interleukin-8 is a potent activator of neutrophils and induces chemotaxis in a CD38/cADPR/ADPR independent fashion. Therefore, we expected that any inhibitor that blocked the CD38-dependent FPR signaling pathway shoud not have an effect on the CD38-independent IL-8 signaling pathway. To test whether suramin and CGS-15943 inhibits the migration of mouse neutrophils to IL-8, as they did to fMLP, we measured the chemotactic response of suramin and CGS-15943-treated neutrophils isolated from bone marrow of wild type C57BL/6 mice. Suramin and CGS-15943 were tested at a concentration of 10μM. We incubated mouse neutrophils in the presence or absence of suramin and CGS-15943 at 10μM and then measured the migration of these cells to fMLP and IL-8 in parallel. Interestingly, unlike what happens with fMLP, suramin did not inhibit the migration of mouse neutrophils to IL-8. By contrast, CGS-15943 inhibits the migration of neutrophils to fMLP and IL-8. This data show that suramin inhibits fMLP signalling pathway, but has no effect on the IL-8 signalling pathway (**Figure 19**). In others words, suramin interferes only with the CD38 dependent signalling pathway while CGS-15943 interferes with both the CD38 dependent and independent signalling pathways. Therefore, we conclude that a receptor that can be inhibited with low dose suramin is likely involved in regulating the CD38 dependent calcium response and chemotaxis.
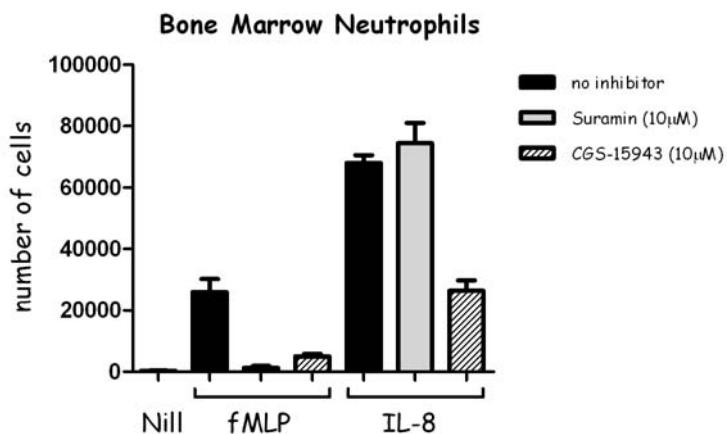
Figure 19. Chemotaxis of mouse bone marrow neutrophils to IL-8 is inhibited by CGS-15943 and not by suramin. Bone marrow neutrophils isolated from *wild-type* C57BL/6J mice were preincubated for 15 min in medium or medium containing suramin and CGS at 10μM. Cells were then placed in the upper wells of chemotaxis chambers that contained medium and medium plus fMLP or IL-8 in the lower wells of the chamber. Transmigrated cells were collected after 45 min and enumerated by flow cytometry. The results are expressed as the mean ±SD of triplicate culture. The data shown are representative of three or more independent experiments.

4. Discussion

CD38 is a multifunctional enzyme widely expressed in hematopoietic and non-hematopoietic tissues [5, 6]. CD38 uses β-NAD$^+$ as a subtrate to catalyze the production of three Ca^{2+}-mobilizing metabolites, ADPR, cADPR, and nicotinic acid dinucleotide phosphate (NAADP$^+$) [10]. cADPR induce intracellular Ca^{2+} release from ryanodine receptor (RyR)-dependent Ca^{2+} stores [15, 16] and NAADP$^+$ induces intracellular Ca^{2+} release from lysosomes, endosomes [26, 27] and the endoplasmic reticulum [28, 29]. ADPR induces extracellular Ca^{2+} influx in myeloid cells by activating TRPM2 [13, 14, 30]. cADPR also has the ability to induce Ca^{2+} entry [20, 121]. The physiological relevance of these metabolites is just beginning to be explored.

Frances Lund´s lab has demonstrated that cADPR induce both intracellular and extracellular Ca^{2+} mobilization and ADPR induce extracellular Ca^{2+} mobilization regulating phagocyte chemotaxis and cell migration of immune cells [23, 37-39]. 8Br-NAD$^+$, 8Br-cADPR

and 8Br-ADPR analogs inhibit mouse neutrophil and DCs chemotaxi, showing that both cADPR and ADPR are obligate components of the process by which CD38 modulates chemotaxis. The molecular mechanism of how these metabolites exerts their activity is not fully understood, however it is clear that the chemotactic response of mouse neutrophils and DCs to multiple chemokines is dependent on Ca^{2+} influx through a plasma membrane channel. Although we do not know the identity of the Ca^{2+}/cation channel activated in response to chemokine receptor ligation, we know that this channel is sensitive to the presence of cADPR antagonist, is dependent on intracellular Ca^{2+} release, can be blocked with an ADPR analog, and it is not a classical SOC like Orail/CRACM. We have hypothesized that ADPR, the main metabolite produced by CD38, directly activates Ca^{2+} influx through TRPM2 channel [39, 54]. Interestingly, low concentrations of cADPR can significantly potentiate the effect of ADPR [42]. It is known that intracellular free Ca^{2+} also enhances ADPR-gating of TRPM2 [44]. It has been postulated that cADPR may synergize directly with ADPR or induce Ca^{2+} release via RyRs and that this free Ca^{2+} synergizes with ADPR to induce Ca^{2+} influx through TRPM2, ADPR alone, or in combination with cADPR, may play a role in CD38-dependent Ca^{2+} influx and cell migration.

It is still difficult to understand how an ectoenzyme, like CD38, regulates Ca^{2+} responses that are activated by metabolites, such as cADPR and ADPR, that appear to function inside cells. cADPR has to bind to RyR-dependent stores to induce Ca^{2+} release, and ADPR has to bind to the cytoplasmic NUDT9-H domain to induce Ca^{2+} entry [49, 122]. However, it has been reported that the extracellular second messengers made by CD38 can be transferred by nucleoside transporters from the outside to the inside of the cell [123], thereby allowing the second messengers to access their cytosolic binding sites.

The first approach that we conducted was to demonstrate whether the TRPM2 channel was involved in the CD38 signalling pathway. Until recently we were not able to do this study because there are no selective inhibitors for TRPM2 channel and *Trpm2^-/-* mice had not been generated. Once we had access to *Trpm2^-/-* mice we tested chemotaxis of severals types of cells to chemokines that signal in a CD38-dependent and CD38-independent manner. We examined the chemotactic response of mouse bone marrow neutrophils from *Trpm2^-/-* mice to fMLP and IL-8. We generated *in vitro* immature and mature DCs from *Trpm2^-/-* mice and checked chemotaxis to SDF-1 and ELC, respectively. SDF-1, ELC and SLC were also analyzed for their chemotactic potential for splenic CD4+ T cells from

Trpm2^{-/-}, and WT mice. Chemotaxis of splenic B cells to SDF-1 and BLC was also tested. Finally we studied the migration of bone marrow monocytes to MCP-1 from both *CD38*^{-/-} and *Trpm2*^{-/-} mice. None of the TRPM2 deficient cells types showed defective migration to the chemokines tested. These results indicate that TRPM2 presumably is not the plasma membrane channel involved in the CD38 signalling pathway (**Figure 20**).
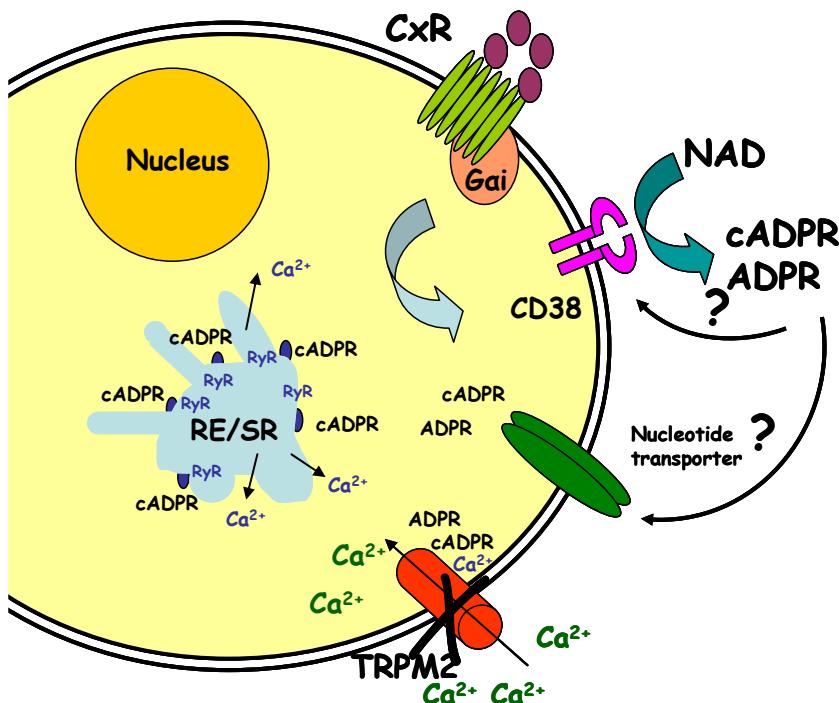
Figure 20. Using *Trpm2*^{-/-} mice we have tested chemotaxis of several types of cells to chemokines that signal in a CD38-dependent manner. The results indicate that TRPM2 presumably is not the plasma membrane channel involved in the CD38 signalling patway.

Clearly, Lund´s lab data show that metabolites made by CD38 play critical roles in regulating leukocyte migration but, the most surprising data is that CD38 controls the chemotaxis to some but not all chemokines since CD38/cADPR/ADPR signalling pathway does not control signalling through all of the known chemokine/chemoattractant receptors. The activation of Ca^{2+} influx by cADPR/ADPR is not required for either Ca^{2+} or chemotactic responses of mouse neutrophils, responding to the CXCR1/CXCR2 agonist IL-8 and MIP2. In addition, *Partida-Sanchez et al.* [37] found that CD38 regulates the migration of dendritic

cells (DC) precursors from the blood to peripheral sites and controls the migration of mature DCs from sites of inflammation to lymph nodes. CD38 and its metabolites modulate calcium mobilization in chemokine-stimulated DCs and are required for the chemotaxis of immature DCs to MCP-1and SDF-1 and mature DCs to SLC and ELC.

It is known that chemokine receptors couple to Gi-containing G proteins and that activation of Gi, with the subsequent release of free βγ subunits, is required for chemotaxis [124]. It has been known for many years that chemokine receptors can also couple to other G proteins, including Gq family members [125]. *Shi et al.* [47] have taken a key step in understanding the signalling pathway of CD38, they revealed that Gαi-2, although necessary, is not sufficient to induce chemotaxis of primary leukocytes to a large array of chemoattractants. Instead, an additional alternative Gαq-coupled pathway must be engaged before primary neutrophils and DCs can migrate, and this second, alternative Gαq dependent pathway is critically important for cell trafficking *in vitro* and, more importantly, *in vivo*, at least in response to inflammatory stimuli. Gαq, like CD38, regulates extracellular calcium entry in chemokine-stimulated cells, playing a role in regulating calcium mobilization after chemokine receptor ligation through a plasma membrane channel. Interestingly, the authors suggest that CD38 and Gαq are coregulators of the same calcium channel. Summarizing, CD38, an alternative Gαq-coupled pathway and extracellular calcium influx are required for the chemotaxis of mouse bone marrow neutrophils to fMLP and are also necessary for chemotaxis of immature DCs to MCP-1and SDF-1 and mature DCs to SLC and ELC.

Given that our hypothesis that TRPM2 was obligate for the CD38 chemokine receptor signalling pathway was incorrect, we decided to investigate whether purinergic receptors could be required for CD38 dependent calcium signalling and chemotaxis. Several studies indicated that nucleotides and nucleotides derivatives may play an important role in chemotaxis through their action on purinergic receptors and interestingly, the P2Y subtype purinergic receptors are coupled to Gαq. As mentioned above, CD38 uses β-NAD$^+$ as a substrate to catalyze the production of three Ca^{2+}-mobilizing metabolites, ADPR, cADPR, and NAADP$^+$. Interestingly, two recent studies report that NAD$^+$ itself may be an agonist for P2Y$_{11}$ receptors in granulocytes and P2Y$_1$ receptors in visceral smooth muscle [59, 126]. NAD$^+$ has been also related with ionotropic P2X receptors, *Grahnert et al.* [61] showed that the extracellular addition of NAD$^+$ promotes a rise on the influx of extracellular calcium in human monocytes and that the Ca^{2+} response is prevented by antagonists acting at ATP

receptors, suggesting the involvement of ATP receptors. Using subtype preferential agonists and antagonists, they identified $P2X_1$, $P2X_4$ and $P2X_7$ receptors being engaged in the NAD-induced rise in Ca^{2+}. NAD^+ unlike ATP lacks the ability to induce activation of a non-selective pore by the $P2X_7$ receptor subtype. Even more interesting is the fact that products of CD38, $NAADP^+$ and ADPR, may act as agonists of P2Y receptors. $NAADP^+$ has been identified as an agonist of the human $P2Y_{11}$ purinergic receptor [127] and triggered a sustained (Ca^{2+}) elevation by influx of extracellular Ca^{2+}. Extracellular ADPR also acts as a primary P2Y and adenosine receptor agonist in primary β cells [48]. The sugar nucleotide UDP-glucose acts as agonist of $P2Y_{14}$ [128], so it is reasonable to think that other sugar-derivatized nucleotides may also act as agonists for purinergic receptors.

In order to address the possibility that purinergic receptors were involved in the CD38 signalling pathway, we first analyzed the expression of purinergic receptors in mouse neutrophils. Our study using quantitative reverse transcriptase polymerase chain reaction (RT-PCR) analysis have shown that mice neutrophils isolated from *wild type*, *Cd38$^{-/-}$* and *Trpm2$^{-/-}$* mice express high levels of $P2X_1$, $P2X_4$, $P2X_7$, $P2Y_6$, $P2Y_{13}$ and A_3 mRNA. A_{2B} and $P2Y_{12}$ are also expressed but to a lesser extent. A_{2A}, $P2Y_4$, $P2Y_{11}$ and $P2X_6$ were not included in this study. Secondly, we tested the effects on mouse neutrophil chemotaxis of the non-selective antagonists, suramin and CGS-15943, and the natural agonist ATP of purinergic receptors. Suramin is a non-selective antagonist at native P2X and P2Y receptors [62]. Suramin can not be considered selective for any particular recombinant P2 receptor subtype. It blocks homomeric $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$, heteromeric $P2X_{2/3}$ and $P2X_{1/5}$ and $P2Y_1$, $P2Y_2$, $P2Y_{11}$ and $P2Y_{13}$ receptors, but is weak or inactive as an antagonist at homomeric $P2X_4$, $P2X_6$ and $P2X_7$, heteromeric $P2X_{4/6}$ and $P2Y_4$ and $P2Y_6$ receptors. However, Suramin when given at high micromolar concentration affects all nucleotide-sensitive P2Y receptors with the exception of $P2Y_4$ receptors. Chemotaxis of mouse bone marrow neutrophils to fMLP is strongly inhibited by 10µM suramin, however the same amount of suramin did not affect the chemotaxis to IL-8. This result means that suramin is acting specifically in fMLP signalling pathway or, in other words, that suramin interferes with CD38 signalling pathway. We also examined the effect of CSG-15943 a highly potent, non selective adenosine receptor agonist, on chemotaxis. Chemotaxis of mouse bone marrow neutrophils to fMLP and IL-8 was strongly inhibited by 10µM of CGS-15943, so the adenosine receptor or receptors that we are blocking with this antagonist are involved in both signalling pathway. The effect of ATP on chemotaxis neutrophils to fMLP is difficult to

explain, as we expected to find that it would block neturophil chemotaxis. However, we showed that mouse neutrophils incubated with ATP only weakly decreased chemotaxis to fMLP. We can not exclude the possibility that the signalling promoted by a theoretical purinergic receptor was interfered with by ATP. The situation is further complicated by the fact that extracellular ATP is rapidly metabolized, and its break-down products, notably ADP and adenosine, have signalling functions of their own through different receptors. It has been reported that activation of the A_3 adenosine receptor suppresses superoxide production and chemotaxis of mouse bone marrow neutrophils to fMLP inhibiting Rac activation [129]. The effect of ATP on chemotaxis neutrophils to IL-8 has not been examined, however it would be interesting to know whether incubation of neutrophils with ATP also produces a slight decrease in chemotaxis to IL-8. All these data show that adenosine receptors likely play a role in the chemotactic response of the neutrophils but these receptors regulate both CD38 dependent and independent signalling pathways. On the other hand, suramin appears to block a receptor that is involved in the CD38 signalling pathway specifically. However, we have to note that suramin presents limitations as a tool for the characterization of P2 receptors because it interacts with various proteases, ecto-nucleotidases and protein kinases as well as G-protein subunits. However it is clear that suramin interrupts the neutrophil migration to fMLP but not IL-8.

Levels of $P2X_2$, $P2X_3$ and $P2X_5$ transcripts are very low in neutrophils. We do not have the data from $P2X_6$ expresion in neutrophils, but we know that $P2X_6$ receptors are suramin insensitive, suggesting that this receptor is not likely to mediate Ca^{2+} influx in chemokine-stimulated neutrophils. However, we have shown previously that mouse neutrophils express high levels of $P2X_1$, $P2X_4$ and $P2X_7$ mRNA. These receptor proteins are co-expressed in most immune cells, including mast cells, B and T lymphocytes, monocytes, macrophages, microglia, and osteoclasts [130]. Cation currents and calcium influx with the features expected for each of these P2X receptors have been demonstrated in all these immune cells. Although the detailed signalling mechanisms have not been established for most P2X receptors subtypes, it is well-known that cytoplasmic Ca^{2+} triggers a variety of intracellular events, in part, through activation of MAPKs, PKC, and calmodulin.

$P2X_1$ ion channels play a significant role in the neutrophils response to chemotactic stimuli. Recently *Lecut et al.* [131] showed the first evidence that $P2X_1$ ion channels contribute to the control of neutrophil chemotaxis. First, the selective $P2X_1$ agonists,

αβMeATP and βγMeATP, are not chemoattractant by themselves, but they caused random migration (chemokinesis) and amplified chemotaxis induced by endogenous (IL-8) and bacterial exogenous chemoattractants. Second, $P2x_1^{-/-}$ neutrophils did not accumulate in the peritoneum upon *E. coli* injection. Third, two-dimensional chemotaxis assays indicated that $P2x_1^{-/-}$ neutrophils moved with diminished speed when placed in a gradient of a bacterial mimetic peptide. In this study, they found that signals elicited upon P2X$_1$ activation involved RhoA, Rho kinase, and myosin L chain phosphorylation. P2X$_1$ seems to be a good candidate among the P2X-type receptors since it is highly expressed in mouse neutrophils, suramin is highly active as a P2X$_1$ antagonist and finally, activation of P2X$_1$ induces a rise in $(Ca^{2+})_i$ caused by an influx of extracellular calcium. Using the most potent and selective antagonist at P2X$_1$ receptors NF449 or $P2x_1^{-/-}$ deficient mice in future experiments, we could demonstrate the possible involvement of P2X$_1$ receptor with CD38 signalling pathway.

P2X$_7$ is expressed predominantly by cells of immune origin and it is critically involved in inflammation; prolonged activation of P2X$_7$ in immune cells such as macrophages leads to release of the pro-inflammatory cytokine interleukin-1β (IL-1β), activation of the inflammasome and, at times, cell death [132, 133]. Knockdown of the P2X$_7$ receptor abolished the ATP-stimulated processing and release of IL-1β in mouse macrophages [78]. Interestingly, P2X$_7$ is indirectly related with CD38. ATP and NAD$^+$ activate the P2X$_7$ purinoceptor although by different mechanisms and with different characteristics. While ATP activates P2X$_7$ directly as a soluble ligand, activation via NAD$^+$ occurs by ART-dependent ADP-ribosylation of the cell surface of P2X7, providing an immobilised permanent ligand. P2X$_7$ activation by either route leads to phosphatidylserine exposure, shedding of CD62L, and ultimately to cell death. Activation by ATP requires high micromolar concentrations of nucleotide and is readily reversible, whereas NAD-dependent stimulation begins at low micromolar concentrations and is more stable. Experiments *in vitro* point to a major role of CD38 in controlling the level of ADP-ribosylation on the surface of naïve T cells by limiting the concentration of available extracellular NAD$^+$ for ART2 [134]. The cytolytic ionotropic ATP receptor P2X$_7$ is frequently coexpressed with the P2X$_4$. *Guo el al.* [135], provided biochemical and electrophysiological evidence for an association between P2X$_4$ and P2X$_7$ that increased the diversity of receptor currents mediated via these two subtypes. Suramin is weak or nearly inactive as a P2X$_7$ and P2X$_4$ receptor antagonist, however, A-740003 is a highly specific and potent antagonist for P2X$_7$ receptors. $P2x7^{-/-}$ mice are also available.

Among the P2Y-type receptors, there are many interesting possibilities of candidates that may be involved in mediating CD38-dependent chemotaxis. In particular, P2Yrs that are couple to Gαq like $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ and P2YRs that are activated by adenine dinucleotides as $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ (**Figure 21**) may be of interest. High levels of $P2Y_6$, $P2Y_{12}$ and $P2Y_{13}$ transcripts are found in bone marrow neutrophils, however, $P2Y_1$, $P2Y_2$, $P2Y_{10}$ and $P2Y_{14}$ receptors are expressed at low levels.
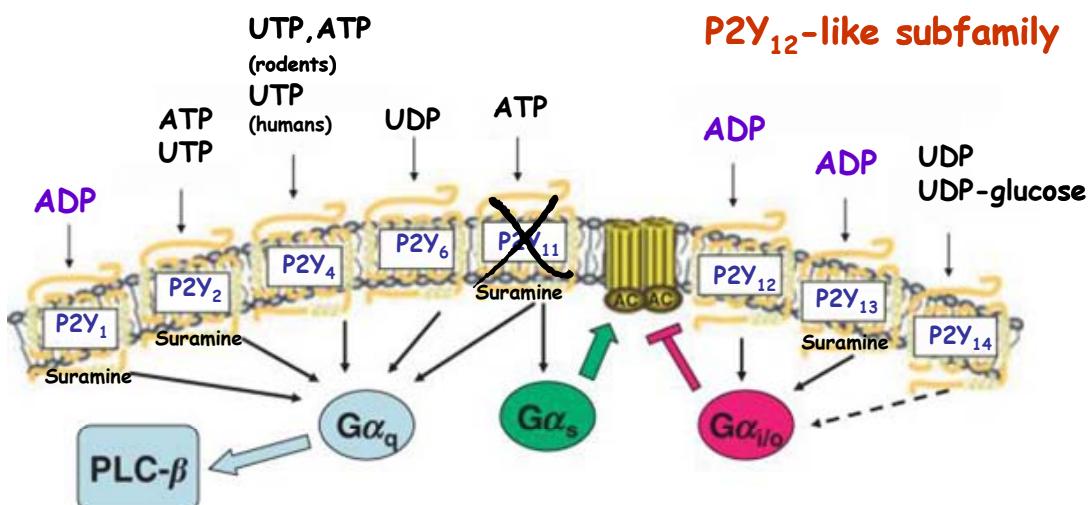

Figure 21. The P2Y receptors. The eight mammalian P2Y receptors and their cognate agonists are indicated. The five members of the $P2Y_1$-like subfamily all couple to heterotrimeric G-protein of the Gαq family and activate phospholipase C-β isozyme. The ATP-activated $P2Y_{11}$ receptor also couples to Gαs resulting in activation of adenylyl cyclase (AC). The three members of the $P2Y_{12}$-like subfamily couple heterotrimeric G proteins of the Gαi/o family and inhibit adenylyl cyclase (AC) activity [136].

The mammalian $P2Y_1$-receptor is selective for adenine nucleotides. P2Y1R is involved in increase of Ca^{2+} levels in response to ADP in mouse peritoneal macrophages [137]. Among its physiological functions in mice are platelet aggregation [138, 139], smooth muscle relaxation [140] and eterosclerosis enhancement [141]. $P2Y_1$-deficient mouse model has been generated which a phenotype showing decresed platelet aggregation and increase

bleading [138, 139]. Interestingly *in vivo* knockdown of the $P2Y_1$ receptor impairs the migration of neural progenitors to the subventricular zone [142]. MRS2179, MRS2279 and MRS 2500 are selective antagonists of $P2Y_1$ receptors. Although P2Y1 transcripts levels are not high in neutrophils, the gene is transcribed. , Thus, P2Y1 seems to be a good candidate to examine further since it is activate by adenine ninucleotides, is coupled to $G\alpha q$ and is inhibited by suramin.

UTP and ATP are full agonists at $P2Y_2$ receptors. When tested under conditions excluding enzymatic conversion of nucleotides, UDP and ADP did not activate the receptor [143]. $P2Y_2$ receptors are involved in the mediation of ion transport in the trachea and the gallbladder [144], activation of luminal K^+ secretion [145] and migration of vascular smooth muscle cells in mice [146]. Also P2Y2R are involved in orientation of human neutrophils chemotaxis [147]. To amplify external signals and migrate in a chemotactic field, human neutrophils release ATP upon stimulation of the formyl peptide receptor (FPR) to amplify chemotactic signals through $P2Y_2$ receptors. Neutrophils rapidly hydrolyze released ATP to adenosine that then acts via A_3-type adenosine receptors [147]. Recently, is has been reported that stimulation of FPR led to this receptor to colocalize with $P2Y_2$ receptors on the cell surface to form a purinergic signalling system that facilitated human neutrophil activation and innate host responses to bacterial infection [148]. IL-8-induced human neutrophils chemotaxis requires also an activation of P2Y receptors, most likely the $P2Y_2$, however, adenosine had no effect on human neutrophil migration towards IL-8 [149]. Moreover, A_3 and $P2Y_2$ receptors control the recruitment of neutrophils to the lungs in a mouse model of sepsis [150]. Surprisingly, according to our data, $P2Y_2$ receptor mRNA is expressed at very low levels by RT-PCR. However, low expression of $P2Y_2$ mRNA may be important biologically in mouse neutrophils. $P2y2^{-/-}$ mice exhibit abolished Ca^{2+} mobilisation in response to UTP. Good selective antagonists for $P2Y_2$ have not been found, however the $P2Y_2$ deficient mice are available.

The human $P2Y_4$-receptor is highly selective for uracil triphosphate derivatives [151, 152]. When studied under conditions chosen to ensure the stability to the nucleotides, the diphosphate nucleotides UDP and ADP were inactive [143]. In contrast to the human $P2Y_4$-receptor, the rodent ortholougs are activated about equally by UTP and ATP. In mouse, they have been involved in regulation of chloride epithelial transport in jejunum [153, 154] and activation of luminal K^+ secretion in colon [145]. $P2y4^{-/-}$ mice exhibit reduced K^+ secretion in

the luminal colonic mucosa [145] and abolish chloride secretory responses in mouse jejunum [154]. This receptor was not included in our study of P2X receptor mRNA expression. There are no selective antagonists for $P2Y_4$.

The $P2Y_6$-receptor prefers UDP as a binding ligand [143]. It has been implicated in Ca^{2+} levels in response to UDP in mouse peritoneal macrophage cells [137] and modulation of intracellular calcium concentrations in satellite glial cells from mouse trigeminal ganglia [155]. Cl^- secretion in trachea [156], protection from apoptosis both *in vivo* and *in vitro* in skeletal muscle [157] and modulation of insulin and glucagon secretion in isolated pancreatic islets and purified β-cells [158] are some examples of physiological functions of these receptors in mouse. *P2y$^{6-/-}$* mice exhibit a reduced macrophage response to UDP and LPS, and the abolition of endothelium-dependent aorta relaxation and of smooth muscle-dependent aorta contraction by UDP [159]. MRS2567 is a selective agonist for $P2Y_6$.

It is interesting to note that $NAADP^+$, one of the products of CD38, has been already identified as agonist of human $P2Y_{11}$ receptors and interestingly, they seem to have a role in maturation and migration of dendritic cells [160]. Because $P2Y_{11}$ transcripts have not been found in rats and mice, it was not included in our study of purinergic receptor expression. This is unfortunate because the $P2Y_{11}$ receptor seemed a good candidate since $P2Y_{11}$ is inhibited by suramin and also is a Gαq-coupled receptor. NF157 was the first good antagonist at $P2Y_{11}$ receptors but it has the same potency at $P2X_1$ receptors. Another analogue, with a higher potency at $P2Y_{11}$ receptor is NF340.

$P2Y_{12}$ receptor is activated by adenine diphosphate derivatives. A role in the vessel wall response to arterial injury and thrombosis [161] and a role in microglial chemotaxis [162, 163] have been associated with mouse $P2Y_{12}$ receptors. *P2y2$^{-/-}$* mice show impaired platelet activation/adhesion in *in vivo* mesenteric arteries [164] and significantly disminished bidirectional branch extension toward sites of cortical damage in the living mouse [162]. AZD6140 is a selective antagonist of $P2Y_{12}$ receptor.

The $P2Y_{13}$-receptor responds to adenine diphosphate analogues. In some experimental system $P2Y_{12}/P2Y_{13}$ receptors were found to be coupled to Ca^{2+} increases. In particular in microglial cells and satellite glial cells from trigeminal ganglia, the ADP-induced $(Ca^{2+})_i$ responses were significantly reduced after pretreatement with Cangrelor, an

antagonist selective for P2Y$_{12}$ and P2Y$_{13}$, thus suggesting that a component of the (Ca^{2+})$_i$ responses evoked by ADP results from P2Y$_{12}$ and P2Y$_{13}$ receptor activation [155, 165]. MRS2211 is a selective antagonist of P2Y$_{13}$ receptors. *P2y13$^{-/-}$* mice have been generated recently.

The P2Y$_{14}$ receptor is activated by UDP-glucose, UDP-galactose and ADP-glucuronic acid. A very recently report shows that UDP is also a potent agonist of this receptor [136]. P2Y$_{14}$ is prominently associated with immune and inflammatory cells, however there is no enough data from mice. These receptors are involved in chemotaxis in human bone marrow stroma cells [166]. Antagonists for P2Y$_{14}$ have not been found, however *P2y14$^{-/-}$* mice have been generated recently.
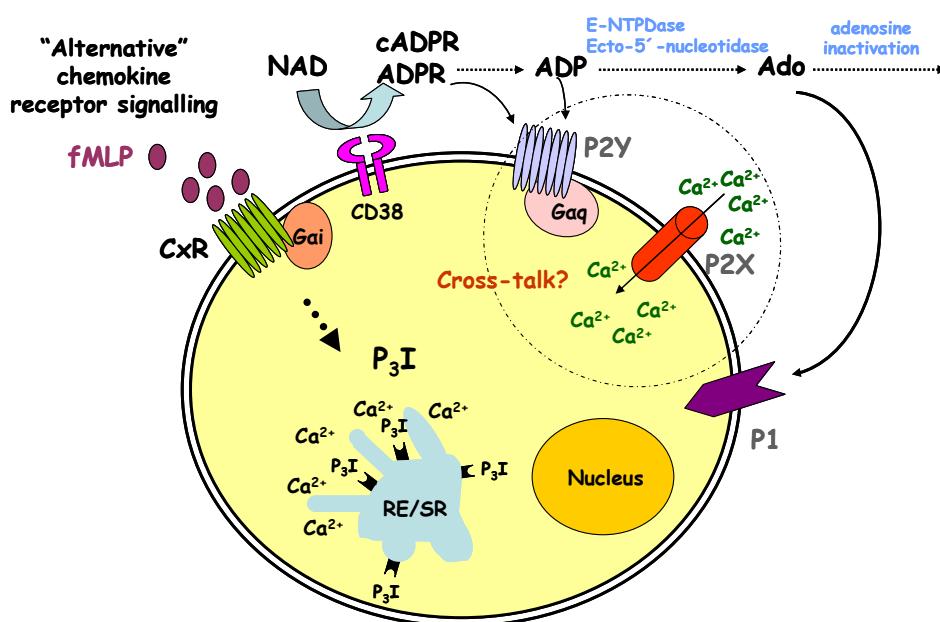
Figure 22. ADPR made by CD38 might directly or indirectly through its breakdown products that can mediate calcium mobilization as purinergic receptors.

It is more than likely that the picture that we face is more complex than expected. The cross-talk between several receptors could lead to the entry of Ca^{2+} into the cells (**Figure 22**). There are many examples of interplay between purinergic receptors. Activation of platelets by extracellular ADP plays a key role in thrombosis and hemostais and it is well established that co-stimulation of P2Y$_1$ and P2Y$_{12}$ receptors is required for aggregation. However, the

mechanism whereby these receptors interact remains poorly resolved. A nice report by *Tolhurst et al.* shows that $P2Y_1$ and $P2Y_{12}$ are required for complete activation of a nonselective cation channel that results in Ca^{2+} and Na^+ entry in marrow-derived megakaryocytes. Furthermore, $P2X_1$ receptors, after the secretion of ATP, can contribute repetively to the ADP-evoked currents and can act to accelerate the P2Y-receptor currents. This complex interplay may allow these 3 receptors to cooperate in the generation of Ca^{2+} influx during platelet activation [167].

In the future, the use of purinergic receptor-deficient mice with the use of specific inhibitors will help us to clarify the mechanisms of CD38-dependent chemotaxis. However, the first thing we plan to do is to demonstrate that suramin inhibits the chemotaxis to all those cytokines that signal in a CD38-dependent manner and not to those cytokines that signal independently of CD38. For now, we only know that suramin inhibits the neutrophils chemotaxis to fMLP but not to IL-8. Although these data are encouraging, it does not mean necessarily that suramin interferes specifically with the CD38 signalling pathway. Indeed, it is possible that suramin inhibits a receptor involved specifically in chemotaxis to fMLP but is not regulated by CD38. Our future experiments should clarify these points.

Finally it would be interesting to highlight the fact that both CD38 and some purinergic receptors are located in lipid rafts. During my stay in M. Zubiaur lab, we isolated lipid rafts from splenocytes and purified B cells from mice and a large percentage of CD38 was found in lipid rafts, so in the same way it is probably that a subset of CD38 is localized in lipid rafts in mouse neutrophils. Local concentrations of nucleotides surrounding lipid rafts are more tightly controlled than in the adjacent membrane and would promote a differential pattern of purinergic signalling in different microdomains. It is easy to predict that composition of lipid rafts containing CD38 will be very different in those neutrophils stimulated with fMLP than those stimulated with IL-8.

Probably CD38, together with other receptors, are involved in an integrative signalling as a multistep coordinated cascade. Without doubt we are at a fascinating point in the study of CD38 signalling mechanisms involved in cell migration.

5. References

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