Universitat Autònoma de Barcelona

Implicación del eosinófilo intestinal y producción del factor liberador de corticotropina en la fisiopatología del Síndrome del Intestino Irritable con predominio de diarrea

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HACEN CONSTAR

Que la memoria titulada **"Implicación del eosinófilo intestinal y producción del factor liberador de corticotropina en la fisiopatología del Síndrome del Intestino Irritable con predominio de diarrea"** presentada por Eloísa Salvo Romero para optar al grado de Doctor, se ha realizado bajo su dirección, y al considerarla concluida, autorizan su presentación para ser juzgada por el tribunal correspondiente.

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Dra. María Vicario Pérez **Dr. Javier Santos Vicente**

“En la vida no existe nada que temer,
solo cosas que comprender”
Marie Curie

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ABREVIATURAS

5-HT, serotonina

5-HTT, transportador serotonina

ACh, acetilcolina

ACTH, hormona corticotropica

APRIL, factor estimulador de la proliferación de células B

ATP, adenosina trifosfato

AVP, arginina vasopresina

BAFF, factor activador de células B

BDNF, factor neurotrófico derivado del cerebro

BMP-2, proteína morfogénica ósea

CAM, molécula de adhesión celular

CCK, colecistoquinina

CCR3, receptor eotaxina 3

CGA, campo de gran aumento

CGRP, péptido relacionado con el gen de la calcitonina

ChAT, colina acetil-transferasa

CHRM1, receptor colinérgico muscarínico 1

CHRM2, receptor colinérgico muscarínico 2

CHRM3, receptor colinérgico muscarínico 3

CLC, proteína charcot-leyden

CRF, factor liberador de corticotropina

CRFR1, receptor 1 del factor liberador de corticotropina

CRFR2, receptor 2 del factor liberador de corticotropina

DF, dispepsia funcional

ECP, proteína catiónica eosinofílica

EDN, neurotoxina derivada del eosinófilo

EEo, esofagitis eosinofílica

EII, enfermedad inflamatoria intestinal

EPO, peroxidasa del eosinófilo

ERGE, enfermedad reflujo esofágico

GALT, tejido linfoide asociado al intestino

GAP, uniones comunicantes

GM-CSF, factor estimulador de colonias de granulocitos y monocitos

HPA, eje hipotálamo-hipofisiario-adrenal

ICAM, molécula de adhesión celular intercelular

IFN-γ, interferón gamma

Ig, inmunoglobulina

IL-, interleucina

JAM, uniones de adhesión

MBP, proteína básica principal

MRI, resonancia magnética estructural

NCAM, molécula de adhesión celular neuronal

NGF, factor de crecimiento nervioso

NK1R, receptor neuroquinina 1

NPY, neuropéptido Y

NT-3, neurotrofina 3

PAR-2, receptor activación proteasas 2

ROS, especies reactivas del oxígeno

SAM, sistema-adreno-medular

SCF, factor de crecimiento de células madre

SII, síndrome del intestino irritable

SII-D, síndrome del intestino irritable predominio diarrea

SII-E, síndrome del intestino irritable predominio estreñimiento

SII-M, síndrome del intestino irritable mixto

SNA, sistema nervioso autónomo

SNARE, receptor de proteína de fijación soluble

SNC, sistema nervioso central

SNE, sistema nervioso entérico

SP, sustancia P

TGF-β, factor de crecimiento transformante β

TGI, tracto gastrointestinal

TNF-α, factor de necrosis tumoral alfa

TRPV1, receptor de potencial transitorio V1

UE, uniones estrechas

VCAM, molécula de adhesión celular vascular

VIP, péptido vasoactivo intestinal

ZO-, zonula ocludens

INTRODUCCIÓN

INTRODUCCIÓN

1 Función barrera intestinal

El tracto gastrointestinal (TGI) constituye una enorme superficie en contacto con el medio externo, con un área aproximada de 250 m^2 y, por ende, continuamente expuesta a antígenos y potenciales patógenos.[1] El TGI desarrolla la función digestiva, mediante la digestión y absorción de los nutrientes, incluyendo el transporte de agua y electrolitos y la secreción de agua a la luz intestinal. Además, es necesaria una función defensiva que impida el paso de sustancias potencialmente nocivas, como microorganismos patógenos, moléculas o factores tóxicos, desde la luz intestinal hacia el medio interno y que, al mismo tiempo, permita el paso selectivo de sustancias que favorecen el desarrollo del sistema inmunitario intestinal y la tolerancia inmunitaria hacia antígenos de la dieta y las bacterias comensales [1]. Estas dos funciones, digestiva y defensiva, son llevadas a cabo gracias a la peculiar anatomía de la mucosa intestinal y, en particular, de la denominada "función barrera intestinal" en la que confluyen diferentes mecanismos, inmunitarios y no inmunitarios, que actúan de forma coordinada para asegurar su correcto funcionamiento.

La capacidad del epitelio intestinal para actuar como una barrera eficaz entre los medios externo e interno está regulada estrechamente por diversos factores y es fundamental para el mantenimiento de la homeostasis intestinal y la salud humana. La disfunción de la barrera y el aumento de la permeabilidad intestinal se asocia con varias enfermedades, tanto intestinales, incluida la enfermedad inflamatoria intestinal (EII), la enfermedad celíaca y el síndrome del intestino irritable (SII), como extraintestinales, como la alergia alimentaria, el autismo o la artritis reumatoide, entre otras (revisado en[2–4]). Sin embargo, si este aumento en la permeabilidad intestinal es un epifenómeno, una manifestación temprana de la enfermedad, o un paso crítico en la patogénesis de la enfermedad, sigue siendo desconocido y ha sido objeto de amplio debate.

1.1 Mecanismos de barrera intestinal

Los elementos que componen la barrera intestinal se clasifican en función de su naturaleza y su localización anatómica, se agrupan en elementos extracelulares y celulares (figura 1).

1.1.1 Elementos extracelulares

En la luz intestinal se encuentra ya la primera línea de defensa del TGI, donde los microorganismos y antígenos son degradados de manera inespecífica por la acción del pH y de las secreciones gástricas, pancreáticas y biliares. Recubriendo el epitelio intestinal se encuentra un microclima consistente en una capa de moco, agua y glicocálix de aproximadamente unas 100 micras de espesor, con propiedades hidrófobas y tensoactivas, que previene la adhesión de microorganismos al epitelio intestinal [5]. Dentro de la capa de moco, se diferencia una capa externa, llamada capa de moco agitada), la cual contiene inmunoglobulina A secretora[6], sintetizada por las células plasmáticas de la mucosa, y productos antimicrobianos como fosfolípidos, mucinas y péptidos como defensinas, secretados por las células de Paneth.[7] Esta capa previene la adhesión a la mucosa y la invasión transepitelial posterior por parte de agentes microbiológicos y contribuye a la retención de secreciones mucosas ricas en péptidos, con actividad frente a bacterias, levaduras, hongos, virus e incluso células tumorales.[8,9] Adherida al epitelio se encuentra la capa de moco interna, no agitada y más densa, llamada también glicocálix, que facilita la absorción de nutrientes, mantiene la hidratación epitelial y protege el revestimiento epitelial de las fuerzas de cizallamiento luminales y de las enzimas digestivas.[10] El glicocálix también participa en la renovación y diferenciación epitelial, así como en el mantenimiento de la tolerancia oral, limitando la inmunogenicidad de los antígenos intestinales mediante la producción de señales tolerogénicas en las células dendríticas.[11] Por otra parte, la secreción de cloro y agua a la luz intestinal, llevada a cabo principalmente por los enterocitos, impide la colonización bacteriana y ralentiza la translocación de antígenos a la *lámina propia* ya que ejerce un efecto de dilución sobre el

contenido intestinal.[12] Finalmente, el peristaltismo, ejercido por las capas musculares del intestino, evacúa el contenido luminal, disminuyendo su tiempo de permanencia y, por lo tanto, el de sustancias potencialmente tóxicas presentes en la luz del intestino.

1.1.2 Elementos celulares

Microbiota intestinal: En la luz intestinal se encuentra también la flora intestinal o microbiota, componente esencial de la barrera intestinal que influye en el metabolismo, proliferación y mantenimiento del propio epitelio intestinal. La flora comensal, además, limita la colonización por parte de agentes patógenos ya que compite por los nutrientes y el nicho ecológico, modifica el pH y produce sustancias que permiten la comunicación entre especies y la optimización de la cantidad de microorganismos beneficiosos[13]. La microbiota intestinal ofrece, además, otras funciones cruciales para el huésped, como la adquisición de nutrientes y la regulación de la energía[14], e influye en procesos tales como la respuesta inflamatoria, la reparación epitelial y la angiogénesis.[15]

Epitelio intestinal: El epitelio intestinal está compuesto por una monocapa de células epiteliales especializadas y polarizadas que se renueva periódicamente cada 3 o 5 días. Las células madre epiteliales intestinales pluripotentes residen en la base de las criptas (criptas de Lieberkühn) y generan células que migran hacia la punta de la vellosidad donde tiene lugar la diferenciación final (Booth *et al.* 2000). Aunque la mayoría de las células que forman la monocapa son enterocitos (alrededor del 80%), la diversidad de funciones que el epitelio intestinal lleva a cabo se pone de manifiesto por la presencia de otros tipos celulares especializados en la secreción de moco (células de Goblet), de defensinas (células de Paneth), de hormonas y neuropéptidos (células enterocromafines) y células especializadas en la captación de antígenos de la luz intestinal, situadas en la superficie de agregados linfoides (células M).[17]. Los enterocitos son elementos clave del revestimiento epitelial y llevan a cabo la función digestiva, metabólica y el mantenimiento de la integridad física de la barrera. También intervienen en el desarrollo de la actividad defensiva ya

que expresan receptores implicados en la respuesta inmunitaria innata[18], actúan como células presentadoras de antígeno no profesionales y liberan varias citocinas y quimiocinas, como la linfopoyetina estromal tímica, el factor de crecimiento transformante-β1 (*transforming growth factor* β1, TGF-β1) [19], interleucina (IL) 25[20], el factor estimulador de la proliferación de células B (*a proliferation inducing ligand*, APRIL) y el factor activador de células B (*B cell activating factor*, BAFF)[21,22], participando en el reclutamiento y la activación de leucocitos y en la regulación de la respuesta inmunitaria local. En la región subepitelial se encuentra la *lámina propia*, que alberga células del sistema inmunitario, del sistema nervioso entérico (SNE) y del tejido conectivo.

Sistema inmunitario intestinal: Las células inmunitarias del tracto gastrointestinal conforman el denominado tejido linfoide asociado al intestino (*gut-associated lymphoid tissue,* GALT), que se divide en dos compartimentos: GALT organizado o inductor de la respuesta inmunitaria y GALT difuso o efector de la respuesta inmunitaria. El GALT organizado está compuesto de estructuras linfoides, principalmente folículos linfoides, placas de Peyer y ganglios mesentéricos.[23] El epitelio que cubre las placas de Peyer contiene células M, que desempeña un papel en la monitorización de la luz intestinal y en el mantenimiento de la función barrera intestinal ya que sus características físicas únicas, como la formación de micropliegues o la reducida capa de moco, facilitan la captura de antígenos y microorganismos luminales y su presentación a las células inmunitarias subyacentes[24]. Por su parte, el GALT difuso se compone de dos poblaciones de leucocitos distribuidos a ambos lados de la membrana basal. Los linfocitos intraepiteliales, mayoritariamente células T $CD8^+$, se encuentran entre las células epiteliales, por encima de la membrana basal, y su función principal es supervisar y responder frente a las bacterias y otros antígenos luminales. Los linfocitos de la *lámina propia* residen junto a otros tipos leucocitarios, tales como eosinófilos, células dendríticas, mastocitos y macrófagos, principalmente. Estos linfocitos constituyen una población heterogénea, siendo aproximadamente el 50% células plasmáticas y el 30% linfocitos T que, a su vez, se pueden dividir en distintos subtipos según el patrón de citocinas secretado.[25]

Estas poblaciones celulares subepiteliales residen en el tejido conectivo junto con neuronas, vasos sanguíneos y fibroblastos. Los fibroblastos mantienen la matriz extracelular, principalmente mediante la secreción de colágeno y metaloproteinasas, y ejercen un papel fundamental en la proliferación del epitelio intestinal gracias a la producción del factor de crecimiento de hepatocitos[26], contribuyendo activamente al mantenimiento de la función barrera intestinal.

Sistema nervioso central y entérico: El sistema nervioso central (SNC) interactúa con el TGI a través del eje cerebro-intestino, comunicándose de forma bidireccional en gran medida a través del sistema nervioso entérico (SNE), para coordinar las funciones digestivas y el mantenimiento de la función barrera intestinal directamente, a través de la liberación de neurotransmisores, e indirectamente a través de la interacción neuro-inmunitaria, como se verá más adelante. El SNE es una subdivisión del sistema nervioso autónomo. Se trata de un sistema local, organizado muy sistemáticamente y con capacidad de operación autónoma, comunicado con el SNC a través de los sistemas simpático y parasimpático, así como sistemas no colinérgicos no adrenérgicos. Estos envían información motora al intestino, al mismo tiempo que éste envía información sensitiva al SNC. El SNE está organizado en una red interconectada de neuronas y células gliales que se agrupan en los ganglios situados en dos grandes plexos: el plexo mientérico (de Auerbach) y el plexo submucoso (de Meissner). La capa mucosa también alberga las redes de células nerviosas y gliales, donde mantiene estrecho contacto con las células epiteliales intestinales y las células inmunitarias de la mucosa.[27]

El SNE se compone de neuronas sensoriales, interneuronas y neuronas motoras, que controlan el peristaltismo, los cambios locales en el flujo de sangre y en la secreción de agua y electrolitos[28], así como en la modulación del sistema inmunitario. Esta comunicación se lleva a cabo a través de mediadores químicos, tales como neuropéptidos, neurohormonas, neurotransmisores, citocinas, quimiocinas, factores de crecimiento y otras moléculas reguladoras.[29]

También contiene células gliales entéricas, que forman una gran red en todas las capas del TGI y sirven como intermediarios en el procesamiento de la neurotransmisión y la información entérica[30], las cuales influyen en la permeabilidad intestinal, en el sistema inmunitario y mantienen la integridad de la barrera epitelial.[31,32]

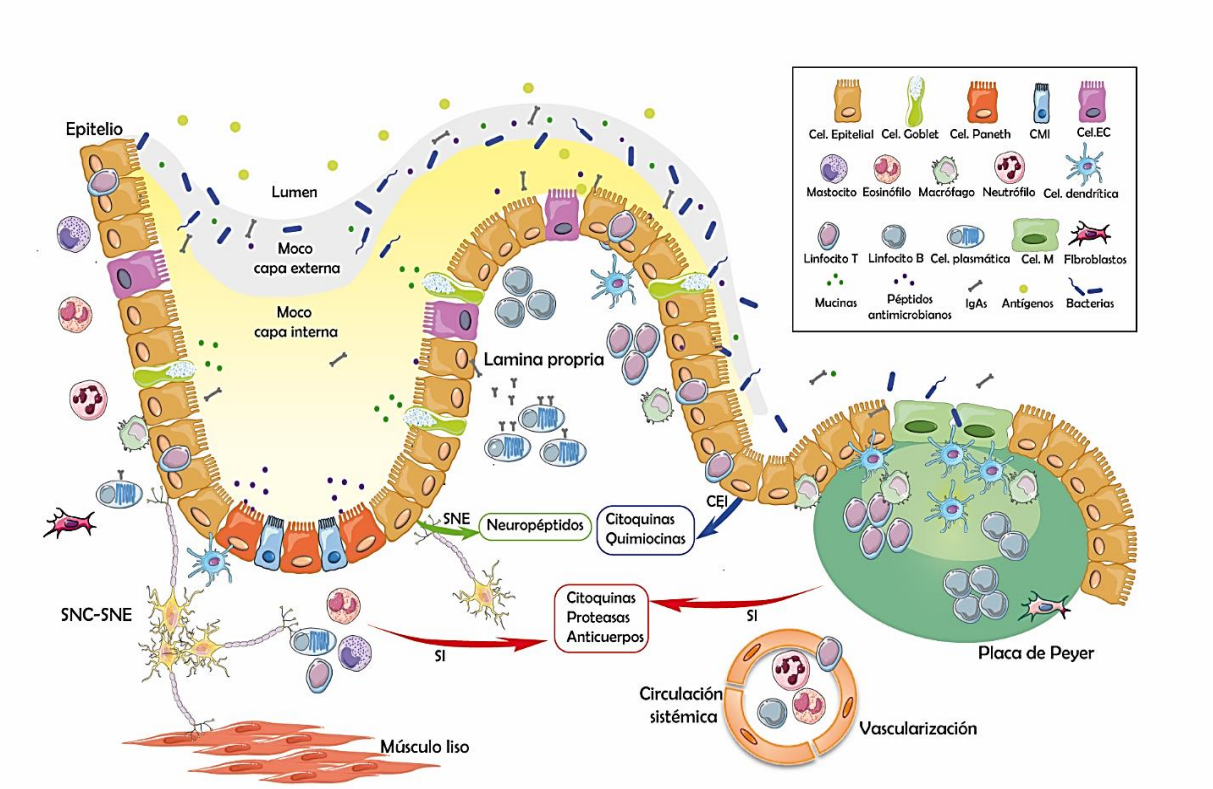


Figura 1: Anatomía y componentes de la barrera intestinal [33]. SNC: sistema nervioso central; SNE: sistema nervioso entérico; SI: sistema inmunitario; CEI: célula epitelial intestinal; CMI: célula madre intestinal; Cel. EC: célula enterocromafín; IgAs: IgA secretora.

1.1.3 El epitelio intestinal

Las células epiteliales forman una monocapa continúa polarizada, donde las membranas de las células individuales están conectadas entre sí y unidas a la membrana basal mediante complejos proteicos que proporcionan al epitelio la integridad estructural y la actividad celular necesarias para llevar a cabo sus funciones específicas. Estas uniones intercelulares se clasifican en tres

grupos funcionales, cada una con su localización anatómica característica: uniones estrechas, uniones de anclaje y uniones comunicantes (figura 2).

Uniones estrechas: El paso de moléculas pequeñas solubles en agua a través del epitelio se realiza a través de las uniones estrechas, las cuales sellan los espacios entre las células epiteliales. Las uniones estrechas (UE) son las uniones intercelulares más apicales y su función es primordial en el mantenimiento de la barrera epitelial y de la polaridad de esta capa, limitando la difusión de iones y la translocación de antígenos luminales desde la región apical hacia la región basolateral de las membranas que limita[34]. Se componen de complejos multi-proteicos constituidos por cuatro familias de proteínas transmembrana: ocludina, claudinas, moléculas de adhesión (*junctional adhesion molecules*, JAM) y tricelulina (figura 2), vinculadas al citoesqueleto por proteínas adaptadoras como *zonula occludens* (ZO)-1, ZO-2 y ZO-3[35], que participan el ensamblaje y desensamblaje de las UE y facilitan la estabilidad y la formación de la barrera epitelial.[36–39]

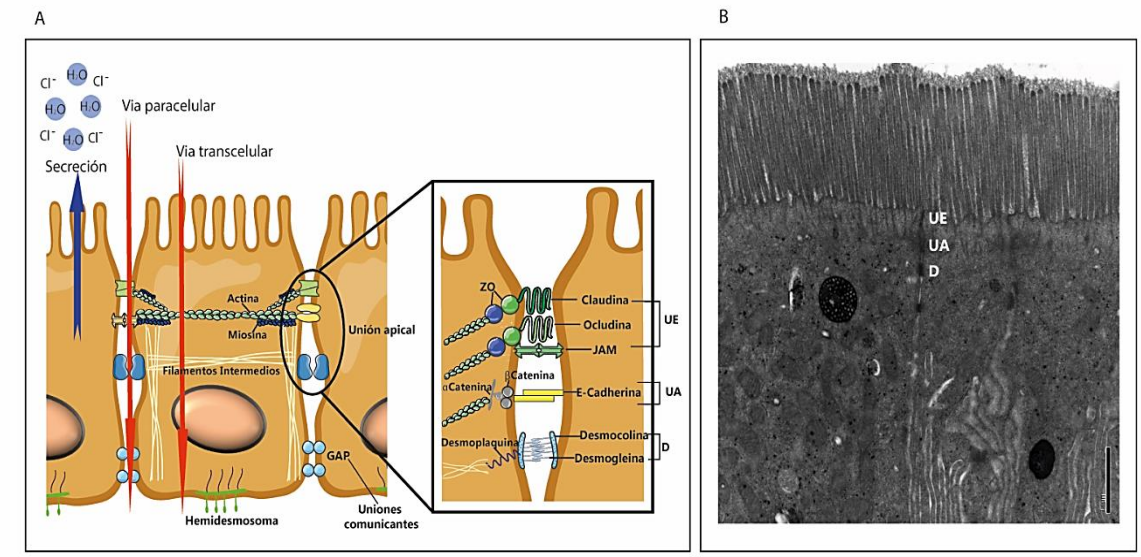
Uniones de anclaje: Las uniones de anclaje conectan el citoesqueleto de cada célula con los de células vecinas o a la matriz extracelular, lo que les permite constituirse como unidades estructurales resistentes. Entre las que desempeñan una función más relevante están:

- *Uniones adherentes:* regulan la adhesión entre células adyacentes mediante receptores de adhesión transmembrana y sus proteínas reguladoras asociadas a la actina. Esta unión del citoesqueleto de actina de ambas células se realiza a través de moléculas de adhesión transmembrana de la súper-familia de las cadherinas y cateninas y de complejos proteicos asociados a éstas, que conectan con el citoesqueleto.[40]

- *Desmosomas:* compuestos principalmente por desmogleína, desmocolina y desmoplaquina, son uniones intercelulares que proporcionan una fuerte adhesión entre las células. Debido a que también vinculan intracelularmente los filamentos intermedios del citoesqueleto de las células vecinas, forman una red transcelular que confiere resistencia mecánica a los tejidos y permite que

las células mantengan su morfología. Además, son centros de señalización y participan en varios procesos celulares como la diferenciación, la proliferación y la morfogénesis.[41]

- Uniones comunicantes: Las uniones comunicantes (*GAP junction*) permiten la comunicación entre los citoplasmas de las células vecinas a través de la formación de un canal que atraviesa las membranas. Están formados por 6 proteínas transmembrana llamadas conexinas, que median el intercambio recíproco de iones y moléculas pequeñas de menos de 1KDa. Las conexinas están consideradas también por tener un papel crucial en el desarrollo, crecimiento y diferenciación de



las células epiteliales, además de estar asociadas a las UE y a las uniones adherentes[42], por lo que desarrollan un papel relevante en el mantenimiento de la función barrera.

Figura 2: Representación de las uniones intercelulares y vías de acceso desde la luz intestinal al interior de la mucosa [33]. UE: uniones estrechas; UA: uniones adherentes; D: desmosoma; ZO: zonula ocludens; GAP: uniones comunicantes

1.2 Permeabilidad intestinal

La permeabilidad intestinal es una característica funcional y cuantificable de la función barrera que define la propiedad del epitelio para ser penetrado por sustancias presentes en la luz intestinal. Los mecanismos moleculares y celulares que regulan el paso de sustancias a través del epitelio

incluyen la interacción de proteínas de las uniones intercelulares, el citoesqueleto de actina y los procesos de endocitosis y de señalización intracelular.

Los enterocitos presentan una elevada actividad transportadora gracias a que poseen canales iónicos, transportadores y bombas en las membranas apical y basolateral y, además, son penetrables por ciertas moléculas por procesos de difusión pasiva. El paso de sustancias desde la luz intestinal se lleva a cabo de forma selectiva mediante dos vías principales: la vía paracelular y la vía transcelular (figura 2).

- La vía paracelular permite el 85% del total del flujo pasivo transepitelial de moléculas a través del espacio entre dos células epiteliales adyacentes y está regulado por las uniones estrechas, que presentan poros de diferente tamaño, limitando la entrada de partículas. Esta vía constituye una barrera efectiva para el paso de antígenos luminales y es determinante en el establecimiento de la permeabilidad intestinal.[43]

- La vía transcelular permite el transporte de solutos a través de la membrana del enterocito. Existen diferentes mecanismos que median el paso de moléculas por la ruta transcelular, como el transporte pasivo, por difusión de compuestos de pequeño tamaño, la difusión facilitada o el transporte activo, mediado por transportadores y diferentes mecanismos de endocitosis, transcitosis y exocitosis para iones, aminoácidos o proteínas y productos bacterianos, para su posterior procesamiento y presentación, como parte de la respuesta defensiva intestinal.[44] Las bacterias, los virus y otras partículas potencialmente antigénicas o tóxicas, pueden aprovechar estos mecanismos de entrada al huésped, mediante endocitosis o fagocitosis, implicando la unión de moléculas a la membrana celular a través de receptores.[45]

La permeabilidad a partículas dependerá, por lo tanto, de su tamaño, carga y naturaleza. Aunque la permeabilidad intestinal varía entre regiones proximales y distales, así como entre las criptas y las vellosidades, los mecanismos moleculares que regulan el paso de sustancias a través del epitelio

son similares a lo largo de todo el intestino e incluyen la interacción de proteínas de las uniones intercelulares, el citoesqueleto de actina y los procesos de endocitosis y de señalización intracelular.[46,47]

Los filamentos de actina forman un cinturón que rodea la zona más apical de la membrana lateral sosteniendo las uniones intercelulares[48] y, junto a las cadenas de miosina asociadas a la actina, que al fosforilarse provocan la contracción del anillo de acto-miosina, tienen también un papel clave en la regulación de la permeabilidad paracelular.[49]

1.3 Interacciones neuro-inmunológicas y función barrera intestinal

La función barrera intestinal está regulada en gran parte por el sistema inmunitario y el sistema nervioso, ya que son las principales interfaces sensoriales del cuerpo y del intestino, que perciben, integran y responden al contenido luminal y a los desafíos ambientales como un estrés psicológico. Estos dos sistemas comparten mecanismos de regulación de genes, señalización, comunicación celular y organización supracelular[50], que de forma coordinada participan en la selección y eliminación de productos tóxicos o antígenos.[51] El funcionamiento del sistema nervioso e inmunitario depende de los contactos célula a célula, y de moléculas solubles que actúan sobre células diana proximales o distantes. Está descrito que el SNE, de forma similar que el SNC, produce una gran variedad de neurotransmisores y neuropéptidos, como acetilcolina (ACh), adenosina trifosfato (ATP), óxido nítrico, péptido intestinal vasoactivo (*vasoactive intestinal peptide*, VIP), taquininas como la sustancia P (SP) y neuropéptido Y (NPY), entre otros[52], y que éstos son también expresados, junto a sus respectivos receptores, en las células inmunitarias.[53,54] Estos neuropéptidos pueden influir en el reclutamiento, la proliferación y la activación de los leucocitos. Por otro lado, las células inflamatorias pueden modular el fenotipo y la función neuronal y reestructurar el número y la distribución anatómica de las terminaciones nerviosas.[55] Además, se ha descrito que prácticamente todas las células inmunitarias de la mucosa intestinal están

inervadas por el SNE[56], aunque principalmente se observan en contacto directo mastocitos y macrófagos[27,57,58], indicando la estrecha interacción de estos dos sistemas (figura 3).

La activación del sistema inmunitario de la mucosa intestinal, en respuesta al reconocimiento de patógenos, proporciona tanto señales iniciales para la inducción de respuestas inflamatorias, como señales para la activación de respuestas del SNC contra-reguladoras que limitan la inflamación. Así, la activación del sistema nervioso periférico en los sitios inflamatorios locales sirve para amplificar y regular la respuesta defensiva innata dirigida a la eliminación local del patógeno, a través de la liberación de neuropéptidos como SP o VIP por las células nerviosas.[59] Esta activación del sistema inmunitario, a través de mediadores proinflamatorios como el factor de necrosis tumoral alfa (*tumor necrosis factor Alpha*, TNF-α), interferón gamma (IFN-ɣ) o interleucina 6 (IL-6), también puede incidir en la función barrera y disminuir la permeabilidad a través de la reorganización de las UE.[60] El mastocito es el principal productor de TNF-α en el intestino[60] y, junto a la liberación de histamina, es capaz, en acción sinérgica, de inducir también un aumento de su secreción en el epitelio[61], además de la activación neuronal. La triptasa y la quimasa, otros mediadores del mastocito, también son capaces de aumentar la permeabilidad y la secreción a través de la activación de receptores de proteasas como PAR-2 *(protease-activator receptor 2)* en el epitelio.[62] Además, los macrófagos, localizados en estrecha localización con las fibras nerviosas, en la región muscular del intestino, regulan directamente la actividad de las neuronas entéricas y la peristalsis a través de la secreción de la proteína morfogénica ósea (*bone morphogenetic protein 2*, BMP-2), en función de las señales recibidas por la microbiota intestinal.[58]

Una vez se ha eliminado el patógeno o el estímulo inductor, la activación del eje hipotálamo-hipofisiario-adrenal (*Hypothalamic pituitary adrenal axis*, HPA), el sistema nervioso simpático y el sistema nervioso parasimpático, amortiguan las respuestas inflamatorias y restablecen la homeostasis intestinal. En el intestino, las señales eferentes del nervio vago atenúan la actividad

de los macrófagos, dando lugar a respuestas antiinflamatorias mediante la liberación de ACh o VIP.[63] Una desregulación de las vías inhibitorias de la activación del SNC/SNE o un exceso de la activación del eje HPA, facilita la activación descontrolada de respuestas inmunitarias que podrían resultar en una pérdida de la función barrera y en enfermedad, como se observa en la EII o en condiciones de estrés crónico.

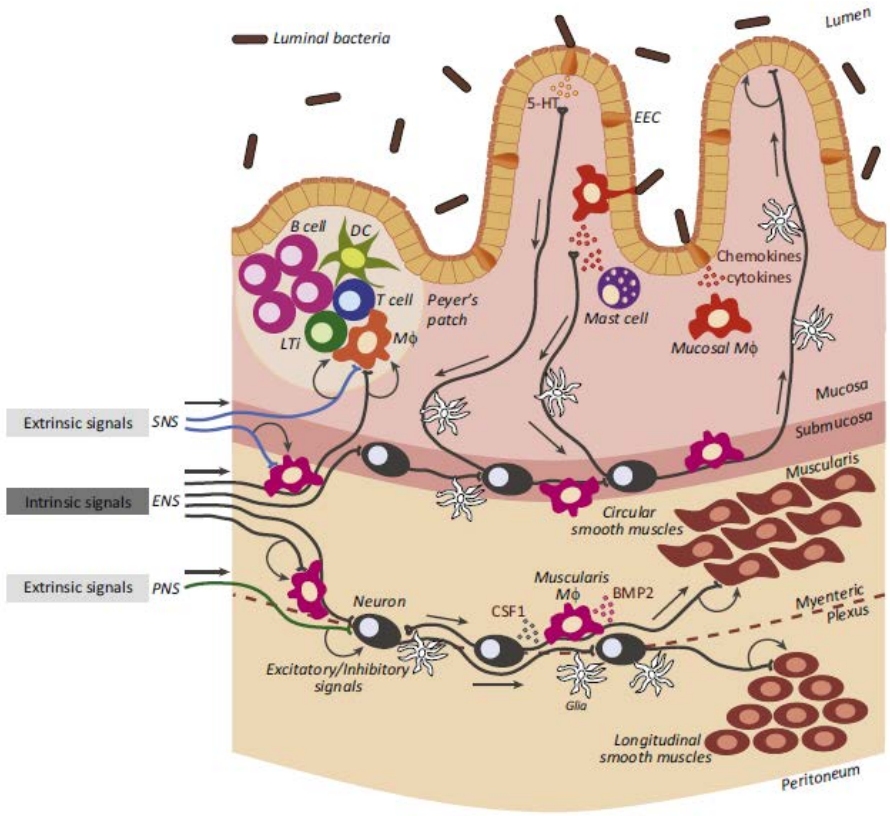


Figura 3: Ilustración esquemática de la organización celular y las conexiones entre el sistema nervioso y el sistema inmunitario.[27]

1.4 Estrés y función barrera intestinal

El estrés se puede definir, de forma simplificada, como la reacción fisiológica coordinada del organismo frente a estímulos que amenazan la homeostasis del individuo. El estrés genera respuestas tanto autonómicas y endocrinas como inmunológicas, a nivel central y periférico, que son coordinadas para mantener la estabilidad del organismo frente a una demanda del ambiente

(estresores externos). Cuando la demanda del ambiente (laboral, social, ambiental, etc.), y reacciones psicológicas al estímulo como el miedo, la agresión o el aislamiento social, es excesiva frente a los recursos de afrontamiento que se posee, se pueden alterar esta respuesta adaptativa y predisponer al desarrollo de nuevas enfermedades o exacerbar enfermedades ya existentes.[64]

1.4.1 Generación de la respuesta al estrés en el sistema nervioso central

La percepción de una amenaza por el individuo activa la respuesta organizada e inmediata de los principales sistemas efectores de defensa: el eje HPA y el sistema simpático-adreno-medular (SAM). El SAM constituye un sistema de respuesta rápida y a corto plazo e implica al sistema nervioso autónomo simpático y a la médula adrenal. Las proyecciones del sistema nervioso autónomo irradian desde la médula espinal y contactan casi todos los órganos, vasos sanguíneos y glándulas sudoríparas del cuerpo. Este sistema se activa durante lo que nuestro cerebro considera o percibe como una alarma. Cuando se activa este sistema, el hipotálamo desencadena la liberación de catecolaminas (adrenalina y noradrenalina) en las glándulas suprarrenales que, a través de los receptores adrenérgicos en las células de los órganos activados por el sistema simpático, intensifican las funciones adaptativas de lucha o huida promoviendo el flujo de sangre y la glucogenólisis.[65] Por otro lado, simultáneamente, la activación del eje HPA estimula a las neuronas parvocelulares del núcleo paraventricular del hipotálamo para secretar hormona o factor liberador de corticotropina (*corticotropin-releasing factor*, CRF) y arginina vasopresina (*arginin vasopressin*, AVP), que son secretados a la circulación portal hipofisiaria desde terminales axonales, los cuales promueven en la hipófisis anterior la síntesis de corticotropina (*adrenocorticotropic hormone*, ACTH). La ACTH circula en sangre e incide sobre la corteza de las glándulas suprarrenales, dando lugar a la producción de corticoides, responsables de mediar mecanismos de retroalimentación negativa en la hipófisis, hipotálamo e hipocampo, así como en el sistema inmunitario, para volver el sistema a su estado basal (figura 4).

El CRF es un importante mediador en la respuesta al estrés. Se ha demostrado experimentalmente que la administración de CRF en el SNC en animales produce respuestas conductuales y fisiológicas similares a las observadas en respuesta al estrés psicológico agudo.[66–68] El estrés crónico o excesivo puede alterar el circuito de estrés central, a través de la comunicación neuro-endocrina y del sistema inmunitario, lo que conduce a una mayor síntesis de CRF, un aumento de la actividad y la sensibilidad en la unidad central-noradrenérgica, una disminución de la regulación de los receptores de glucocorticoides centrales y una reducción de la actividad inhibitoria de este sistema.[66,69], pudiendo desencadenar en un desorden físico o psicológico (figura 4).[70] Esta desregulación se ha visto implicada en distintas patologías como la depresión[71], la ansiedad[72] y el síndrome del intestino irritable (SII).[73]

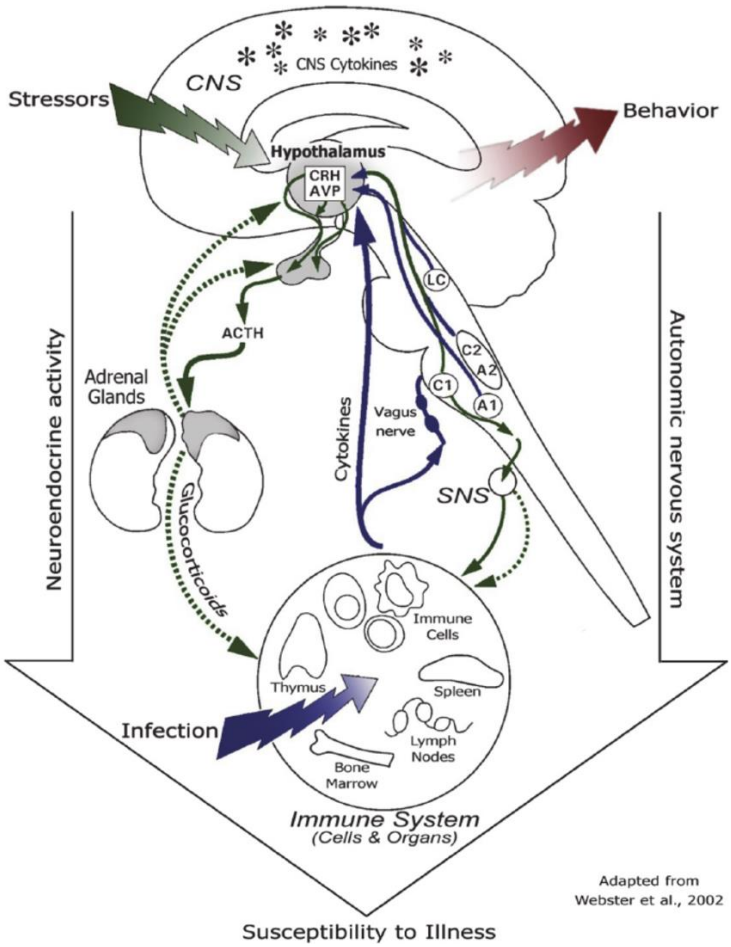


Figura 4. Diagrama de las rutas de comunicación entre el cerebro y el sistema inmune, incluido el eje HPA, el sistema nervioso simpático y la retroalimentación de citocinas al cerebro.[74]

1.4.2 Respuesta al estrés en el sistema gastrointestinal

La influencia del estrés, tanto agudo como crónico, en los síntomas y el curso de las enfermedades intestinales está cada vez más reconocida. El principal mediador de la respuesta al estrés es el CRF, cuyos efectos sobre la función del TGI han sido demostrados tanto a nivel del SNC como periférico, con efectos tanto en la motilidad y sensibilidad visceral, como en la permeabilidad intestinal[75] (figura 5).

La presencia de los receptores de CRF, los subtipos 1 y 2 (CRF-R1 y CRF-R2) se ha descrito en todo el TGI en roedores[76,7778] y en humanos[79], aunque su localización y función se ha estudiado principalmente en el colon e íleon [76,80]. Los receptores se localizan principalmente en leucocitos de la *lámina propia* como mastocitos y macrófagos, así como en el plexo mientérico y submucoso.[81–83] Su activación en el TGI en respuesta al estrés se ha relacionado con cambios en la motilidad e hipersensibilidad visceral. Asimismo, tanto la activación periférica como intra-cerebro-ventricular de CRF-R1, produce un aumento de las contracciones gástricas, de la motilidad del colon y de la sensibilidad visceral, junto a un aumento de la activación de los mastocitos.[76,84,85] Estos cambios motores son bloqueados por antagonistas no selectivos como el CRF9-41-helical.[86,78] Por otro lado, y de forma contrapuesta, la activación de CRF-R2 inhibe el vaciamiento gástrico, suprime la estimulación de la función motora del colon y protege del dolor visceral inducido por la distensión colónica.[87,88]

Tanto el estrés agudo como el estrés crónico, o la administración periférica de CRF, alteran la secreción y la permeabilidad epitelial. En humanos, tanto un estrés agudo (psicológico como físico), como la administración periférica de CRF, induce, en voluntarios sanos, un aumento de la secreción de agua en el yeyuno, un incremento de la permeabilidad intestinal, junto a un incremento en el eje HPA, reflejado por aumento de la concentración de cortisol en sangre. Este incremento de la permeabilidad es revertido si previamente se administra cromoglicato de sodio, un estabilizador del

mastocito.[89] En ratas también induce la liberación de mucina[90] y el aumento de la secreción de iones y agua y de la permeabilidad intestinal a macromoléculas[86,89,91], implicando los nervios adrenérgicos y colinérgicos y a los mastocitos.[92] Los cambios secretores producidos por el estrés agudo o la administración periférica de CRF son inhibidos con el tratamiento con CRF_{9-41} -helical[86] y son mediados por ambos receptores.[93]

La disfunción de la barrera intestinal observada en situaciones de estrés crónico puede implicar un aumento en la captación luminal de antígenos o de translocación bacteriana, con el consiguiente inicio o perpetuación de la respuesta inflamatoria en la mucosa, tal y como se observa en algunas enfermedades y trastornos digestivos.

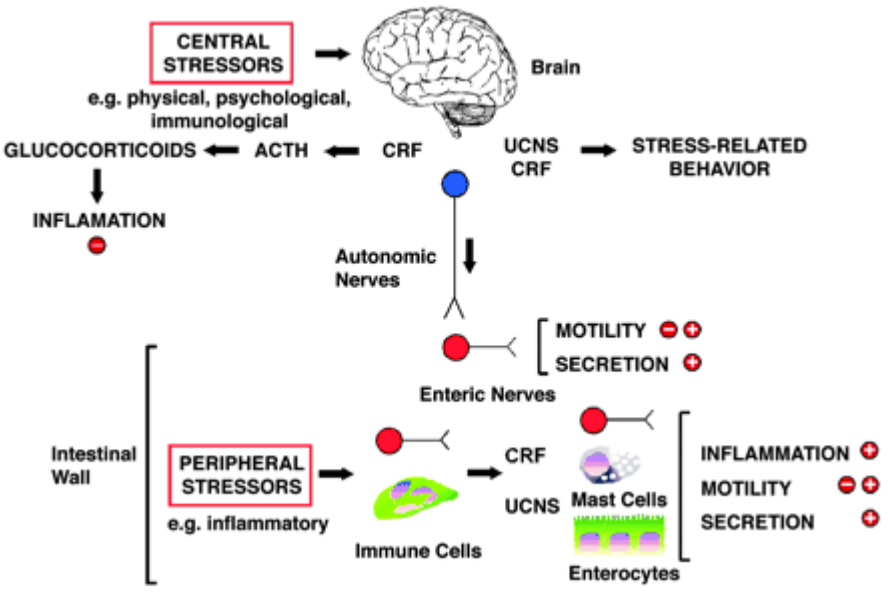


Figura 5. Alteraciones inducidas por estrés en las funciones gastrointestinales a través del sistema CRF.[94]

2 Síndrome del intestino irritable

El SII es el trastorno funcional digestivo crónico más común, con una prevalencia de hasta el 15% de la población en países desarrollados.[95] Se caracteriza por la presencia de dolor abdominal asociado a un cambio en el número y/o consistencia de las deposiciones[96], y presenta una predominancia en el sexo femenino, de 1,67 veces respecto al masculino.[97] El SII constituye uno de los mayores problemas actuales de salud en el mundo por su gran impacto socio-económico[98] y por el elevado impacto que genera en la calidad de vida. Su diagnóstico se fundamenta en criterios derivados de las manifestaciones clínicas principales, debido a que todavía no existen marcadores biológicos útiles. Tampoco se observan cambios macroscópicos durante la endoscopia ni en la histología convencional. Además, la etiología y la fisiopatología de este trastorno no están bien establecidas y tampoco existen opciones terapéuticas satisfactorias.

2.1 Manifestaciones clínicas y diagnóstico

Debido a la falta de biomarcadores, y a la heterogeneidad de sus manifestaciones clínicas, existen claras dificultades a la hora de diagnosticar el SII. Actualmente, el diagnóstico se basa en la exclusión de otras patologías que cursan con síntomas similares y en el cumplimiento de criterios clínicos, siendo los más utilizados los de Roma (The Rome Criteria, 1988). Esta guía ha sido actualizada a lo largo de los años y hoy en día se utiliza el criterio de Roma IV.[96]

El diagnóstico del SII se fundamenta en la presencia de dolor asociado a un cambio en la frecuencia o consistencia de las deposiciones. Actualmente, el SII se diagnostica cuando el dolor está presente al menos un día a la semana asociado con dos o más de las siguientes características: a la defecación, a un cambio en la frecuencia de las deposiciones y/o a un cambio en la consistencia. Los criterios deben cumplirse durante los últimos tres meses y los síntomas, haber comenzado un mínimo de seis meses antes del diagnóstico [96]. También es muy frecuente la hinchazón y la distensión abdominal. El SII se divide en 4 subtipos clínicos en función del hábito deposicional:

predominio diarrea (SII-D), predominio de estreñimiento (SII-E), mixto (SII-M) e inclasificable (anormalidad en las heces pero sin cumplir criterios de diarrea).[99]

El SII presenta a menudo una relación con otros trastornos funcionales digestivos, como enfermedad por reflujo gastroesofágico (ERGE) o dispepsia funcional (DF), alcanzando el 47% y el 15-42% de solapamiento, respectivamente, con una prevalencia mayor que en la población general, siendo de 11-29% en DF[100,101] y de 9%-26% en Europa para la ERGE.

2.2 Etiopatogenia

Aunque se desconoce la base fisiopatológica del inicio del SII, se han identificado algunos factores determinantes de la aparición, el desarrollo y su severidad. Se postula que el origen del SII es multifactorial y que depende de interacciones complejas entre el huésped y el medioambiente. Entre los factores inherentes al huésped se incluyen la edad, el género, la predisposición genética y factores socioculturales. Los factores ambientales incluyen el estrés psicológico, la dieta, las infecciones gastrointestinales y el consumo de antibióticos.

2.2.1 Factores genéticos:

Diversos estudios sugieren la existencia de agregación familiar en el SII, dado que se observa una probabilidad de 2 a 3 veces mayor de presentar un trastorno similar y que el trastorno afecte también con mayor frecuencia a gemelos homo- y heterocigotos.[102,103] Se han descrito varios polimorfismos en genes de pacientes con SII relacionados con la neurotransmisión[104], con la función neuro-inmunomoduladora[105] y con la actividad enzimática de la sucrasa isomaltasa.[106] Entre ellos, el más estudiado es el sistema serotoninérgico, en el que se han descrito polimorfismos de diferentes proteínas relacionadas con la serotonina (5-HT). El más estudiado es el polimorfismo del gen del transportador de la serotonina (*serotonin transporter*, SERT o 5-HTT) en la región promotora[107,108], aunque no se ha relacionado como un factor de riesgo del SII. Sin embargo, en los últimos años se ha postulado que el SII podría responder, más que a un solo polimorfismo, a un modelo de trastorno poligénico y multifactorial[109], en el que la susceptibilidad

genética es un factor que contribuye al desarrollo del síndrome, siendo necesaria la contribución de otros factores ambientales.

2.2.2 Factores ambientales:

Las infecciones intestinales, víricas o bacterianas, son uno de los factores de riesgo más relacionados con el desarrollo del SII y se calcula que un 10% de individuos que contrae una infección gastrointestinal desarrolla síntomas compatibles con el SII.[110,111] También se han relacionado otros factores que modifican la microbiota intestinal con el desarrollo del SII.[112] De hecho, se detecta un sobre-crecimiento bacteriano casi hasta en el 84% de los pacientes utilizando la prueba de aliento, resultando en una mejoría de los síntomas tras la erradicación del sobre-crecimiento.[113] También se han identificado cambios en la composición de la microbiota en los pacientes con SII, observando diferencias con controles sanos en muestras fecales.[114] Además, diversos estudios muestran que la microbiota intestinal alterada (rica en Veillonella y Lactobacillus) contribuye a los síntomas del SII a través del aumento de los niveles de ácidos orgánicos como ácido acético o propiónico[115], o la presencia de especies metanógenas y enterotipos enriquecidos con especies de Clostridiales o Prevotella asociados a la severidad de los síntomas del SII.[116]

Por otro lado, en los últimos años se ha evidenciado la influencia de algunos constituyentes de la dieta sobre los síntomas del SII, ya que alrededor de un 60% de los pacientes con SII refiere un empeoramiento de los síntomas tras las comidas[117] y que una dieta baja en productos fermentables como mono- di- u oligo-sacáridos mejora los síntomas digestivos.[118] La dieta proporciona sustratos para la fermentación microbiana y, como la composición de la microbiota intestinal se ha visto alterada en los pacientes con SII, el vínculo entre la dieta, la composición de la microbiota y los productos de fermentación microbiana podría tener un papel esencial en la etiología del SII.

2.2.3 Factores psicosociales:

Numerosos estudios describen una elevada prevalencia de comorbilidades psiquiátricos en pacientes con SII, principalmente ansiedad y depresión (40-50% más respecto a sujetos sanos)[119,120], pero también presentan neuroticismo, así como adaptación ineficiente a situaciones dramáticas.[121]

Por otra parte, la existencia de experiencias traumáticas como el abuso sexual, el maltratos u otros acontecimientos, se ha relacionado, en estudios epidemiológicos, con la aparición posterior del SII.[122–125] Además, el estrés psicosocial, agudo o crónico, también se observa no sólo en relación con la aparición del SII, sino también con la gravedad o exacerbación de los síntomas digestivos.[126,127]

2.3 Fisiopatología

2.3.1 Alteración motilidad intestinal

La alteración del hábito deposicional refleja cambios en la función motora intestinal. Las alteraciones de la motilidad incluyen un aumento del tiempo de tránsito tanto en el intestino delgado como en el colon en el SII-D (un 26% de los pacientes) y un enlentecimiento en el SII-E (un 33% de los pacientes).[128] Sin embargo, a pesar de que las alteraciones de la motilidad intestinal se han considerado un factor de gran relevancia en el SII, no existe un patrón motor específico del SII, sino más bien alteraciones de carácter cuantitativo respecto a personas sanas. A su vez, el cambio en la respuesta motora se ha observado en relación a diversos estímulos exógenos, incluyendo la ingesta de alimento y el estrés psicológico.[129,130] Se ha postulado que esta disfunción motora resulta de una alteración de las vías de neurotransmisión implicadas en la regulación de la función del sistema nervioso entérico. Esto justifica el estudio de los diferentes neuropéptidos y hormonas gastrointestinales cuya secreción está modificada en respuesta al estrés, entre otros: CRF, VIP y colecistoquinina (*cholecystokinin,* CCK).[131,132] De hecho, en algunos pacientes con SII-D se ha observado un aumento de la concentración de 5-HT en plasma, mediador clave de los reflejos

secretores y peristálticos[133], así como una importante mejoría clínica mediante el uso de antagonistas 5-HT3 en formas graves del SII-D o de agonistas HT4 en el SII-E.[134] Sin embargo, las alteraciones motoras observadas en los pacientes con SII no son específicas, consistentes, ni correlacionan totalmente con los síntomas, por lo que no pueden considerarse marcadores útiles para su diagnóstico.[135]

2.3.2 Hipersensibilidad visceral

Numerosos estudios han descrito la presencia de hipersensibilidad visceral en respuesta a estímulos físicos y fisiológicos en pacientes con SII. Las sensaciones viscerales abdominales son mediadas por vías aferentes en el SNC, por lo que la alteración de las vías aferentes del eje cerebro-intestino, el procesamiento del dolor en el SNC y una elevada respuesta a estímulos ambientales podrían ser mecanismos implicados en la hipersensibilidad visceral.[136] En los últimos años, diversos estudios han demostrado la relación entre la alteración en los mecanismos de control y procesamiento del dolor en el SNC y las manifestaciones clínicas características del SII.[137–139] En este sentido, se ha identificado un aumento del receptor de potencial transitorio V1 (*transient receptor potential cation channel V1*, TRPV1) en las fibras nerviosas aferentes de la mucosa intestinal, aumento que correlaciona con la puntuación en dolor abdominal mediante cuestionarios validados.[140] También se ha observado un aumento de las fibras nerviosas además de mayor presencia de daño ultraestructural en las fibras neuronales de la mucosa (principalmente balonización de mitocondrias y axones) y un aumento del factor neurotrófico derivado del cerebro (brain-derived neurotrophic factor, BDNF) en la mucosa de los pacientes con SII, este último correlacionado con el dolor abdominal.[141] La activación o un funcionamiento anómalo en las neuronas sensitivas primarias da lugar a liberación de neuropéptidos como SP o el péptido relacionado con el gen de la calcitonina (calcitonin gene-related peptide, *CGRP*) en el tejido. En el intestino humano, los mastocitos se encuentran cerca de estas fibras nerviosas TRPV1.[142] Se ha sugerido que esta estrecha asociación espacial, junto con una determinada activación de los

mastocitos intestinales, es de relevancia funcional para la actividad neuromuscular y para la alteración de la percepción del dolor en respuesta a estímulos como infecciones y en respuesta al estrés.[143,144]

Es importante resaltar que cada vez surgen más evidencias que muestran alteraciones en los mecanismos de control y de procesamiento del dolor a nivel central y la relación entre estas anomalías y las manifestaciones clínicas del SII[137,145], otorgando un papel predominante al SNC en la generación de hipersensibilidad visceral en este trastorno.

2.3.3 Alteración de la permeabilidad intestinal

La barrera intestinal y la activación inmunitaria desempeñan un papel importante en la fisiopatología del SII. En este sentido, el aumento de la permeabilidad intestinal se ha observado en numerosos estudios en pacientes con SII[146,147], lo que contribuye, mediante la exposición a antígenos procedentes de la dieta o microorganismos, a un aumento del proceso inflamatorio.[148] Se ha observado que la permeabilidad intestinal es distinta según el subtipo de SII, siendo los pacientes con SII-D y SII post-infeccioso los que presentan una alteración de la permeabilidad más prominente.[149,150] Además, esta disfunción de la barrera se ha relacionado con cambios en la integridad de las UE que, a su vez, se asocian con la activación del mastocito de la mucosa intestinal y con la sintomatología, principalmente en el SII-D.[151] Las principales alteraciones estructurales epiteliales son las identificadas sobre proteínas de la UE con disminución de ZO y ocludina, así como un incremento en claudina-2 y en la fosforilación de la cadena ligera de la miosina.[152–154]

Los mediadores presentes en el tejido de estos pacientes, como las serin-proteasas, tal como se demuestra en sobrenadantes de biopsias de colon y en las heces de paciente con SII-D, también se han relacionado con cambios en la permeabilidad intestinal y una subsecuente percepción anormal del dolor (alodinia) en modelos experimentales.[155] Existe, además, una correlación directa entre el

aumento de la permeabilidad y la intensidad del dolor abdominal en los pacientes con SII, principalmente en el SII-D.[156] Sin embargo, ello no implica que la alteración de la función barrera produzca *per se* dolor, sino que, como consecuencia de ésta, el fácil acceso del contenido luminal a la mucosa puede originar respuestas inflamatorias locales y la modulación de la función sensitivo-motora. Por lo tanto, la alteración de la permeabilidad no implica necesariamente la aparición del SII o de sus manifestaciones clínicas características. La progresión desde el aumento de la permeabilidad intestinal hasta la aparición de la enfermedad o de los síntomas implica un desequilibrio de los diversos factores que mantienen la función barrera, siendo el sistema inmunitario el principal candidato a ejercer un mayor efecto sobre ésta, dada la asociación entre inflamación y disfunción de la barrera en diferentes enfermedades digestivas y su sintomatología.

2.3.4 Sistema nervioso

El modelo biopsicosocial aplicado a la comprensión de la fisiopatología del SII asume que los factores psicosociales, que interactúan con los cambios neuroendocrinos e inmunitarios, tanto periféricos como centrales, pueden inducir y modular la gravedad de los síntomas, e influir en la experiencia de la enfermedad y en la calidad de vida de los estos pacientes. Como se ha descrito anteriormente, el estrés y la ansiedad son factores determinantes en el SII. Recientemente, se han empleado técnicas de imagen para detectar anormalidades en la estructura y el funcionamiento del cerebro y sus posibles implicaciones en la patología del SII. Sólo existe un estudio de resonancia magnética estructural (MRI)[157], en el que se observó el adelgazamiento en el cateterismo anterior y en la corteza insular en los pacientes con SII, áreas del cerebro involucradas en la percepción del dolor, la atención y en la percepción de los estados internos del cuerpo. Estos resultados fueron confirmados posteriormente por resonancia magnética funcional.[158] También se han observado diferencias morfométricas cerebrales entre pacientes mujeres con SII y sujetos sanos, en términos de aumento regional y disminución en la densidad de materia gris, principalmente en las áreas cerebrales involucradas en la atención y modulación de la emoción, así como en las redes que

procesan la información interoceptiva. Actualmente no es posible discernir si estos cambios son un factor predisponente para el desarrollo del SII o un cambio secundario después de la percepción de señales viscerales repetidas.[159] Los trastornos en del sistema nervioso autonómico (SNA), identificados por la disminución de la actividad simpática y el aumento de la parasimpática, y los reflejos autonómicos alterados, a menudo se describen en pacientes con SII, y explican el nivel de percepción de los estímulos gastrointestinales y los síntomas extra-intestinales (figura 6).[160,161]

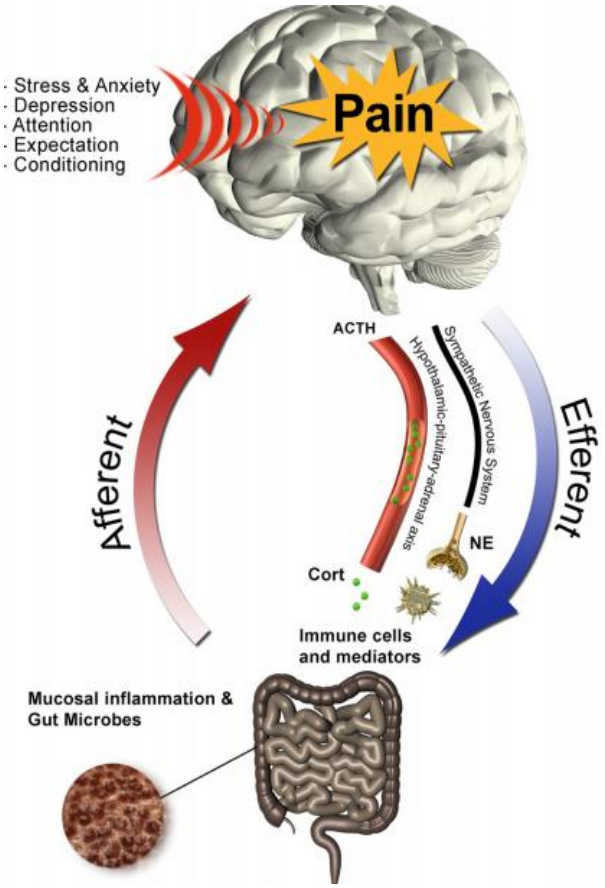


Figura 6. Vías periféricas y centrales del eje cerebro-intestino en la hiperalgesia del SII (Elsenbruch, 2011)

2.3.5 Microinflamación intestinal

En los últimos años, múltiples evidencias corroboran la existencia de una inflamación mucosa de bajo grado, o micro-inflamación, en el SII. Esta inflamación, sin embargo, no se observa durante el

procedimiento endoscópico ni durante el examen histológico convencional de biopsias de la mucosa intestinal. Es mediante técnicas específicas que se ha descrito a lo largo del intestino, desde el yeyuno hasta el recto, y en diferentes localizaciones, desde la mucosa, la muscularis mucosa y los nervios entéricos, la presencia de distintas estirpes inmunitarias en mayor proporción respecto a controles sanos.[162,163] A pesar de que no se conoce con exactitud el grado de inflamación necesario para alterar la función intestinal, sí se ha observado un infiltrado diferente según el subtipo clínico de SII. En el SII-D predomina un infiltrado de mastocitos y linfocitos en la *lámina propia*[164], mientras que en el SII-PI se observa un mayor infiltrado de linfocitos T, macrófagos activos y mayor proliferación de células enterocromafines productoras de serotonina en el epitelio.[165] En los subtipos SII-E y SII-M también se ha observado un incremento de linfocitos T y mastocitos en la *lámina propia*, sin embargo no hay tantos estudios que lo confirmen[136,163] No obstante, también existe una discrepancia entre los distintos estudios en cuanto al aumento del número de células en la mucosa intestinal. En este sentido hay estudios que muestran mayor o menor infiltrado de linfocitos T y B en las diferentes porciones del intestino, mientras que otros no observan diferencias significativas con respecto al grupo control[163,166,167] También, con respecto a los mastocitos, el número varía según los estudios y el segmento, observándose también recuentos normales, aumentados o disminuidos en diferentes cohortes de SII. Esto se debe, probablemente en parte, a la ausencia de la estandarización en la metodología usada en la cuantificación celular, a la variación interindividual, a los criterios utilizados en la selección de los pacientes y/o al segmento de intestino evaluado.[168]

Esta infiltración y activación celular se asocia, en algunos subgrupos de pacientes, con el aumento de la expresión de moléculas proinflamatorias en la mucosa intestinal, como la interleucina 1 beta (IL-1β) y la triptasa y, en ocasiones, con la presencia en sangre periférica de citocinas proinflamatorias como el TNFα y la IL-6. También se ha observado una disminución de citocinas anti-inflamatorias como la IL-10 y el TGF-β.[153,169,170]

La persistencia de la activación inmunitaria en la mucosa intestinal podría ser el origen de la mayor excitabilidad de las terminaciones nerviosas en la mucosa intestinal, contribuyendo a iniciar o perpetuar la hipersensibilidad visceral en pacientes con SII. Esta hipótesis se ve reforzada por los estudios realizados en animales de experimentación en los que se ha podido comprobar que incluso la inflamación leve o anatómicamente distante puede provocar cambios persistentes en los nervios entéricos y en la función muscular digestiva, siendo la activación inmunitaria el mecanismo responsable de la sensibilización de las terminaciones aferentes de la pared intestinal, modificando el estado basal del nociceptor y alterando la respuesta habitual frente al estímulo.[171,172] Además, la severidad del dolor abdominal en los pacientes con SII se correlaciona positivamente con el número de unidades mastocito-nervio, sobre todo nervios inmunorreactivos a SP[173] y con la liberación por parte del mastocito de mediadores neuromoduladores, como la histamina y la triptasa, que son capaces de actuar sobre las terminaciones nerviosas aferentes. El origen de la micro-inflamación mucosa es desconocido, pero podría ser consecuencia de la alteración de la función de barrera del epitelio intestinal[174], sin embargo se desconoce la secuencia de estos fenómenos.

3 El eosinófilo

3.1 Origen: de la médula ósea a residente de tejido

El eosinófilo es un leucocito polimorfonuclear de tipo granulocito que se origina en la médula ósea a partir de células madre hematopoyéticas multipotentes. Madura bajo la influencia de los factores de transcripción GATA-1, GATA-2, PU.1 y C/EBP[175] y su proliferación depende de diversos factores de crecimiento. Entre ellos se encuentran la IL-3, cuya función es estimular la proliferación de todas las células de la línea mieloide en conjunción con otras citocinas[176], el factor estimulador de colonias de granulocitos y monocitos (*granulocyte-monocyte colony stimulating factor*, GM-CSF), el cual juega un papel importante en el reclutamiento, maduración y expresión de moléculas de

adhesión en el eosinófilo y en otras células hematopoyéticas[177], así como en el bloqueo de la apoptosis[178] y la IL-5, el factor más específico del eosinófilo, responsable de su expansión y su salida de la médula ósea, así como de su diferenciación, supervivencia y activación.[179] A lo largo de su maduración, el eosinófilo adquiere características morfológicas, fenotípicas y funcionales específicas. Una vez maduro (tiempo medio de 2 a 6 días), ingresa en la sangre, donde circula en bajas cantidades, de 12 a 18 horas antes de migrar hacia los tejidos, donde se encuentra en una cifra aproximadamente cien veces mayor que en la circulación sanguínea.[180] Los eosinófilos se distribuyen en diferentes órganos, pero se encuentran en mayor densidad en aquellos en los que interactúan con el ambiente externo, como los tractos respiratorio bajo, gastrointestinal y genitourinario; y en menor proporción en el timo, en el útero y en las glándulas mamarias. Una vez en los tejidos, pueden sobrevivir desde días hasta semanas, bajo el efecto de citocinas liberadas local o sistémicamente.[181]

La extravasación de eosinófilos de sangre a tejidos está dirigida por citocinas (IL-4, IL-5 e IL-13), quimiocinas (eotaxina y RANTES) y moléculas de adhesión. Estos se expresan en el microambiente local por las células epiteliales, endoteliales e inmunitarias[182], incluyendo los propios eosinófilos, que tienen la capacidad de regular su acumulación en los tejidos y sus funciones efectoras de manera autocrina y paracrina, gracias a que expresan receptores críticos para su supervivencia, reclutamiento y activación, como receptores de citocinas (IL-5R, GM-CSFR), de quimiocinas (receptor de eotaxina, CCR3), liberados también por los propios eosinófilos, y de inmunoglobulinas (IgE, IgA e IgG), leucotrienos y receptores para TGF-β.[182]

3.2 Características del eosinófilo

3.2.1 Morfología y composición de sus gránulos

El eosinófilo maduro tiene un tamaño de 12 a 14 µm, un núcleo bilobulado característico y un citoplasma con distintos tipos de gránulos compartimentados repletos de diferentes

mediadores[183](figura 7). Los principales y más abundantes (95% de la granulación) son los denominados gránulos específicos, redondeados y de un tamaño que oscila entre 0,5 y 1,5 µm, que ocupan todo el citoplasma de la célula y se tiñen de color naranja-rosado con el colorante eosina debido a su elevada afinidad (por su carácter básico). Además, los eosinófilos contienen gránulos primarios, que contienen principalmente la proteína charcot-leyden (CLC), y gránulos pequeños o microgránulos, que contienen otros mediadores no catiónicos, como la arilsulfatasa, y que no dan coloración con la eosina.

Los eosinófilos generan y almacenan gran diversidad de proteínas (figura 7), siendo las cuatro proteínas catiónicas principales las más características de esta célula, junto a una gran variedad de citocinas, quimiocinas, factores de crecimiento, mediadores lipídicos y neuropéptidos (tabla 1).[182,184] Las proteínas eosinofílicas catiónicas son: proteína básica principal (m*ajor basic protein*, MBP); proteína catiónica eosinofílica (*eosinophilc cationic protein,* ECP); neurotoxina derivada de eosinófilos (*eosinophil derived neurotoxin*, EDN); y la peroxidasa del eosinófilo (e*osinophil peroxidase,* EPO). En el citoplasma del eosinófilo también se encuentran cuerpos lipídicos sin membrana, que son la fuente principal del ácido araquidónico. Éstos, además, contienen ciclooxigenasa y 5-lipooxigenasa que son requeridas para sintetizar prostaglandinas y leucotrienos. Normalmente se encuentran pocos o ningún cuerpo lipídico cuando se observan bajo microscopía electrónica de transmisión, sin embargo, cuando el eosinófilo se encuentra más activado, por ejemplo en procesos inflamatorios, puede llegar a contener varios de ellos.[184] Además, los eosinófilos presentan una gran variedad de receptores de citocinas, quimiocinas, mediadores lipídicos y neuropéptidos[185], en su superficie que facilitan las interacciones con el ambiente mediante la síntesis y la secreción de mediadores específicos[186] (figura 7)

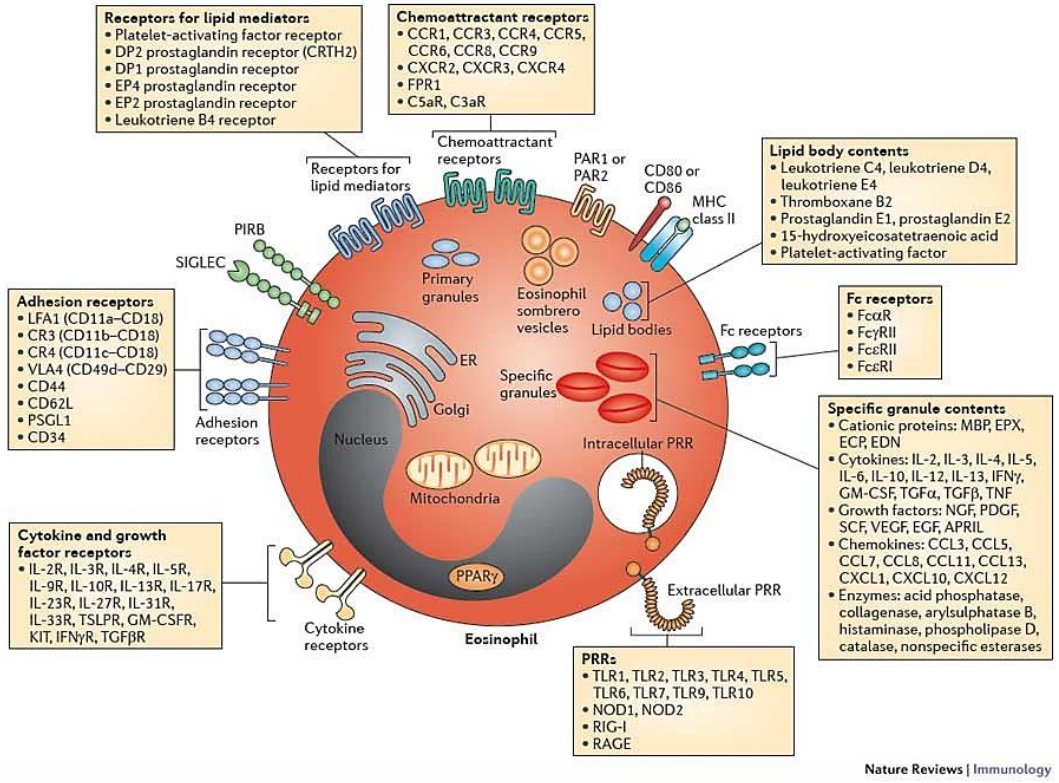


Figura 7. Características y expresión de receptores y mediadores en los eosinófilos

Tabla 1: Mediadores derivados del eosinófilo y función biológica

Tipo de mediador	Función	Mediador	Referencia
Proteínas catiónicas	Defensa huésped/ homeostasis función barrera	ECP, EDN, EPO, MBP	187
Citocinas y factores de crecimiento	Inmunoregulación sistema inmunitario adaptativo	**Th1:** IFN-γ, IL-2, IL-12 **Th2:** IL-4, IL-5, IL-13, IL-9, IL-25 **Th17:** IL-17A, IL-17F **Treg:** IL-10, TGF-β	188
	Inmunoregulación sistema inmunitario innato	GM-CSF, IL-3, IL-4, IL-5, IL-13, SCF	
	Cambio de isotipo de células B y mantenimiento de células plasmáticas	APRIL, IL-4, IL-6	
	Remodelación y reparación tisular	TGF-α, TGF-β, IL-1β, IL-13, PDGF-B, VEGF	
Mediadores lipídicos	Lípidos proinflamatorios	LTC4, PAF, PGE, PGF1, TxA2	189
	Lípidos que resuelven la proliferación	PD1, RvE3	190

Neuropéptidos y neurotrofinas	Supervivencia, desarrollo y función nerviosa	CGRP, CRF, *NGF, NT-3,* SP, VIP	[191] [192] [193]

3.2.2 Mecanismos de degranulación

La secreción es un proceso celular fundamental subyacente a diferentes eventos fisiológicos y patológicos. Los eosinófilos liberan el contenido de sus gránulos específicos de cuatro maneras diferentes: a través de exocitosis, clásica y compuesta, por degranulación fragmentaria o tipo *piecemeal* y por citólisis (figura 8). Además, los eosinófilos pueden activarse y degranular rápidamente para liberar mediadores pre-formados y de nueva síntesis, gracias a la presencia de componentes vesiculares específicos dentro de estos gránulos compartimentados, que permite cambios dinámicos en su estructura y contenido.[194] El proceso de activación y secreción de los eosinófilos puede verse reflejado en cambios en la morfología, en la expresión de proteínas en la membrana, en el tipo de vaciado de los gránulos y en la expresión de diferentes combinaciones de los receptores SNARE (acrónimo derivado de "SNAP Soluble NSF Attachment-Protein REceptor" o receptor de proteína de fijación soluble de NSF), receptores que median la secreción del contenido mediante el acoplamiento de los gránulos o vesículas para su fusión en la membrana citoplasmática.[195] Entre las SNARE, las proteínas de membrana descritas en el eosinófilo son la SNAP23 y SNAP25 y sintaxina 4. De la familia de las proteínas de membrana asociadas a vesículas (*vesicle-associated membrane protein*, VAMP), las descritas en los eosinófilos son las VAMP2, VAMP7 y VAMP8. [200,201]

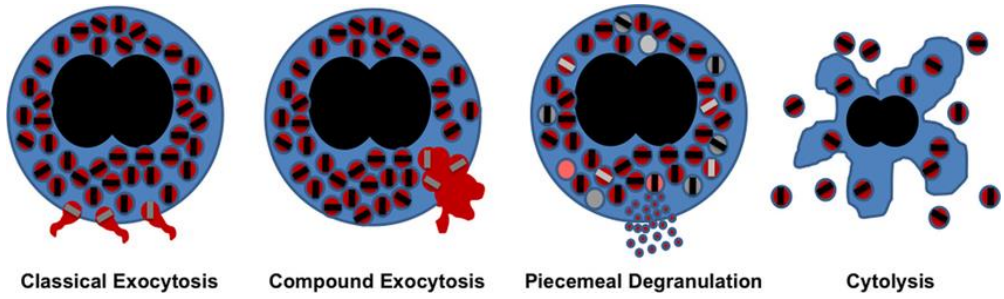


Figura 8. Mecanismos de degranulación en el eosinófilo[196]

La exocitosis clásica, es el proceso de liberación mediante la cual los gránulos liberan sus contenidos enteros después de la fusión de los gránulos con la membrana plasmática, incluida la exocitosis compuesta, que también implica primero la fusión intracelular gránulo-gránulo antes de la liberación extracelular[197] (figura 8). La liberación al medio es gracias a la participación de las proteínas VAMP7 y VAMP8 / SNAP23/ Sintaxina 4 que forman el complejo entre la membrana granular y la membrana plasmática (figura 9).[198]

La degranulación fragmentaria (*piecemeal degranulation*, PMD), es un proceso de liberación selectiva de mediadores a través de vesículas de transporte, como las "vesículas tipo sombrero" en el citoplasma[199], relacionadas con la expresión de componentes SNARE específicos como VAMP2, que se localiza predominantemente en pequeñas vesículas secretoras (figura 9).[200,201] Este proceso se identifica gracias a la presencia de gránulos parcialmente vacíos mientras que otros permanecen intactos.

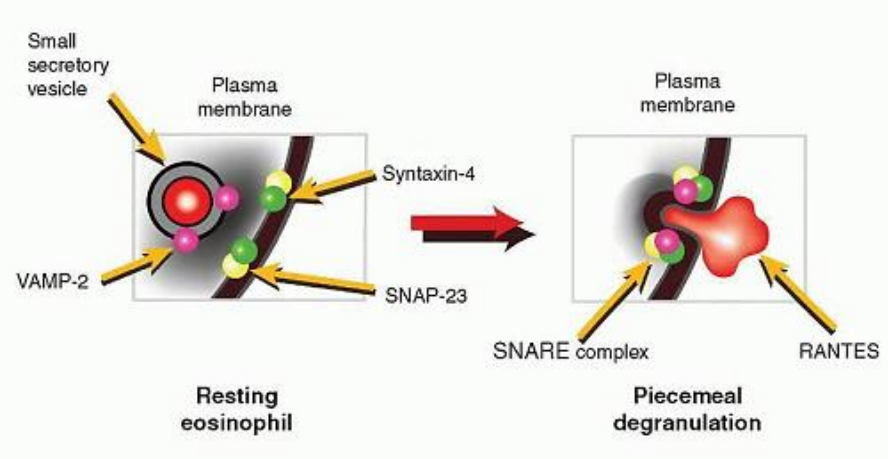


Figura 9. Detalle del modelo esquemático de la liberación de RANTES mediante degranulación tipo *piecemeal* y la participación de las proteínas SNARE (VAMP2, SNAP23 y Syntaxin4)

Finalmente, la deposición extracelular de gránulos se realiza en el programa de degranulación citolítica, donde los eosinófilos sufren la rotura de la membrana celular y los gránulos libres de las

células se depositan dentro del tejido y pueden seguir funcionando como orgánulos competentes con capacidad secretora[202] (figura 8).

3.3 El eosinófilo intestinal

La población mayoritaria de eosinófilos se encuentra en el TGI, localizándose principalmente en la *lámina propia* ya durante el período prenatal. Ello sugiere, a diferencia de otros leucocitos, que la presencia de eosinófilos intestinales es independiente de la presencia de la flora bacteriana. El tráfico de eosinófilos es diferente al de otros leucocitos, siendo la molécula más característica la eotaxina, expresada constitutivamente por las células endoteliales y epiteliales del TGI.[203]

3.3.1 Infiltrado y fenotipo del eosinófilo

En condiciones fisiológicas se ha demostrado que los eosinófilos residen en todos los compartimentos del TGI, excepto en el esófago, principalmente en la mucosa, aunque algún eosinófilo intraepitelial está presente en el intestino delgado.[204,205] En el estómago hay poca información con respecto al rango normal, aunque algún estudio determinó una media de 4 ± 4 eosinófilos por campo de gran aumento (CGA). En el colon existe un gradiente decreciente proximal a distal, habiendo recuentos de 10 a 70 eosinófilos en el ciego y de 1 a 30 en el recto por campo de gran aumento, aunque tampoco se ha alcanzado un consenso en los rangos normales de esta población en los diferentes segmentos intestinales.[204,206]

En cuanto al fenotipo, existen diferencias entre eosinófilos de sangre y los residentes de tejido intestinal, dado que los eosinófilos circulan ya maduros en sangre y acaban de diferenciarse en el tejido. Según el medio en el que se encuentren, adquirirán características funcionales y morfológicas diferentes. Estudios en modelos de ratón han caracterizado algunas de estas diferencias fenotípicas en la expresión de marcadores de membrana entre eosinófilos residentes del TGI y los de sangre[207], como la expresión del receptor CD63, asociado con degranulación, y una expresión abundante de CD22, un marcador Siglec específico de células B.[208] Sin embargo, los

estudios en humanos son mucho más limitados debido a la dificultad de aislar un número adecuado de células de una porción pequeña de intestino y, a pesar de las similitudes descritas con los eosinófilos de ratón, también existen ciertas diferencias tanto genéticas como moleculares y morfológicas que podrían no reflejar las mismas diferencias fenotípicas.

3.3.2 Funciones de los eosinófilos en el intestino

Los eosinófilos, al ser una fuente de numerosos mediadores con efectos proinflamatorios y reguladores, son capaces de regular diferentes funciones fisiológicas en el TGI, incluyendo la respuesta inmunitaria local, la función de barrera epitelial y, directa o indirectamente reguladas por su interacción con nervios (figura 10).

3.3.2.1 Función inmunoreguladora

Los eosinófilos humanos pueden producir, almacenar y liberar rápidamente más de 30 citocinas distintas y quimiocinas capaces de generar diferentes respuestas inmunitarias.[209] Los eosinófilos son mediadores de respuestas defensivas tanto innatas como adaptativas, y participan tanto en respuestas fisiológicas como inflamatorias, ya que pueden intercambiar señales con casi todas las células residentes de la mucosa.[210]

Las interacciones eosinófilo-célula epitelial representan un mecanismo regulador bidireccional que modula las respuestas inmunitarias locales. Las células epiteliales tienen la capacidad de activar los eosinófilos a través de la secreción de varias citocinas y quimiocinas, como eotaxinas e IL-5 y la generación de especies reactivas de oxígeno (ROS).[185] Además, la liberación de leucotrienos y citocinas por los eosinófilos representa un mecanismo de señalización local por el cual los eosinófilos modulan funciones en las células epiteliales.

Los eosinófilos también están presentes en los lugares de inflamación con perfil predominante de citoquinas Th2, participando en el mantenimiento de la respuesta en el microambiente local, y

potenciando las funciones de células de memoria Th2 mediante la generación de citocinas como la interleucina-25 (IL-25).[211] Pero, a pesar de su asociación general con las respuestas inmunitarias de Th2, también almacenan y secretan citocinas asociadas a Th1 (IFN-γ, IL-12), a T reguladoras (IL-10, TGF- B) y a Th17.[209] Además, los eosinófilos promueven la actividad de las células B, ya que participan en la disminución del umbral de activación requerido para la producción de IgM mediante IL-4[212] y desempeñan una función esencial en el mantenimiento, la generación y el cambio de isotipo de linfocitos B y producción de inmunoglobulinas (Ig) en la *lámina propia*, principalmente mediante la expresión del ligando inductor de la proliferación APRIL.[213]

Los eosinófilos también tienen efectos sobre las células inmunitarias innatas, especialmente sobre los mastocitos. Los eosinófilos pueden promover el crecimiento, la supervivencia y la activación de los mastocitos, mediante la liberación de varias citocinas y también mediante proteínas como MBP, el factor de células madre (s*tem cell factor,* SCF) y el factor de crecimiento nervioso (*nerve growth factor,* NGF).[214]

3.3.2.2 Función barrera intestinal

Los eosinófilos pueden influir significativamente, tanto fisiológica como patológicamente, en la función de barrera intestinal (figura 5). El tipo de efecto que puede ejercer el eosinófilo sobre la función barrera está directamente relacionado con la proporción entre eosinófilos y células epiteliales. Si hay un elevado ratio se observa una disminución de la función de barrera epitelial, dependiente de MBP[215], pero por otra parte, una relación muy baja de eosinófilos, que es lo normal en condiciones fisiológicas (por ejemplo ratio de 0,01) ayuda a la función barrera, activando por ejemplo, a través también de MBP, la secreción de prostaglandina y de iones en las células epiteliales.[216,217] La depleción de eosinófilos en el intestino promueve una barrera defectuosa contribuyendo a un aumento de la permeabilidad[218], por lo que es necesaria una

mínima presencia de estas células para un correcto funcionamiento de la barrera, siendo sin embargo el número de eosinófilos determinante en tipo de función que desempeñará.

El eosinófilo participa también en el mantenimiento de la homeostasis de la barrera intestinal influyendo en la microbiota y en la producción de moco. Aunque no hay estudios en el TGI, estudios en células epiteliales de las vías respiratorias sugieren un papel de las proteínas catiónicas en la regulación de la secreción de moco, inhibiendo y estimulando la secreción de moco, por MBP y ECP respectivamente[219], e inhibiendo, mediante MBP y EPO, la liberación de mucina de células caliciformes.[220] Además, los eosinófilos del TGI tienen la capacidad de sintetizar y liberar rápidamente "trampas bactericidas extracelulares" que son estructuras que contienen ADN mitocondrial, de una manera reactiva dependiente de especies de oxígeno, pero independiente de la muerte de eosinófilos, creando una segunda barrera física que limita la invasión bacteriana.[221]

Los eosinófilos contribuyen a la remodelación y reparación de tejidos, como parte del proceso de renovación normal o cuando hay muerte celular o daño en el tejido. Las proteínas catiónicas eosinofílicas como EDN promueven la proliferación de fibroblastos[222] y MBP, en sinergia con IL-5 y TGF-β, e inducen la expresión de fibroblastos y la secreción de IL-6 e IL-11.[223] Además, los eosinófilos son fuente de una amplia gama de proteínas y citocinas implicadas en la fibrogénesis y la angiogénesis, tales como el TGFβ, la metaloproteasa-9, la angiogenina e interleucinas que activan varias células mesenquimales e inducen la síntesis de proteínas de la matriz extracelular.[224]

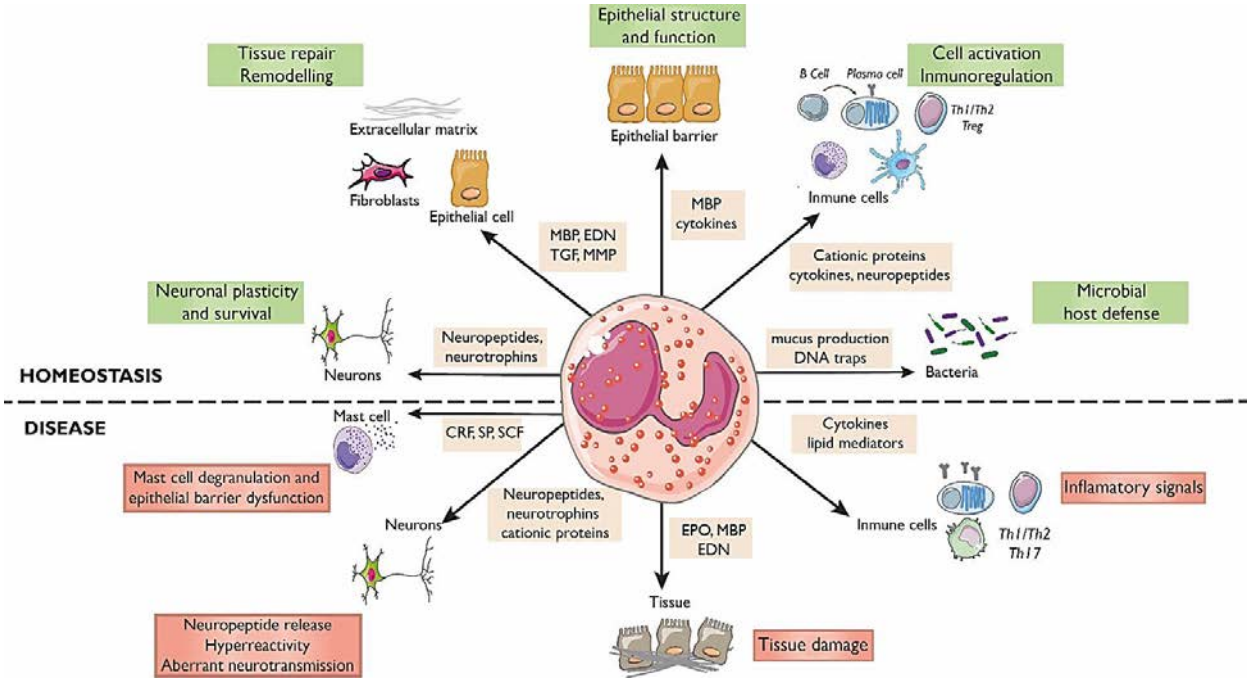


Figura 10. Papel de los eosinófilos sobre la función barrera intestinal en homeostasis y enfermedad. CRF: corticotropin-releasing factor; EDN: eosinophil-derived neurotoxin; EPO: eosinophil peroxidase; MBP: major basic protein; MMP: matrix metalloproteinases; SCF: stem cell factor; TGF: transforming growth factor

3.3.2.3 Interacción neuro-inmunológica

Los eosinófilos pueden sintetizar, almacenar y liberar neuropéptidos, tales como SP, VIP, CGRP y neurotrofinas tales como NGF y neurotrofina-3 (NT-3), que participan en las interacciones neuro-inmunitarias que modulan la actividad funcional y la supervivencia de los nervios periféricos.[225] Estos neuro-mediadores también pueden modular la actividad de otras células inmunitarias, incluyendo los propios eosinófilos, debido a la expresión de receptores de neurotransmisores (figura 11). Los nervios reclutan activamente y establecen contacto directo con los eosinófilos produciendo una gama de efectos en ambos tipos de células[182] (figura 11). Esta interacción eosinófilo-nervio es bidireccional y tiene importantes efectos fisiológicos, en la regulación de la homeostasis y en diversas enfermedades inflamatorias asociadas con eosinófilos.

- *Activación de células neuronales por los eosinófilos*: Los eosinófilos pueden actuar sobre los nervios aumentando la liberación y el contenido de los neurotransmisores, alterando el

crecimiento nervioso y aumentando la supervivencia de las neuronas.[226–228] Se ha demostrado, tanto en estudios *in vitro* como *in vivo*, que los eosinófilos son reclutados y activados por los nervios a través de taquininas que, a través de MBP, inducen la liberación de SP en las neuronas sensoriales[226,229] y causan una hiper-respuesta del receptor vagal M2, contribuyendo a la liberación de ACh, hiperactividad y/o remodelación neuronal.[230] Los eosinófilos pueden inducir también la plasticidad neural, la capacidad de las sinapsis neurales para modificar la fuerza de la conexión dependiendo de la experiencia. Así, los eosinófilos inducen una mayor actividad neural asociada con el dolor y cambios en la motilidad entérica en el trastorno inflamatorio, observado en neuronas aferentes primarias de la vía aérea[231], y también pueden mediar la pérdida de la actividad neuronal y de daño nervioso en las neuropatías[232], mecanismos ambos que conducen a una neurotransmisión aberrante.

- *Activación de los eosinófilos por células neuronales:* Muchos estudios tanto *in vitro* como *in vivo* han demostrado que los nervios pueden reclutar eosinófilos mediante la liberación de neuropéptidos, bien directamente, mediante unión a los receptores en eosinófilos[233]; o bien indirectamente, participando en el cebado en aumentar la quimiotaxis hacia otros factores quimiotácticos.[234] Después del reclutamiento, los eosinófilos se adhieren a las células nerviosas a través de las moléculas de adhesión celular (CAM), principalmente las CAM vasculares, intercelulares y neurales (VCAM, ICAM y NCAM), induciendo la activación y degranulación de los eosinófilos.[235,236] La expresión de ICAM en las células nerviosas es inducible por lo que citocinas inflamatorias y otros mediadores pueden regular su expresión[230,235], aumentando la adhesión y degranulación de los eosinófilos.[237] La importancia de la adhesión de los eosinófilos en los nervios se evidencia por la presencia de eosinófilos degranulados adheridos a los nervios colinérgicos en estados patológicos como en la EII y la alergia.[236,238] Los neuromediadores pueden inducir también la

producción y liberación de especies ROS en los eosinófilos, que a su vez pueden modular la actividad de nervios vagales aferentes.[239] Ello genera reacciones exageradas como tos o broncoconstricción en los pulmones. Además, el contacto eosinófilo-nervio induce ROS y la consecuente retracción de las neuritas.[240] Al mismo tiempo, los nervios también pueden producir y liberar ROS, con efectos sobre los eosinófilos, como estimular la degranulación [241], siendo la producción de ROS un mecanismo compartido y una señal bidireccional entre los nervios y los eosinófilos adheridos.

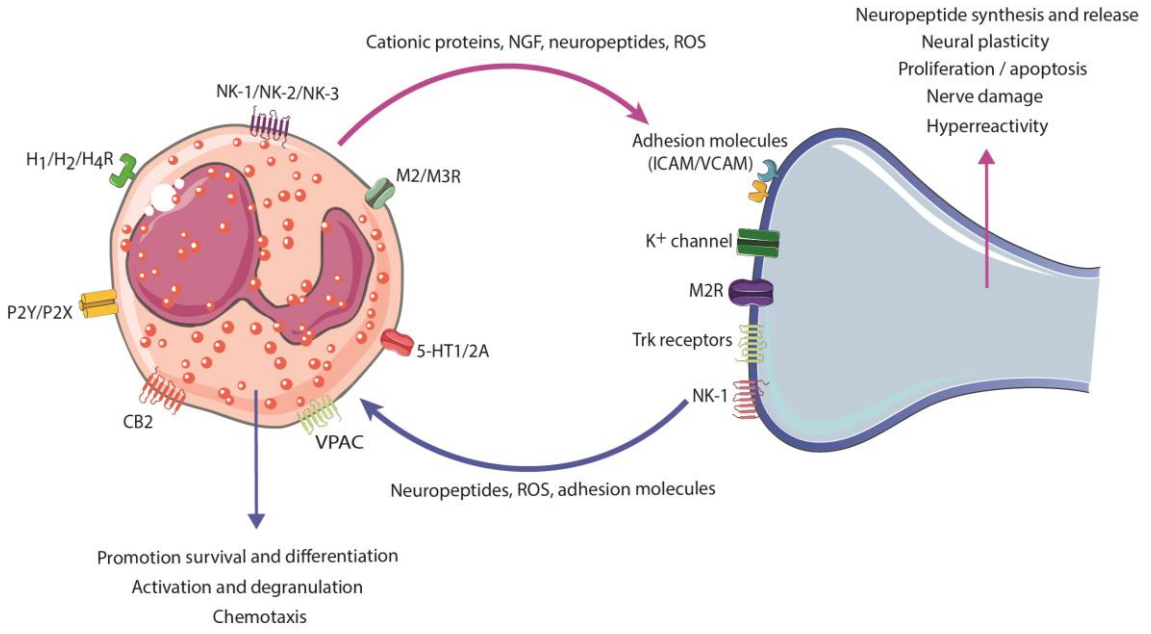


Figura 11: Representación esquemática de la interacción entre eosinófilo y célula nerviosa a través de los receptores y mediadores expresados en ambas células

3.3.3 El eosinófilo en enfermedades gastrointestinales

3.3.3.1 Enfermedades gastrointestinales eosinofílicas primarias

Este grupo de patologías se caracterizan por la inflamación e infiltración de eosinófilos en el TGI sin causa conocida de eosinofilia. Incluye la Esofagitis Eosinofílica (EEo), gastritis eosinofílica, gastroenteritis eosinofílica, enteritis eosinofílica y colitis eosinofílica. Los síntomas varían

dependiendo del segmento intestinal involucrado e incluyen dolor abdominal, diarrea, disfagia, dismotilidad y vómitos.[242]

La evidencia apoya que estas enfermedades están fuertemente asociadas con una causa alérgica, considerando que aproximadamente el 75% de los pacientes son atópicos[243], existe una degranulación de mastocitos en el tejido[244] y que una dieta libre de alérgenos puede disminuir la gravedad de la enfermedad en muchos casos.[245] En la alergia no dependiente de IgE, considerada una EGID secundaria, el eosinófilo participa, en la inflamación de la respuesta alérgica activando la respuesta Th2.[246] Los mediadores de los eosinófilos juegan un papel en la fisiopatología, como se ha demostrado por la deposición extracelular de MBP y ECP en el intestino delgado de pacientes con gastroenteritis eosinofílica[247] y EoE[248], correlacionando el nivel de eosinófilos y la gravedad de la enfermedad. También se ha asociado la interacción entre eosinófilos y el SNE en la patogénesis de la enfermedad, ya que se han observado muy cerca de los nervios entéricos dañados en animales con alergia provocada[249], y en pacientes con síntomas obstructivos persistentes, asociados con el plexo mientérico.[250] También se han observado características indicativas de necrosis axonal, tales como hinchazón de cámaras axonales agrandadas y pérdida de organelos internos en pacientes con EGID.[251]

El aumento de la activación y degranulación de eosinófilos están presentes también en EoE[248] y los mediadores de los gránulos parecen ser los responsables de las lesiones y daños en los tejidos, pudiendo sensibilizar el nervio sensorial que conduce a los síntomas.[252]

Los eosinófilos se infiltran en las capas serosa, muscular y mucosa del tracto GI durante las EGIDs. La presencia de eosinófilos en estas diferentes capas se asocia con síntomas clínicos. Un mayor infiltrado en la mucosa se manifiesta con diarrea, vómitos, hemorragia o malabsorción; un infiltrado en la musculatura a menudo se asocia con síntomas obstructivos y la presencia en la capa serosa de eosinófilos se presenta con ascitis.[253]

Actualmente los tratamientos para la eosinofilia y la activación inflamatoria que pueden ser útiles son los agentes que bloquean la eotaxina-1, como el anticuerpo anti-eotaxina-1 humanizado y el antagonismo de CCR3 (receptor de eotaxina-1). Además, el bloqueo de IL-5 también puede tener utilidad terapéutica. De hecho, un estudio preliminar reciente ha reportado un papel beneficioso para anti-IL-5 en EoE.[254]

3.3.3.2 Enfermedad inflamatoria intestinal (EII)

El papel de los eosinófilos en EII ha sido de gran interés durante los últimos años debido a la acumulación y activación de los eosinófilos en la mucosa intestinal inflamada.[255] Los eosinófilos parecen estar implicados en la promoción de la inflamación y en los cambios en la motilidad durante los estados inflamatorios de la EII a través de la liberación de diversas proteínas catiónicas, tales como ECP y MBP, y citocinas, aumentando el daño tisular y la activación de otras células inmunológicas.[256,257] Los eosinófilos activados además se localizan cerca de los nervios inmunorreactivos para SP y colina acetil-transferasa (ChAT) en pacientes con enfermedad de Crohn.[238] Se ha correlacionado también el número de eosinófilos activados con una mayor permeabilidad de la barrera intestinal en pacientes con colitis ulcerosa. Estos eosinófilos contienen CRF y expresan los receptores colinérgicos muscarínicos M2 y M3[258], sugiriendo la participación de esta célula por interacciones neuro-inmunológicas, ya que el sistema CRF hipotalámico y periférico parece estar desregulado en estos pacientes, jugando el estrés un papel importante en la fisiopatología y el curso de la enfermedad.[259]

3.3.3.3 Trastornos gastrointestinales funcionales

En la DF, el infiltrado de eosinófilos en el duodeno en un subgrupo de pacientes que experimentan saciedad temprana y plenitud postprandial, correlaciona con la presencia de dolor abdominal.[260] Además, en el 40% de los pacientes, la inflamación duodenal eosinofílica se ha observado con presencia de clústers y en algunos casos, degranulados cerca de las terminaciones nerviosas. Esta

interacción eosinófilo-nervio en el duodeno se asocia con la alteración de la motilidad y la distensión gástrica en estudios en animales.[261]

En el SII, a diferencia de la DF, no se ha observado presencia de un mayor infiltrado de eosinófilos, o de sus proteínas catiónicas a nivel local.[262,263] Sin embargo, se sabe poco sobre el estado de activación de los eosinófilos en el SII y sobre los efectos de los mediadores derivados de eosinófilos en la fisiopatología, a pesar de su relevancia potencial y de su papel en la DF.

Se postula, como se ha mencionado anteriormente, que el CRF periférico puede desempeñar un papel importante en la disfunción intestinal y en los síntomas en estos pacientes.[85] El eosinófilo, siendo célula que produce y responde a neuropéptidos[182,225,233] y es fuente de CRF en el intestino[192,258], podría tener un papel relevante en el SII. Se ha demostrado que el mastocito tiene un papel relevante en la disfunción de la barrera en el SII, y en parte mediado por la activación de los receptores de CRF.[264–266]

La proximidad de los mastocitos y los linfocitos en el SII y los eosinófilos en DF a las fibras nerviosas entéricas ofrece un mecanismo razonable por el cual las células inmunitarias, en particular el eje eosinófilo-mastocito, serían funcionalmente relevantes para la función sensorial o motora intestinal, para causar síntomas en respuesta a insultos tales como infecciones, antígenos y estrés.

HIPÓTESIS

HIPÓTESIS

En el marco de modelo biopsicosocial del SII, el sistema inmunitario intestinal podría estar respondiendo a estímulos de estrés favoreciendo la disfunción intestinal y la aparición de los síntomas. El eosinófilo, como célula residente intestinal, con un papel clave en diversas enfermedades gastrointestinales, y capaz de interaccionar con el sistema nervioso, se identifica como célula candidata a mediar los efectos del estrés sobre la función digestiva.

En esta tesis se postula la siguiente hipótesis:

“La activación por estrés del eosinófilo de la mucosa intestinal contribuye a la disfunción intestinal en el SII y participa en la generación y exacerbación de los síntomas característicos mediante la liberación de CRF”

1 OBJETIVOS

OBJETIVOS

Objetivo principal:

Establecer el papel de la activación del eosinófilo por mecanismos neuro-immunológicos asociados al estrés en la fisiopatología y manifestaciones clínicas del SII-D. Para ello, se plantean estudios en pacientes con esta patología y en grupo control, y se desarrolla un modelo *in vitro* de respuesta a factores de estrés en eosinófilos.

Objetivos específicos:

1. Evaluar el número, el estado de activación y contenido de CRF de los eosinófilos de la mucosa del yeyuno de pacientes con sindrome del intestino irritable con predominio diarrea, y determinar su implicación en la severidad clínica.
2. Evaluar, en un modelo *in vitro*, la respuesta subyacente al estrés en el eosinófilo para determinar la utilidad de los modelos celulares en el estudio de los mecanismos asociados al SII. . Para ello, se plantean los siguientes objetivos específicos:
    a. Evaluar el fenotipo y la funcionalidad de dos líneas celulares eosinofílicas.
    b. Analizar el estado de activación y mecanismos de secreción de los eosinófilos en respuesta a factores de estrés
    c. Determinar la producción y liberación de CRF en los eosinófilos en respuesta a estímulos de estrés

.

1

CAPÍTULO 1

CAPÍTULO 1

"Corticotropin-releasing factor content in mucosal eosinophils is associated with clinical severity in diarrhea-irritable bowel syndrome"

AUTHORS

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AFILIATION

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Keywords: eosinophil, corticotropin-releasing factor, diarrhea-predominant Irritable Bowel Syndrome

INTRODUCTION

Eosinophils are multifunctional granulocytes resident of the gastrointestinal (GI) mucosa except the esophagus. These cells play a prominent role in the physiology and host responses contributing to mucosal homeostasis and inflammation trough the release of a wide plethora of mediators including cationic proteins, cytokines, chemokines, growth factors and lipids.[1–3] Eosinophils have classically been implicated in allergic disease, and parasite and bacterial responses[4–7], in which they develop a prominent defensive role. In the GI tract, the presence of abnormal counts of eosinophil features several diseases such as eosinophil gastrointestinal disorders (EGIDs)[8] and inflammatory bowel disease (IBD)[9,10], and has recently been associated with functional dyspepsia (FD) pathophysiology.[11,12] Even though its contribution to GI pathology seems to be mediated mostly by inflammatory mechanisms, eosinophils also synthesize neuro-hormones and peptides and express receptors that mediate neuro-immune responses which may also contribute to disease mechanisms. In fact, intestinal mucosal eosinophils contain substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP) and corticotropin-releasing factor (CRF)[1,13–15] and, as most immune cells in the *lamina propria*, locate near nerves in pathological conditions[9,16], which enables bidirectional communication between the immune and nervous systems. This interaction may be relevant in stress-mediated GI dysfunction, particularly in functional GI disorders (FGID), however the contribution of eosinophils is not well described.

The role of stress is well recognized on GI symptom generation, especially in patients with FGID such as irritable bowel syndrome (IBS) and FD, where stress and anxiety appear as important comorbidity factors.[17] The main mediator of the stress response is central CRF, which coordinates responses of the autonomic and the behavioral systems[18,19]. Peripheral CRF can also contribute to changes in gut motility and intestinal permeability, mediated in part via recruitment and activation of mast cells, mimicking gut disturbances and symptoms of IBS patients.[20,21] Interestingly, and contrary to what is classically established, i.e. the only involvement of the large bowel, growing evidence points to a significant contribution of the small bowel to the origin of IBS symptoms.[22–24] The small intestine

discriminates between nutrients and harmful antigens from the load of foreign antigens is exposed to, and loss of function may facilitate the passage of potentially noxious substances to the mucosa, thus activating the immune system.[25] Recent studies in our group revealed the presence of low-grade mucosal inflammation and immune activation in the jejunal mucosa, together with an increase in psychological stress in diarrhea-predominant IBS (IBS-D) patients.[26–28] Moreover, mast cells and plasma cells activation is associated with impaired epithelial barrier function and with clinical manifestations.[27,29] In this scenario, eosinophils appear as mediators of local responses, either by inflammatory or neuro-hormonal mechanisms, which can promote immune activation, neural stimulation and barrier dysfunction. However, the role of eosinophils and the mechanisms underlying the molecular basis of these abnormalities are still unclear.

We hypothesized that jejunal mucosal eosinophils contribute to mucosal inflammation and GI dysfunction in IBS-D through CRF production. We aimed to identify jejunal eosinophil activity and contribution to IBS-D pathophysiology.

METHODS

Participants and clinical assessment

Newly diagnosed diarrhea-predominant IBS (IBS-D) patients fulfilling the Rome III criteria[30] were prospectively recruited from the outpatient gastroenterology clinic. Healthy controls (HC) were recruited from the general population by public advertising. Prior to entering the study, candidates were asked to complete structured clinical questionnaires (see below) to characterize digestive symptoms and to exclude other GI diseases in patients, and to verify the lack of symptoms in HC. Dyspepsia was also evaluated following the Rome III criteria. Past episodes of infectious gastroenteritis and GI comorbidities were assessed by means of broad biochemical and serological tests including anti-transglutaminase antibodies and thyroid hormones. Inclusion and exclusion criteria are summarized in supplementary table S1.

All candidates were also evaluated by an allergist to discard food and respiratory allergy. A battery of skin prick tests (SPT) (Laboratorios Leti SA, Barcelona, Spain) for 22 common food allergens and 12 inhalants was performed prior to the biopsy collection using histamine and saline as positive and negative controls, respectively. Positivity to food allergens by SPT or consistency to clinical history (digestive and/or extra-digestive symptoms associated with exposure to certain food components) were excluded. Clinical questionnaires were completed daily (10 days) by all participants and recorded: 1) pain severity (by a 100-point visual analogue scale, VAS)[31]; 2) pain frequency (number of days with pain)[31]; 3) stool frequency (maximum number of bowel movements); 4) stool form (by the Bristol Stool Chart score)[32]; and 5) distention using a 6 point VAS[31]. Background stress and depression levels were assessed using the validated Spanish versions of the Modified Social Readjustment Scale of Holmes-Rahe,[33] the Perceived Stress Scale of Cohen,[34] and the Beck's Depression Inventory[35], respectively. The severity of IBS was evaluated by the irritable bowel syndrome severity scoring system (Francis Score), which includes 5 questions, related to intensity of pain, frequency of pain, bowel habits, distention and quality of life, in a prompted visual analogue scale. Each question generates a maximum score of 100, leading to a total possible score of 500[31]. Written informed

consent was obtained from each participant. The study protocol was approved by the Ethics Committee at Hospital Vall d´Hebron [PR(AG)76/2006].

Experimental design and procedures

Clinical assessment and a jejunal biopsy were obtained in all participants. Tissue samples were obtained as follows: a single mucosal biopsy per participant was collected from the proximal jejunum, 5–10 cm distal to the Treitz's angle, using a Watson's capsule, orally inserted under fluoroscopic control, by suction with a 50mL syringe, between 8-10 am and after an overnight fast. Biopsy was immediately split into two similar pieces with a sterile scalpel. One fragment was fixed in 4% buffered formalin and embedded in paraffin for further microscopic examination of the tissue architecture and the inflammatory infiltrate, following routine procedures and immunohistochemistry for mast cells (CD117), eosinophils (hematoxylin eosin and major basic protein, MBP) and intraepithelial lymphocytes (IELs, CD3). The remaining fragment was placed either in RNAse-free tubes containing 500 µL of RNA Later Solution (Ambion, Thermo Fisher Scientific) and stored at -80ºC until processed for RNA isolation and gene expression analysis, or in fixative buffer (see below) for ultrastructure and immuno-gold labeling by transmission electron microscopy (TEM) and stored at 4ºC until processed. Samples for each experimental procedure were coded and analyzed blindly by one or two different investigators.

Analytical procedures

RNA isolation

Total RNA was isolated using the RNAeasy Mini Kit (Qiagen). First, biopsies were homogenized in RLT lysis buffer in the FastPrep Mixer (MP Biomedicals) for 2 cycles of 10 seconds at 6.5 speed alternating with incubations in ice for 10 seconds to prevent heating of samples. After tissue lysis, homogenates were centrifuged 3 min at maximum speed and transferred to a new tube. Lysates were mixed with 70% ethanol and placed on RNA isolation columns, following manufacturer's instructions. DNase treatment (Qiagen) was performed on columns at room temperature. RNA was eluted in 30µL of RNase-free water and stored at -80ºC until necessary. Prior to gene analysis, RNA quantity and quality were confirmed by capillary electrophoresis (Agilent 2100 Bioanalyzer; Agilent Technologies). Only samples with values of RIN ≥ 7 were included in the analysis.

Microarray analysis and pathway analysis

Five jejunal biopsy samples per group were blindly selected for this analysis, to compare gene expression profiles between groups. Microarray technology (Affymetrix GeneChip Human Genome HG-U133 Plus 2.0 arrays (Affymetrix)) was used for gene expression analysis according to manufacturer's instructions. The images were captured using Affymetrix Genechip Scanner 3000, and the resulting images were processed with Microarray Analysis Suite 5.0 (Affymetrix). Raw expression values obtained directly from CEL files were pre-processed using the RMA (Robust Multi-Chip Average) method. The complete dataset is available at the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; accession number GSE14841). To further select differentially expressed genes, a comparative analysis and linear models for microarray data (LIMMA) were performed for the entire probe set. The selection of differentially expressed genes between conditions was based on a linear model analysis with empirical Bayes moderation of the variance estimates.[36] To control errors derived from multiple simultaneous testing (one per gene), *P*-values were adjusted to obtain strong control over the "false discovery rate" (FDR) using the Benjamini and

Hochberg method.[37] Differentially-expressed genes (P<0.05), with a mean fold change of ≤0.7 and ≥1.4, were submitted to pathway and network analysis using the Ingenuity Pathway Analysis methodology (IPA 7.0, Ingenuity Systems). For network analysis, IPA computed a score (p-score=-log10(p-value)) according to the fit of the set of supplied focus genes and a list of biological functions stored in the IPKB. A score >3 (P<0.001) indicates a >99.9% confidence that a focus gene network was not generated by chance alone. The functional analysis identified biological functions and canonical signaling pathways most significant to the data set. The significance of the association between the data set and functions/pathways was determined based on the ratio of the number of genes from the data set that map to the function/pathway divided by the total number of genes that map to the function/pathway, and on a P-value calculated using Fischer's exact test determining the probability that the association between the dataset and the function/pathway is explained by chance alone.

c-DNA synthesis and quantitative real time PCR

cDNA synthesis was performed using 1 µg of total RNA with the High Capacity Reverse Transcription Reagents Kit (Applied Biosystems, Thermo-Fisher Scientific) following manufacturer's protocol. Gene expression was assessed for a selection of genes involved in eosinophil chemotaxis, pro-inflammatory activity and secretory responses by qPCR using validated TaqMan® Gene Expression Assays (GEA) (Applied Biosystems). Due to the low abundance of eosinophil-specific genes in mucosal samples, pre-amplification of cDNA was performed before qPCR when required (see table 1) with the PreAmp Master Mix following the manufacturer's instructions (Applied Biosystems). TaqMan primer sets used are described in table 1:

Gene assay	Gene name	Specie	Taqman assay	Preampl.
Eosinophil chemotactic proteins				
CCL11	C-C motif chemokine ligand 11	Homo sapiens	*Hs00237013_m1*	No
CCL24	C-C motif chemokine ligand 24	Homo sapiens	*Hs00171082_m1*	Yes
CCL26	C-C motif chemokine ligand 26	Homo sapiens	*Hs00171146_m1*	Yes
Eosinophil cationic proteins				
CLC	Charcot-Leyden crystal galectin	Homo sapiens	*Hs00171342_m1*	Yes
EPX	Eosinophil peroxidase	Homo sapiens	*Hs00946094_m1*	Yes
PRG2	Proteoglycan 2, pro eosinophil major basic protein	Homo sapiens	*Hs00794928_m1*	Yes
RNASE2	Ribonuclease A family member 2	Homo sapiens	*Hs00795553_s1*	Yes
RNASE3	Ribonuclease A family member 3	Homo sapiens	*Hs01923184_s1*	Yes
Secretory activity proteins				
SNAP23	Synaptosome associated protein 23	Homo sapiens	*Hs01047496_m1*	Yes
STX4	Syntaxin 4	Homo sapiens	*Hs00190266_m1*	Yes
VAMP2	Vesicle associated membrane protein 2	Homo sapiens	*Hs00360269_m1*	Yes
Stress-related proteins				
CRHR1	Corticotropin-releasing hormone receptor 1	Homo sapiens	*Hs00366363_m1*	Yes
CRHR2	Corticotropin-releasing hormone receptor 2	Homo sapiens	*Hs00266401_m1*	Yes
Endogenous control gene				
18S	Eukaryotic 18S rRNA	Homo sapiens	*Hs99999901_s1*	No

Table 1. Gene expression assay probes used for qPCR analysis. Preampl. indicates pre-amplification of cDNA.

Gene expression was analyzed by qPCR on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems). Each sample, including distilled water as negative control, was run in triplicate and data were analyzed by the $2^{-\Delta\Delta Ct}$ method. The expression of each gene was normalized to the endogenous control, and the fold-change was calculated individually respect to the average of the HC group.

Immunohistochemistry

Tissue sections (4µm) were processed following standard procedures. First, tissue samples, after deparaffination at 60ºC 1h, were hydrated in decreased order of alcohol solutions. After antigen retrieval in citrate solution at 120ºC 20min, an incubation with endogenous peroxidase blocking and protein blocking was performed, followed by incubation with anti-human MBP, CD3 or CD117 for eosinophils, IELs or mast cell identification, respectively (see supplementary table S4 for supplier and concentration details). Slides were further incubated with the appropriate peroxidase-conjugated secondary antibody and developed with the Vectastain ABC kit (Vector Laboratories, United Kingdom). Positive cells were quantified in 8-10 non-overlapping fields and results are expressed as the number of cells per high-power field (HPF), per 100 epithelial cells or per mm^2, using the CellSens-1.7 software (Olympus BX61).

Processing of tissue samples for transmission electron microscopy and immuno-labelling

- *Conventional TEM analysis*: Tissue fragments were immersed in a fixative solution of 2.5% (v/v) glutaraldehyde + 2% paraformaldehyde (w/v) (PFA) in 0.2M phosphate buffer (PB) for at least 48 hours. Tissues were then post-fixed in 1% (w/v) osmium tetroxide (Sigma) followed by washes in deionized water and dehydration sequentially in acetone. Samples were then embedded in Eponate 12 resin (Ted Pella Inc., USA) and polymerized at 60ºC.

- *Nano-immunogold TEM analysis*: Tissue fragments were fixed in 2% PFA in 0.2M PB for at least 48h followed by washes in PB and glycine. Tissues were cryoprotected by passage through a graded series of sucrose solutions of 0.7, 1.4 and 2.3 M in PB. After hydration and immersion in methanol containing 0.5 uranyl acetate, samples were embedded in acrylic Lowicryl HM20 resin (Polysciences Inc., Warrington, USA) and polymerized. Semithin sections (1 µm thick) for both conventional and nano-immunogold analysis were obtained with a Leica ultracut UCT microtome (Leica Microsystems GmbH, Wetzlar, Germany), stained with 1% (w/v) aqueous toluidine blue solution and examined with a light microscope to identify well-oriented areas (comprising epithelium and *lamina propria*) for further analysis. Ultrathin sections (70 nm thick) were cut with a diamond knife (45, Diatome, Biel,

Switzerland), placed on grids and contrasted with conventional uranyl acetate (30 min) and Reynolds lead citrate (5 min) solutions.

Eosinophil ultrastructure analysis

The general structure of each tissue was evaluated and only samples containing both intact epithelium and *lamina propria* (assuring good fixation) were evaluated. Mucosal eosinophils were identified based on their specific morphology in non-overlapping fields, in at least 10 ultrathin cuts. The analysis included the evaluation of the presence, type and degree of degranulation in eosinophils at 1,000-15,000x magnification. Piecemeal degranulation (PMD) (loss of intragranular electro-density without signs of intergranular or granule-to-cell membrane fusion) was identified as a marker of cell activation. Each individual eosinophil was evaluated, and each specific granule was examined and classified as either intact (no signs of degranulation) or activated (PMD). In order to express the degree of degranulation for each individual eosinophil, the percentage of granules with loss of content respect to the total number of granules was calculated. Therefore, based on "loss of content" eosinophils were categorized into: resting (no degranulated granules), low (0-<20% degranulated granules) moderate (20-60%) and high (>60%), as previously described.[38] Morphological analysis of eosinophils included quantification of: total area of eosinophil cell, nucleus, cytoplasm and granules; degranulated area of cytoplasmic granules; and number of cytoplasmic granules and lipid bodies. Tissues were observed with a TEM Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) equipped with a Gatan Ultrascan ES1000 CCD Camera, at magnification 10.000-100.000x. Morphological analysis was performed using the ImageJ® software.

Nano-immunogold labelling analysis and validation:

CRF labelling was performed over tissue cuts on coper grids, following standard procedures. Grids were blocked for 25 min in 1% bovine serum albumin (BSA; Sigma) and glycine 20mM in PB and incubated with the primary human CRF antibody in 1% BSA/PBS for 2h, with a determined pH of incubation according to the antibody used (see supplementary table S5). Sections were incubated with the secondary antibody coupled to nano-gold particles of 10 or 15 nm (supplementary table S5).

Validation of CRF antibody specificity and reproducibility in human jejunal samples was confirmed by testing antibodies in rat-brain extracts and in transfected HEK cells followed by comparinson of the signal obtained with different sets of primary anti-human CRF antibodies (supplementary methods chapter 1). Negative controls included sections in which incubation was with BSA/PBS instead of primary antibody.

Statistical analysis

Data are expressed as median (range) or mean ± standard deviation. Normality of data distribution was tested by the D'Agostino and Pearson omnibus normality test. Normally distributed parametric data were compared by the unpaired Student's t test (two-tailed); otherwise, the Mann-Whitney U test was used, using GraphPad Prism 6.0 software. Categorical variables were compared using the Fisher's exact test or the Chi-square test. Relationships between clinical variables and gene expression were assessed by Spearman's rho correlation. *P* values of ≤ 0.05 were considered significant and were adjusted for multiple comparisons using the Benjamini and Hochberg method[39] and the application of correction is indicated in each table and figure legends.

RESULTS

Study population

Twenty-five HC and 42 IBS-D patients were included in the study. There were no differences in age between participants; however, the number of women in IBS-D was higher than in HC (*P*<0.05). The HC group had similar stress level in the last year as compared with IBS-D patients. However, IBS-D patients exhibited higher level of stress in the last month (*P*<0.0001) and of depression (*P*<0.0001) than HC subjects (table 2). In the IBS-D group, 53% had dyspepsia, with similar frequency and intensity of abdominal pain, number of bowel movements and stool form than non-dyspeptic patients with IBS-D (see supplementary table S2 for individual clinical data).

	HC (n=25)	**IBS-D (n=43)**	***P* value**
Gender, F:M	11:14	30:13	**0.036 ***
Age, years	35 (30-39)	37 (34-41)	0.242
Atopy, yes: no	6:14	7:8	0.253
Intensity of abdominal pain, score	0	47.69 ± 24.54	-
Frequency of abdominal pain, score	0	5.48 (3-10)	-
Bowel movements (n)	1 (1-2)	4 (1-12)	**<0,0001****
Stool form, Bristol score	3.3 (2-5)	5.5 (2-7)	**<0,0001****
Dyspepsia	-	23/43	
Distention	-	47.5 (0-100)	-
Holmes-Rahe Scale	116 (29-267)	179 (25-889)	0.237
Cohen Scale	16 (4-28)	25 (9-46)	**<0,0001****
Beck's Depression Index	3 (0-22)	11 (0-36)	**<0,0001****

Table 2. Clinical and demographic characteristics of participants. Values represent the median (range) or the mean ± SD. *P* Values considered significant are shown in bold. *P* value adjusted (false discovery rate): * *P* <0.05; ** *P* <0.001. F, female; M, male; HC, healthy controls; IBS-D, diarrhea-predominant IBS.

The intestinal mucosa of IBS-D patients shows a distinctive transcriptional profile linked to alterations in eosinophil signaling and activation

In a previous work, we observed differential gene expression comparing healthy controls with IBS-D by microarray[26,40]. Analysis of microarray data, revealed a total of 286 differentially expressed genes in IBS-D patients compared to HC (FDR-adjusted *P* value<0.05). [26,40] IPA analysis identified canonical signaling pathways key for intestinal barrier function[26], as well as immunological functions as playing an important role in IBS-D. Interestingly, biological functions related to eosinophil chemotaxis appear as differentially expressed between the two groups (*P*<0.05) (table 3). Moreover, biological functions associated to the transcriptome also showed functions indirectly related to eosinophil survival, chemotaxis and activation as differentially expressed between groups

Ingenuity canonical pathway	*P* value	Ratio*	Molecules
Caveolar-mediated Endocytosis	0,0002	8,54E-02	*B2M, RAB5C, COPA* (includes EG:1314), *ITGAV, INSR, MAP3K2, COPG*
Tight-junction signaling	0.0006	5,49E-02	*TJP2, PPP2R5C, MPP5, TJP1, PVRL3, CTNNA1, INADL, CTNNB1*
PTEN signaling	0.0008	6,67E-02	*RAC2, MAGI1, SOS1, FOXO3, BMPR2, INSR, TNFRSF11A*
Ephrin receptor signaling	0.0020	4,62E-02	*VEGFA, RAC2, ACTR2, NGEF, SOS1, CRK, GNB1L, GNG12, EFNA1*
ERK/MAPK signaling	0.0030	4,69E-02	*ETS1, RAC2, PPP2R5C, PIK3C2A, SOS1, MAPKAPK5, PPP1CB, CRK,*
Integrin signaling	0.0040	4,43E-02	*RAC2, ACTR2, ARF1, PIK3C2A, SOS1, ITGAV, RHOU, PPP1CB, RAP2A*
NFAT in Regulation Immune Response	0.0080	4,17E-02	*CDE3, PIK3C2A, CSNK1G2, ITPR2, SOS1, GNBL1, GNG12, PPP3CA*
TNF-β signaling	0.0100	5,81E-02	*SOS1, BMPR2, HNF4A, ACVR1C, ACVR2A*
Reg. of Actin-based motility by Rho	0.0100	5,38E-02	*RAC2, ACTR2, RHOU, PPP1CB, GSN*
PI3K/AKT signaling	0.0100	4,38E-02	*PPP2R5C, YWHAE, HSP90AB1, SOS1, FOXO3, CTNNB1*
VEGF signaling	0.0200	5,15E-02	*VEGFA, YWHAE, PIK3C2A, SOS1, FOXO3*
SAPK/JNK signaling	0.0100	5,21E-02	*RAC2, PIK3C2A, SOS1, CRK, MAP3K2*
PPAR signaling	0.0200	4,90E-02	*HSP90AB1, IL1RN, SOS1, INSR, CITED2*
Antigen Presentation Pathway	0.0200	7,69E-02	*B2M, CALR, CD74*
Axonal Guidance signaling	0.0200	2,97E-02	*VEGFA, RAC2, ACTR2, NGEF, PIK3C2A, SOS1, SEMA6A, CRK*
GM-CSF signaling	0.0200	5,97E-02	*ETS1, PIK3C2A, SOS1, PPP3CA*
Actin Cytoskeleton signaling	0.0200	3,38E-02	*RAC2, ACTR2, PIK3C2A, SOS1, PPP1CB, CRK, GSN, GNG12*
Leukocyte Extravasation signaling	0.0300	3,59E-02	*RAC2, MMP28, PIk3C2A, CTNNA1, CRK, CTNNB1, CTTN*
NFkB signaling	0.0300	4,00E-02	*PIK3C2A, IL1RN, TGFA, BMPR2, INSR, TNFRSF11A*
B cell receptor signaling	0.0400	3,87E-02	*ETS1, RAC2, PIK3C2A, SOS1, PPP3CA, MAP3K2*
Clathrin-mediated endocytosis	0.0400	3,59E-02	*VEGFA, ACTR2, PIK3C2A, RAB5C, CTTN, PPP3CA*
CD28 signaling in T helper cells	0.0400	4,00E-02	*ACTR2, CD3E, PIK3C2A, ITPR2, PPP3CA*
CCR3 signaling in Eosinophils	**0.0400**	4,13E-02	***PIK3C2A, ITPR2, PPP1CB, GNB1L, GNG12***
EGF signaling	0.0500	5,77E-02	*PIK3C2A, ITPR2, SOS1*

Table 3: Functional analysis of canonical pathways associated to the differential transcriptome in IBS-D *versus* HC. List of categories and function annotation of genes identified, including eosinophil signaling. *P* values are indicated. *P* value adjusted (false discovery rate). Ratio*: number of genes in the analysis that are associated with the canonical pathway divided by the total number of genes that map to the canonical pathway. The genes from the input data set that belong to each canonical pathway are also indicated.

Mucosal jejunal eosinophils are found in similar quantity in HC and IBS-D patients

To examine tissue eosinophil infiltration and location, eosinophil counts were analyzed only in specimens with well-preserved villous architecture. Under the routine histopathological study, samples did not show lymphoid follicles and had a discrete lymphoplasmacytic infiltrate in the *lamina propria.* The number of intraepithelial $CD3^+$ cells was similar in both groups [H 25.2±10.2; IBS-D

26.3±18.5). The number of mucosal eosinophils was similar in both groups and were found scattered along the crypts in the *lamina propria* (figure 1).

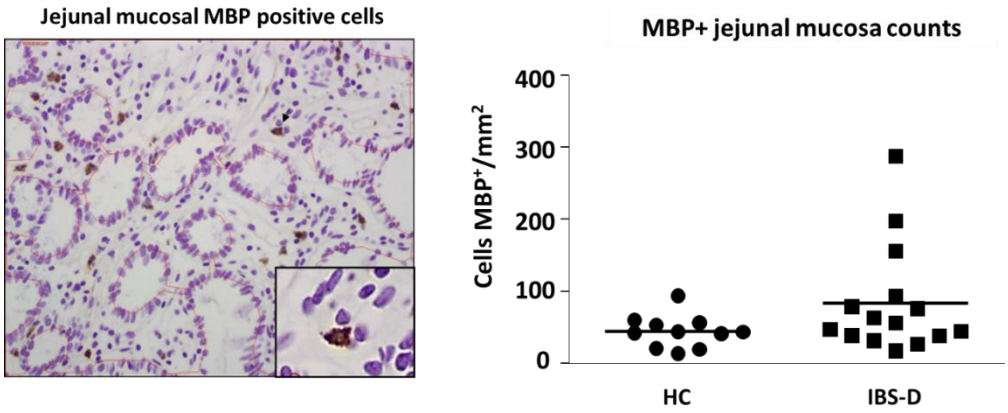


Figure 1. Distribution and counts of jejunal eosinophils in HC and IBS-D groups. Representative image of MBP-positive staining in the *lamina propria,* and an insert illustrating positive staining. Positive cell counts are represented per mm^2 in each group. Magnification 400x. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

Mucosal jejunal eosinophils show a low-inflammatory activation profile in IBS-D patients

For microarray data and IPA analysis validation, genes involved in eosinophil chemotaxis, activation and secretory activity were further quantified by qPCR. Related to eosinophil homing to the gut, eotaxin receptor *CCR3* and eotaxins 2 and 3 (*CCL24* and *CCL26*, respectively), were down-regulated in IBS-D patients, with no differences in eotaxin 1 (*CCL11*) (figure 2).

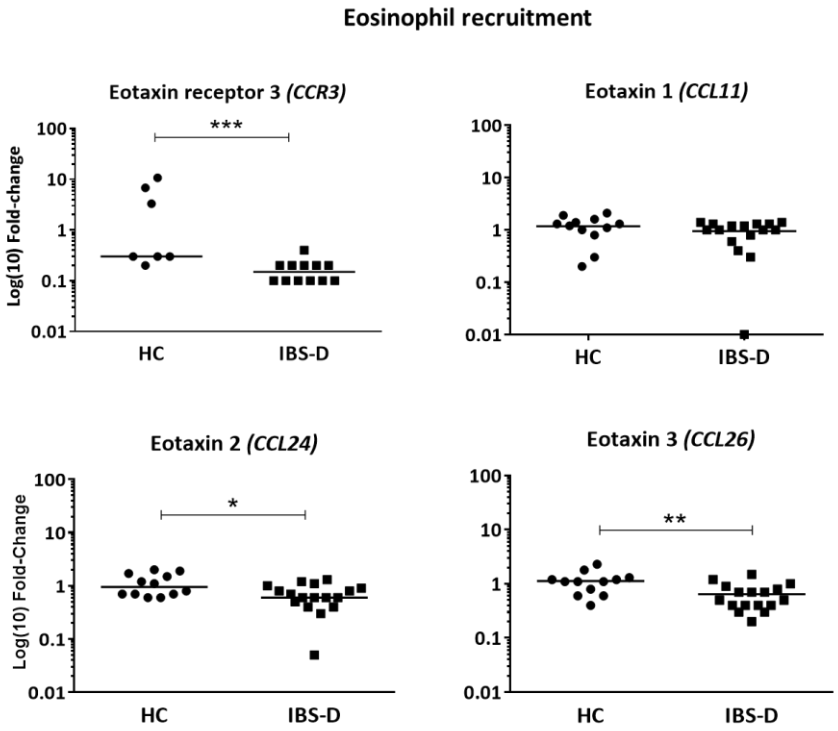


Figure 2. Representation of the expression of genes associated with recruitment of eosinophils in biopsy samples from HC and IBS-D groups. Expression analysis of the eosinophil-related chemotactic genes C-C chemokine receptor 3 (*CCR3*), C-C motif chemokine 11, 24 and 26 (*CL11*, *CCL24* and *CCL26,* eotaxins 1, 2 and 3, respectively) in IBS-D and HC groups. Individual values represent the fold change with respect to the average of the HC group. The 18S was used as an endogenous gene. *P* values are indicated: **P*<0.05, ** *P* <0.01 and *** *P* <0.001. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

Gene expression of eosinophil pro-inflammatory cationic proteins, as indicators of eosinophil activation: eosinophil-derived neurotoxin (EDN (*RNASE2)*), eosinophil cationic protein (ECP (*RNASE3)*) and eosinophil peroxidase (EPO (*EPX)*), were also down-regulated in the intestinal mucosa of IBS-D patients, while no changes in *PRG2* and *CLC* were observed compared to HC (figure 3).

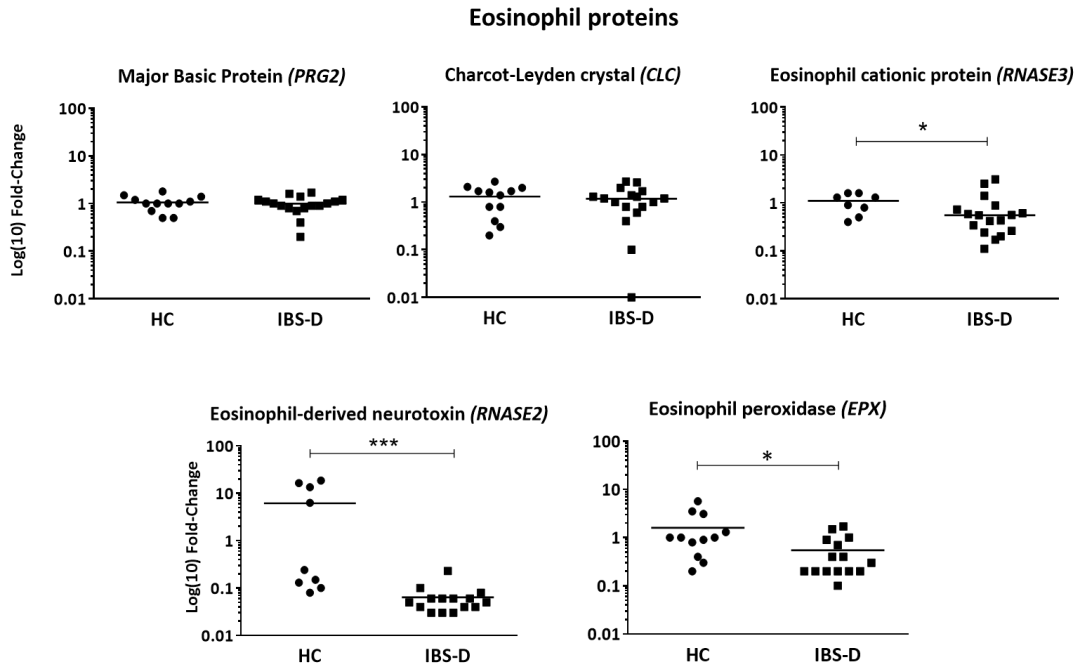


Figure 3. Representation of the expression of genes associated with pro-inflammatory activity of eosinophils, in biopsy samples from HC and IBS-D groups. Expression analysis of genes indicative of activation of eosinophils: eosinophil major basic protein (Proteoglycan 2, *PRG2)*, Charcot-Leyden crystal galectin *(CLC),* eosinophil-derived neurotoxin (ribonuclease A family member 2, *RNASE2*), eosinophil cationic protein (ribonuclease A family member 3, *RNASE3*) and eosinophil peroxidase (EPX). Individual values represent the fold change with respect to the average of the HC group. The 18S was used as an endogenous gene. *P* Values are indicated: * *P* <0.05 and *** *P* <0.001. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

To further assess secretory activity in the jejunal mucosa, the expression of SNAREs genes involved in eosinophil secretion process were assessed. *SNAP23,* a membrane protein related to vesicular transport, was up-regulated in IBS-D, suggesting an increased secretory activity in this group, despite no differences were observed in other eosinophil-related SNARE proteins syntaxin 4 (*STX4)* and vesicular-associated membrane protein 2 (*VAMP2)* (figure 5).

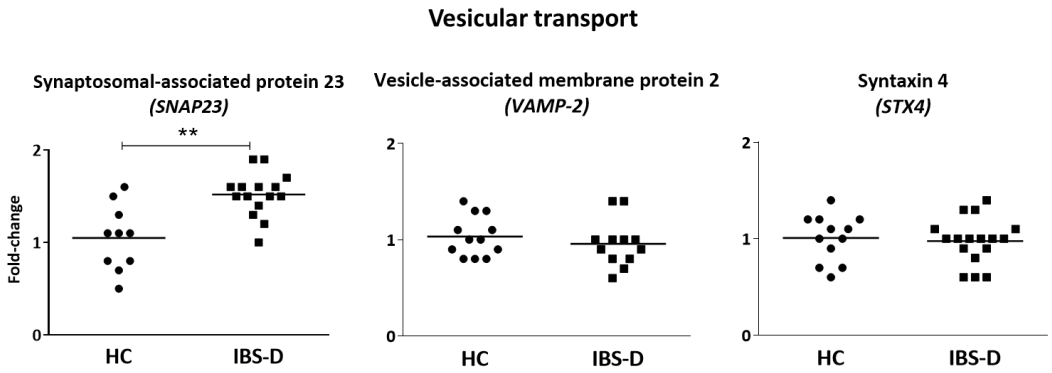
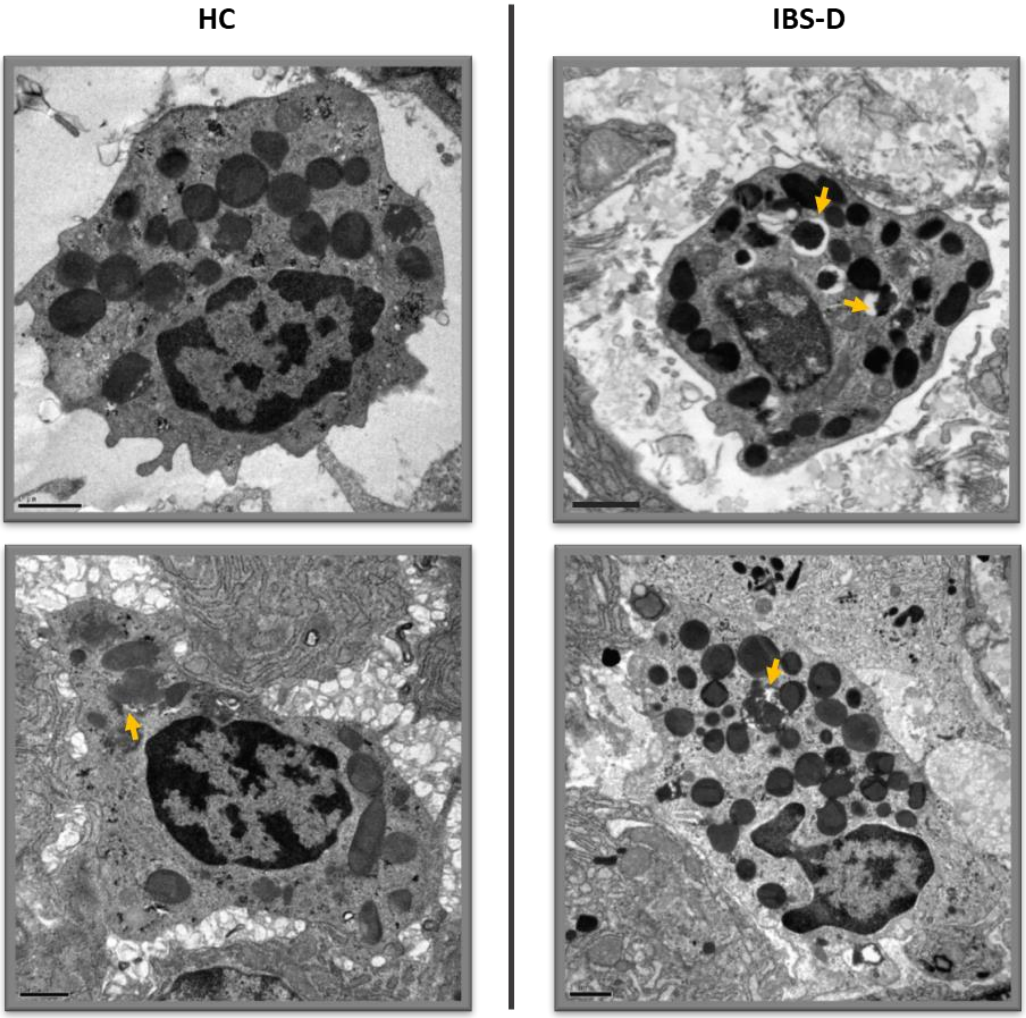


Figure 4. **Representation of the expression of genes associated with secretory activity in biopsy samples from HC and IBS-D groups.** Quantitative gene expression analysis of the secretory activity-related genes Synaptosomal-associated protein 23 (*SNAP23*), Vesicle-associated membrane protein 2 (*VAMP2*) and Syntaxin4 (*STX4*) in HC and IBS-D groups. Individual values represent the fold change with respect to the average of the HC group. The 18S was used as an endogenous gene. *P* Values are indicated: ** *P* <0.01. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

Eosinophils from IBS-D patients show ultrastructural signs of activation in the jejunal mucosa

Qualitative analysis of the eosinophil ultrastructure revealed pronounced signs of activation and secretion in the IBS-D group, as indicated by higher frequency of PMD with selective fragmentation of cytoplasmic granules (figure 5 A) and higher frequency of vesico-tubular structures such as “sombrero-like” vesicles (figure 5 B).

A.



B.

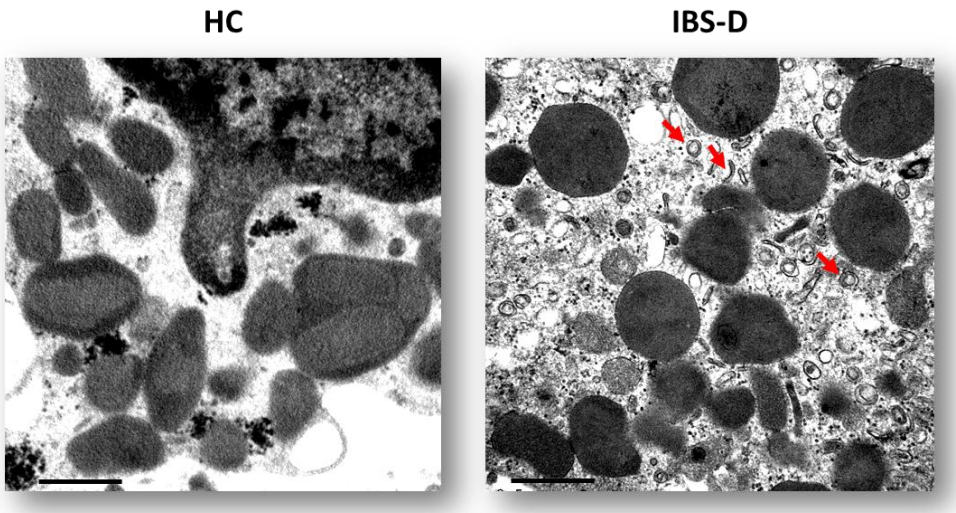


Figure 5. Eosinophil ultrastructure revealing a secretory profile and activation in mucosal jejunal samples from HC and IBS-D groups. (A) Representative images of jejunal mucosal eosinophil ultrastructure. Orange arrows indicate loss of

granular content (B) Higher magnification of jejunal mucosal cytoplasm containing granules and secretory structures. Red arrows indicate sombrero-like vesicles and tubular structures indicative of active secretion. Bars indicate magnification (top panels: 1μm; low panels: 0.5μm)

By the use of a PMD index previously defined as the percentage of granules with PMD[38], eosinophils were classified as resting eosinophils, or displaying low, moderate or high PMD degranulation. A similar number of eosinophils [60 eos HC; 73 eos IBS-D] and cytoplasmic granules [1,665 granules in HC; 1,785 granules in IBS-D] were analyzed in both groups. Visual analysis revealed the presence of resting eosinophils, and also low and moderate level of degranulation in both groups. High level of degranulation was not observed in any eosinophil from the groups analyzed. However, when quantifying the degree of eosinophil degranulation, the percentages were significantly different, having HC 62% of resting and 38% of degranulated eosinophils, of which 91% showed a low degree of PMD; while the IBS-D group showed 17% of resting and 83% of degranulated eosinophils, in which 72% eosinophils display low PMD and 28% moderate PMD ((figure 6; *P*<0.0001)

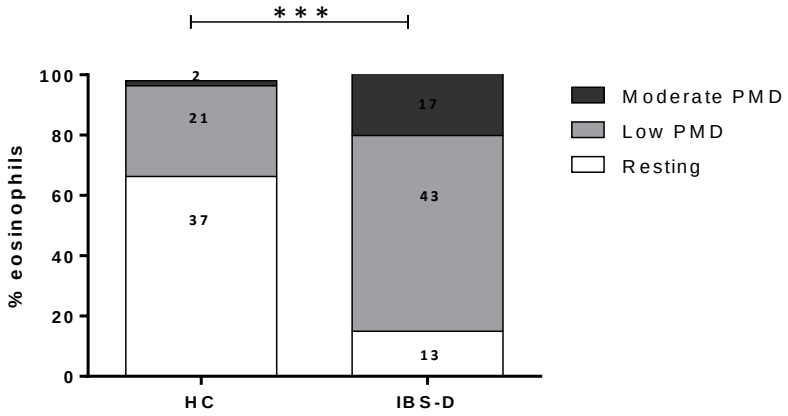


Figure 6. **Quantification of eosinophil PMD-pattern of degranulation in HC and IBS-D groups.** Visual analysis of eosinophil degranulation patterns expressed as proportions of eosinophils displaying different level of degranulation. Resting eosinophils: 0% altered granules; low piecemeal degranulation (PMD): 0–20%; moderate PMD: 21-60%. The number of eosinophils in each category is indicated. Categorical variables were compared using the Fisher's exact test. ****P*<0.0001. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

Quantitative analysis of activation markers showed no differences in the number of cytoplasmic granules and lipid bodies per eosinophil between groups (figure 7). However, the area occupied by the granules (i.e. cytoplasmic granular density) was higher in the IBS-D group (*P* <0.05), with also larger degranulated area (*P* <0.0001) compared to control subjects. (figure 7)

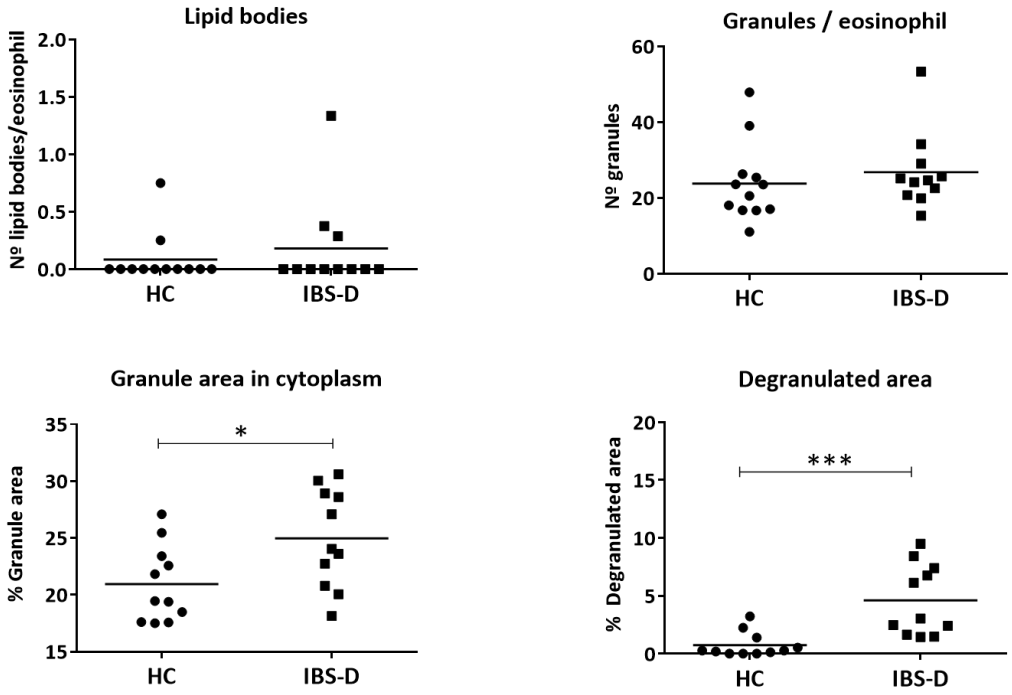


Figure 7. Ultrastructural quantitative analysis of eosinophil activation in jejunal samples from HC and IBS-D groups. Representation of the number of lipid bodies, number of granules, granule area, and degranulated area of granules of jejunal eosinophils. Images were analyzed with the ImageJ software. *P* values are indicated: * *P* <0.05 and *** *P* <0.001. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

Jejunal mucosal eosinophil granules of IBS-D present higher CRF expression than HC

A detailed analysis of all the *lamina propria* resident cells, revealed that positivity to CRF was identified only in eosinophils and not in plasma cells, macrophages, dendritic cells mast cells, lymphocytes or mast cells (figure 8).

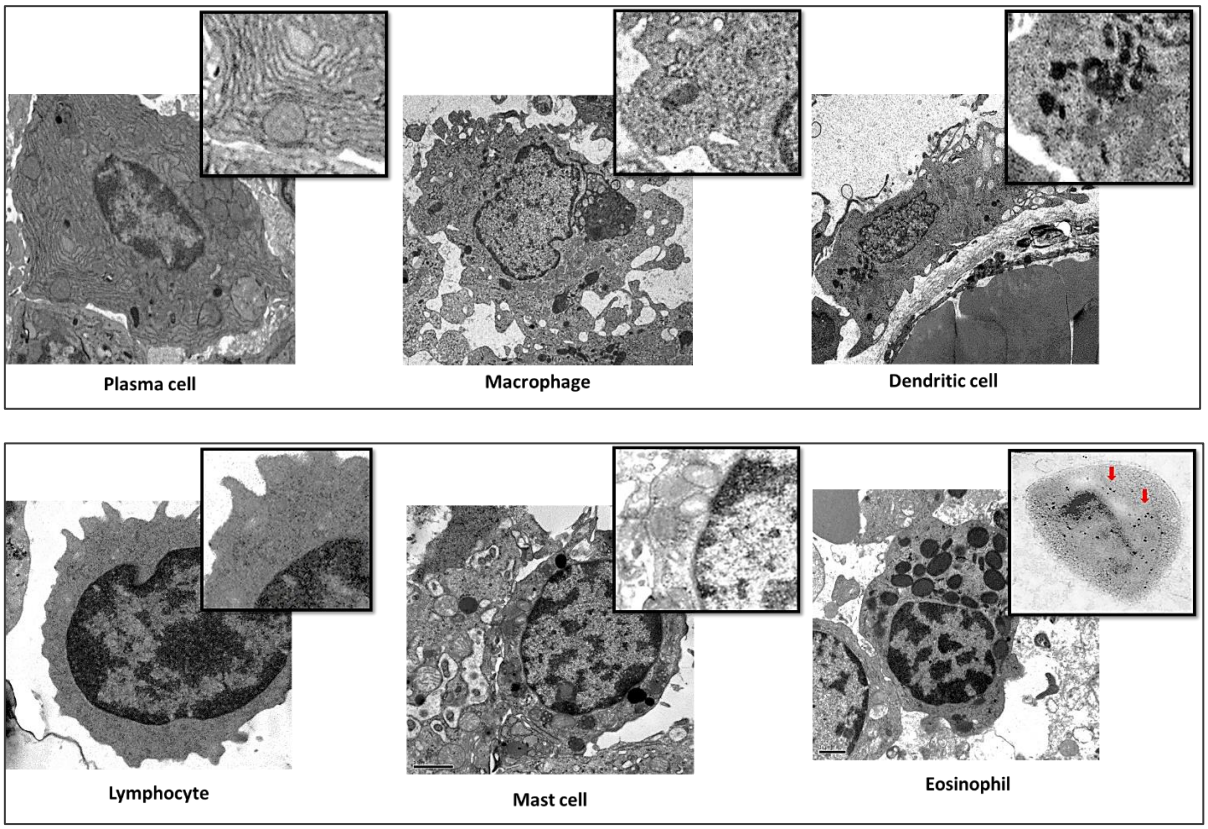


Figure 8. Representative micrographs of the *lamina propria* resident cells in the jejunal mucosa and localization of CRF positivity. Arrow indicates CRF nano-gold particles in eosinophil granules (magnification 10.000x). Insert in each photomicrograph represents a detail of the cell's cytoplasm in each cell type or granule with CRF nano-immunogold particles in the eosinophil.

CRF was revealed by gold-electron-dense particles and was found in cytoplasmic granules of eosinophils in both, IBS-D patients and HC (figure 9 A). Similar number of eosinophils per sample and granules per eosinophil were analyzed between groups (supplementary figure S8). CRF location in eosinophil granules was identified in specific (contain crystal core) and primary (without crystal core) granules, in both HC and IBS-D, although it was more frequently observed in specific granules (figure 9B)

A higher CRF content per granule was found in eosinophils from IBS-D samples (figure 9 C left) which positively correlated with the percentage of degranulated area (figure 9 C right).

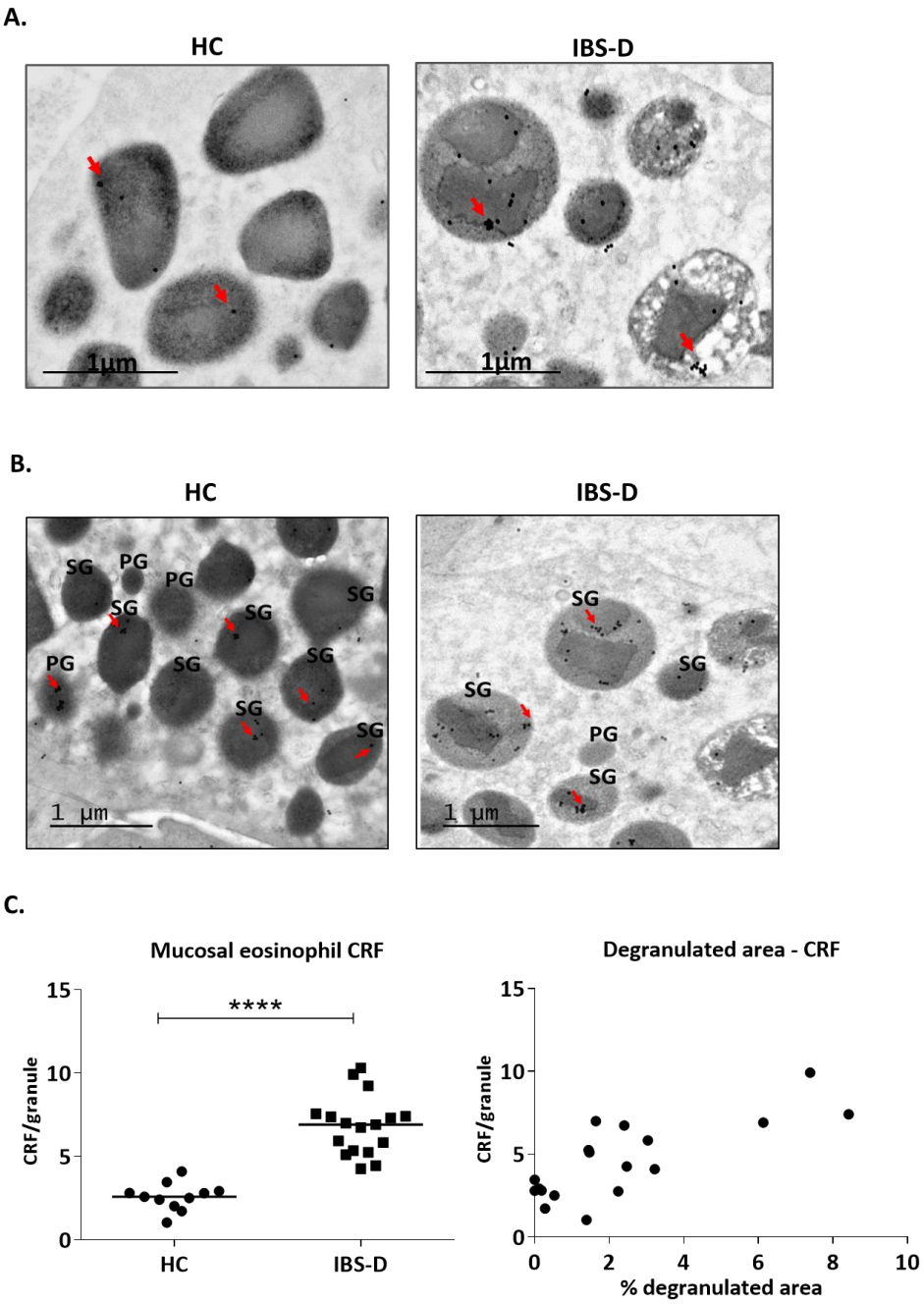


Figure 9. Identification and quantification of nano-immunogold labeling for CRF in jejunal eosinophils from HC and IBS-D patients. **A.** Representative micrographs of CRF positivity in eosinophil granules from both groups. **B**. Eosinophil cytoplasm images of CRF localization in eosinophil specific and primary granules in HC and IBS-D mucosal samples**.** Arrow indicate CRF nano-immunogold particle in eosinophil granules. **C.** Left: CRF content in eosinophil granules from HC and IBS-D groups. *P* value is indicated: *** *P* <0.001. Right: Correlation between CRF content and degranulated area of eosinophils from HC and IBS-D groups. r_s and *P* value according to Spearman rank correlation.SG: specific granule; PG: primary granule. HC: healthy control; IBS-D: diarrhea-predominant irritable bowel syndrome.

Association between mucosal eosinophil CRF content and IBS-D clinical manifestations

To gain insight into eosinophil-derived CRF connection to intestinal dysfunction, we then analyzed the association between eosinophil CRF granular content and major clinical manifestations. A significant correlation was found between CRF content and IBS-D symptoms including abdominal pain intensity, IBS severity, distention, number of bowel movements per day and stool form. Psychological stress and depression also significantly correlated with CRF content (table 3).

CRF/granule	r_s	*P value*	FDR	HC	IBS-D
Intensity of pain	**0,799**	**0,0002**	**0,001*** **	-	17
Frequency of pain	0,3812	0,1313	0,184	-	17
IBS Severity	**0,5343**	**0,0291**	**0,058***	-	17
Distention	**0,4856**	**0,0499**	**0,087***	-	17
Dyspepsia	0,3114	0,2454	0,312	-	17
Bowel movements	**0,6161**	**0,0013**	**0,005****	7	17
Stool form	**0,6055**	**0,0017**	**0,005**	7	17
Stress (last month)	**0,6557**	**0,0001**	**0,001*** **	12	17
Stress (last year)	0,3478	0,0645	0,100	12	17
Depression score	**0,7191**	**<0,0001**	**0,001*** **	12	17
Mast cell (CD117) /hpf	-0,1411	0,4826	0,563	11	16
Eosinophil /mm^2	0,1404	0,4849	0,522	11	16
Atopy	-0,06747	0,7433	0,743	10	16
Sex	**0,4435**	**0,016**	**0,037***	12	17

Table 3. Correlation between CRF content in eosinophils and clinical parameters. *P* values considered significant are shown in bold. *P* value adjusted (false discovery rate (FDR)): * *P* <0.05; ** *P* <0.01 and *** *P* <0.001. HC, healthy controls; IBS-D, diarrhea-predominant IBS; A FDR <10% was accepted as significant.

Expression of CRFR receptor1 is decreased in the jejunal mucosa of IBS-D patients and correlates with major clinical and psychological manifestations

Finally, the expression of CRF receptors (CRHR) in the jejunal mucosa was analyzed in both experimental groups. *CRHR1* expression was down-regulated in IBS-D patients, while no changes in *CRHR2* were detected (figure 10). Moreover, the CRFR1 gene downregulation association with clinical

manifestations showed negatively correlation with intensity of pain and stool form, as well as with psychological stress of last year and depression levels (table 4).

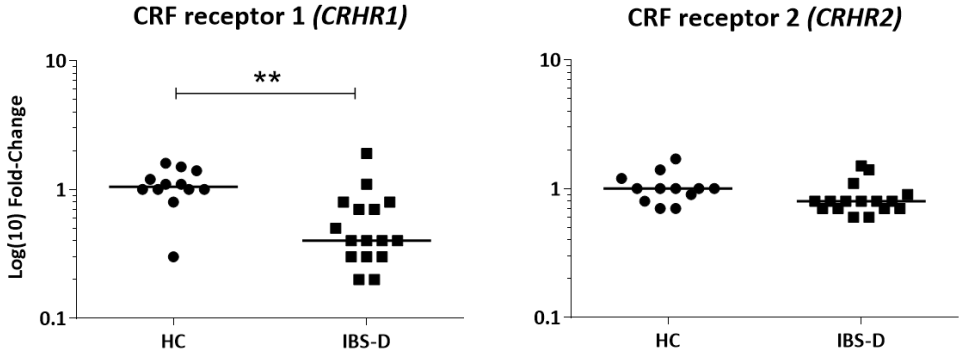


Figure 10. CRF receptors expression in the jejunal mucosa of HC and IBS-D. mRNA expression of corticotropin-releasing factor (*CRF*) receptors (*CRHR1* and *CRHR2)* was measured by qPCR in HC controls and IBS-D patients. Individual values represent the fold change with respect to the average in the HC group. Normalization was performed using the 18s rRNA gene as the endogenous control. *P* values are indicated ** *P* <0.01. HC, healthy controls; IBS-D, diarrhea-predominant IBS.

CRFR1 expression levels	r_s	*P value*	FDR	HC	IBS-D
Intensity of pain	**-0.6157**	**0.0109**	**0.0681***	-	17
Stool form	**-0.5743**	**0.0042**	**0.0357****	7	17
Stress (last year)	**-0.5561**	**0.0026**	**0.0357****	12	16
Depression score	**-0.4646**	**0.0143**	**0.0620***	11	16
Stress (last month)	-0.3552	0.075	0.2108	11	16

Table 4. Correlation between CRFR1 gene expression in jejunal samples from HC and IBS-D and clinical parameters. *P* values considered significant are shown in bold. *P* value adjusted (false discovery rate (FDR)): * *P* <0.05; ** *P* <0.01. HC, healthy controls; IBS-D, diarrhea-predominant IBS; A FDR <10% was accepted as significant.

DISCUSSION

The present study demonstrates, for the first time, increased content of CRF in jejunal eosinophils of IBS-D respect to healthy controls, in association with clinical severity and stress and depression levels. Interestingly, eosinophils infiltrating the jejunal mucosa of these patients are similar in number than in physiological conditions and display a low pro-inflammatory profile along with increased CRF granular content and degranulation degree. These findings, together with previous reports, suggest that local eosinophils play a role in GI dysfunction, possibly contributing to stress-mediated mechanisms.

The analysis of integrative pathways associated with the transcriptome of IBS-D identified eosinophil chemotaxis, activation and survival, as significant pathways differentially expressed. Eosinophils are generally thought to act as pro-inflammatory cells in disease conditions due to their content in pleiotropic cytokines, chemokines as well as toxic cytoplasmic cationic proteins.[41] Increased intestinal mucosal eosinophil numbers with a pro-inflammatory profile is associated with up-regulation of eosinophil signaling trough eotaxin-CCR3 axis and supports a role of eosinophils in mediating intestinal inflammation. Nevertheless, in IBS, despite the fact that microinflammation and hyperplasia of mast cells, plasma cells and lymphocytes have been described in the jejunal mucosa[28,42,43], we observed comparable eosinophils counts between groups, as previously reported in colonic tissue.[44] Additionally, we also observed down-regulation of *CCL24* and *CCL26* (eotaxins 2 and 3, respectively) and *CCR3* gene expression in IBS-D, together with a decrease in *EPX* (EPO), *RNASE2* (EDN) and *RNASE3* (ECP) cationic proteins gene expression. Our results, in contrast to what is observed in inflammatory diseases, suggests specific conditions for eosinophil recruitment and activation which may account for the presence of normal counts. Whether these findings indicate the existence of homeostatic loops, in part via eotaxin-CCR3 pathway, that prevent eosinophil-mediated inflammatory activity in the mucosa of IBS needs to be explored.

Apart from the inflammatory cationic proteins, eosinophils, as intestinal resident cells, can modulate inflammatory responses through the extracellular release of a wide variety of other granule-derived products.[45] The up-regulation of SNAP-23 gene expression in IBS-D patients suggests an increase in secretory activity in this group, and the proteins VAMP2, syntaxin4 and SNAP23 were described to be related in piecemeal degranulation (PMD) in eosinophils[46,47], but also mast cells and neutrophils.[48,49] PMD is a secretory mechanism by which cells release small regulated amount of mediators, and can be identified by the loss of granular density without fusion of intergranular membranes and an increase in secretory vesicles.[50] In fact, assessment of eosinophil activation by ultrastructural analysis has been performed in the colonic mucosa from ulcerative colitis patients, reporting a majority of eosinophils with low and moderate PMD.[51] In the present study, both visual and quantitative analysis demonstrated higher prevalence and degree of degranulation of eosinophils in IBS-D compared with HC, with a majority of eosinophils displaying low PMD degranulation. As eosinophils can release a wide-variety of mediators upon activation apart of cationic proteins, increased secretory activity, could result in multiple different effects.[52]

On the other hand, the analysis of the number and area of the granules has also been described as a relevant event in the eosinophil activation state. Hypodense eosinophils (that contain less or smaller granules) are particularly important in the pathophysiology of various inflammatory diseases such as hyper eosinophilic syndrome (HES)[53] or allergic rhinitis[54], and are described to be more activated with enhanced eosinophil cytotoxic activity and degranulation.[53,55] Here we report that eosinophils from IBS-D patients display increased granule area but with total number of granules similar to control subjects, not fulfilling, therefore, hypodense characteristics. In concordance to gene expression data and morphological phenotype, these results come to reinforce that eosinophils from IBS-D are activated, but not in a pro-inflammatory way as observed in other inflammatory GI diseases.

Emerging research is now identifying the eosinophil as key player in stress-mediated intestinal dysfunction, and not necessarily through the release of pro-inflammatory proteins, as they can

respond, store and release a wide variety of neuropeptides and CRF.[1,2,56,57] IBS is presumed to be a disorder of the brain–gut axis associated with exaggerated response to stress. About 50% of IBS patients display psychiatric comorbidities such as anxiety and depression and suffer more chronic stress than the healthy population.[58] Central CRF system is a key mediator of the stress response, both in the brain and in the gut.[59,60] Evidence from clinical and experimental studies showed that psychological stresses have marked impact on intestinal sensitivity, motility, secretion and permeability[21,61], being mucosal immune activation and alterations in central and peripheral neurons the plausible underlying mechanisms.[62,63] In addition, recent studies showed that peripheral administration of CRF aggravated visceral sensorimotor function, visceral hyperalgesia as well as activation of mast cells and serotonin response in IBS patients[64], similar to those reported in models of chronic psychological stress[65], pointing to important contribution of local CRF signaling in the gut to the GI response to stress. Although it has been described that peripheral CRF is found in the enteric nerves and immune cells such as eosinophils[15] and dendritic cells[66], its source in the human jejunal mucosa had not been investigated before. Here, we provide evidence that, among mucosal immune cells, CRF in jejunal mucosa is found only in eosinophil granules. Remarkably, quantification of CRF particles showed significant higher amount of CRF per granule in eosinophils from IBS-D patients compared to controls, which also correlates with psychological-related disorders, such as depression and stress levels. In an animal model, the increase in CRF expression in eosinophils parallels the stress time period, decreasing gradually after removal of stress[13], and in humans CRF is also related to depression.[67] Eosinophil express a wide variety of neuropeptide receptors and can interact directly with nerves in homeostasis and disease. In fact, eosinophil stimulation with SP or CCh induce eosinophils to express and release CRF[13,15]. Thus, during stress and/or depression, activation and release of SP, ACh from the nervous endings are potentially important in neuro-immune responses to stress in IBS [68–70], and may activate eosinophils to produce CRF in the intestine.

We also found that the amount of CRF per granule strongly correlates with IBS clinical manifestations, including pain intensity, IBS severity and bowel habits, and with the degree of degranulation in eosinophils, thereby suggesting local CRF production with clinical implications.

Although much of the influence of CRF on GI function is mediated centrally, jejunal eosinophil-derived CRF correlation with cardinal clinical symptoms suggests that organ-specific activation of CRF receptors is important for stress-induced changes in bowel function. A candidate in mediating the effects of CRF on the pathophysiology of IBS is the mast cell. We and others have previously reported that stress can deeply affect permeability via mast cell activation.[71,72] In fact, CRF receptors (CRFR1 and CRFR2) are widely distributed in the GI tract[73], principally in enteric neurons and *lamina propria* cells, predominantly mast cells in the colonic mucosa[74]. Moreover, impaired intestinal permeability, and the expression of tight junction proteins, have been shown to correlate with pain/discomfort and/ or bowel habit in IBS.[26,75,76]

Altogether, it reinforces the hypothesis that peripheral CRF acts as the signaling system between eosinophils and mast cells in the pathogenesis of barrier dysfunction and symptom generation in IBS-D. Nevertheless, we observed no changes in CRFR2 expression, and a downregulation of CRFR1 in the jejunal mucosa of IBS-D patients, which negatively correlated with clinical symptoms such as pain and stool consistency and with stress levels of last year and depression. To our knowledge, the presence of CRF receptors in the human jejunum and its specific function has not been established yet. Both, CRFR1 and CRFR2 are involved in altered secretory state and permeability, respectively, in the colonic mucosa of rats[65], an effect mediated in part by mast cells[77,78]. However, and interestingly, it has been reported that, in an *in vitro* model, mast cell overstimulation with CRF leads to downregulation of CRFR1.[79] This finding may explain the fact that CRFR1 gene expression is decreased in IBS-D and negatively correlates with stress and depression levels, possibly because of chronic overstimulation. Nonetheless, our findings regarding CRFRs expression in jejunal mast cells deserve future studies in order to determine its contribution to GI physiology.

In conclusion, our study revealed distinctive eosinophil activity in IBS-D, featured by higher CRF content respect to health, in association with clinical manifestations. These data, together with previous observations, suggest a role of eosinophils in the pathophysiology of IBS-D via peripheral CRF. Identification and targeting the CRF-mediated targets in the jejunal mucosal may be a useful approach in the management of IBS patients and other functional GI disorders.

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CAPÍTULO 2

CAPÍTULO 2

"Eosinophil activation by neuromediators: an in vitro model to assess stress-mediated corticotropin releasing factor involvement in functional disorders"

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Keywords: eosinophil, in vitro, neuromediators, corticotropin-releasing factor

INTRODUCTION

Stress responses in diarrhea-predominant IBS are strongly associated with intestinal dysmotility, visceral hyperalgesia, microinflammation and epithelial barrier dysfunction.[1,2] Even though stress-induced pathophysiology is not well understood, central and/or peripheral CRF system is the most studied neurobiological common denominator of IBS symptoms susceptible to stress[3]. Central CRF release, through hypothalamic–pituitary–adrenal (HPA) axis, mediates autonomic, hormonal, and behavioral reflexes that inhibits inflammatory responses at regional level, and influence the gut modulating motility and secretion.[4–6] Moreover, within the gastrointestinal tract, peripheral stressors or inflammatory stimuli induce the release of CRF and neuropeptides, such as substance P (SP), from peripheral nervous system and immune cells[7,8], with effects on immune activation and gut function.[6] Mucosal immune activation is implicated in IBS-D pathophysiology and has been associated with molecular and structural alterations in intestinal barrier[9–11], thus favoring the passage of antigens. In this context, prolonged stress-induced immune activation in the GI tract could favor sensitization of visceral afferent terminals, inflammation and symptom exacerbation in IBS.[6,11,12]

Eosinophils are resident cells in the intestinal mucosa and are involved in initiation and contribution of diverse GI inflammatory conditions, in part through neuro-immune interactions.[13–15] Eosinophils produce and respond to a wide variety of neuromediators such as SP and acetylcholine (ACh), with effects on eosinophil chemotaxis[16], as well as CRF production and release.[17,13] Consequently, eosinophil-dependent regulation of immune and inflammatory pathways in stress-related GI disorders may be mediated by locally released CRF in the intestinal mucosa in response to stress-derived factors from nerve and immune cells. Results obtained in chapter 1 of this thesis showed that eosinophils from IBS-D patients display distinctive activation and increased CRF content in their granules in association with the clinical manifestations and stress and depression levels, suggesting an important contribution of eosinophils in stress-induced bowel dysfunction. However, the

mechanisms of CRF secretion and the activation profile of eosinophils in response to stress factors have not been defined yet.

Therefore, the goals of the present study were to evaluate eosinophil activation and CRF expression in an *in vitro* model in response to stress factors, to better understand mechanisms implicated in IBS-D. To accomplish this aims, we first evaluated the phenotype and functionality of two eosinophil cell lines. We then assessed the inflammatory activation profile, the secretory mechanisms involved and CRF expression and release in response to neuromediators.

METHODS

Eosinophil-like cell lines and culture conditions

AML14.3D10 cells

Acute human myeloid leukemia subclone AML14.3D10 (ATCC, CRL-12079), which comes from the parental AML cell line. These cells are reported to maintain an advanced state of differentiation and a high rate of proliferation in the absence of cytokine supplementation.[18] AML14.3D10 cells were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% penicillin- streptomycin (Gibco), 0.05mM 2-mercaptoethanol (Gibco) and 10% geneticin G418 (Merck).

15HL-60 cells

Human myeloid leukemia 15HL-60 (ATCC, CRL-1964) established through long-term culture of the HL-60 cell line.[19] 15HL-60 cells were maintained in culture in RPMI 1640 with 10% FBS, 1% penicillin-streptomycin and 1% L-Glut as manufacturer's instructions. However, cells were cultured in medium with a modified pH (set at 7.8), as indicated in previous studies[19], achieving higher reproducibility between cell passages. These cells are reported to be differentiated into an advanced eosinophil-like phenotype by either butyric acid or sodium butyrate treatment[20,21], as described below. Undifferentiated cells (referred to as 15HL-60 Und) were used as the control condition. Therefore, differentiation was performed with two different methods, according to literature, as follows:

- *15HL-60 differentiated with butyric acid* (referred to as 15HL-60 Bi): Cells were treated with 0.5mM of butyric acid and 0.5ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) at a density of $1x10^5$ cells/mL, without changing the culture media for the next 7 days, as previously described.[13]

- *15HL-60 differentiated with sodium butyrate* (referred to as 15HL-60 Ba): Cells were treated with 0.5mM of sodium butyrate at a density of $3.5x10^5$ cells/mL, without changing the culture media for the next 7 days, as previously described.[22]

Experimental design

To further delineate how eosinophil activation may be involved in the etiopathology of IBS-D, we assessed the mechanisms of CRF secretion and the type and degree of activation of eosinophils in response to stress factors in an *in vitro* cell model. In order to accomplish this aims, this study has been designed in two phases, as follows (see experimental diagram in figure 1).

Phase 1:

Eosinophil-like cell lines were first evaluated both, phenotypically and functionally, in order to stablish the best experimental tool to be used in the second phase of the study.

First, phenotypic features of AML14.3D10, 15HL-60 Und, 15HL-60 Bi and 15HL-60 Ba cells were evaluated and compared. Terminally differentiated eosinophils were used as control for morphological and ultrastructural features and obtained either from blood or from jejunal mucosal samples (from chapter 1); while for gene and protein expression relative expression between cell lines were compared, due to the impossibility to obtain enough material from blood or tissue isolated eosinophils to use as reference control. CRF and expression of receptors of neuropeptides selected for posterior stimulation were also evaluated as part of the phenotypic and functional analysis of cell lines.

Description of the analyses performed are described below (further methodological details can be found in the Material and Methods section). Cell pellets, obtained after collection and centrifugation in round-bottom tubes at 300g for 7min, were collected and gathered into 4 types of analysis.

- Morphologic and ultrastructural evaluation: cells were stained with hematoxylin-eosin (H&) for further microscopic examination under a light microscope or processed for ultrastructural procedures and visualized by transmission electron microscopy (TEM).
- Protein expression analysis of eosinophil-related proteins: cells were evaluated for major basic protein (MBP) localization by immunofluorescence (IF), and for MBP and plasma membrane integrin-beta 7 (ITGB7) expression quantification by flow cytometry analysis (FCA).
- Gene expression analysis of eosinophil-specific proteins: cells were analyzed for expression of eosinophil specific proteins by quantitative PCR (qPCR)
- Expression analysis of CRF and neuropeptide receptors:
  - CRF gene and protein expression: cells were analyzed for each experimental assay by qPCR or by IF, respectively. In addition, CRF protein expression was validated with three different antibodies and measured by FCA (protocols described in supplementary methods chapter 2).
  - Expression analysis of receptors for substance P (SP), acetylcholine (ACh), and lipopolysaccharide (LPS) (see table 1 for receptors details): cells were collected and processed for receptor expression and localization by IF assay.

Phase 2.

To further explore associated mechanisms through neuro-immune interactions in IBS-D pathophysiology, eosinophil activation and CRF production in response to stress-like stimulation were analyzed. Stimulation by lipopolysaccharide (LPS) as a control of inflammatory-related eosinophil activation was also evaluated and compared.

Eosinophil-like cells were stimulated with the neuromediators SP and carbachol (CCh), and with bacterial-derived lipopolysaccharide (LPS) as a control, in a time course stimulation (methodological details can be found in the material and methods section). Non-stimulated cells were used as the basal condition control. Cells stimulated with vehicle were used as a control to stimulation. After stimulation, cell pellets were collected as previously described, and kept at 4ºC. When required,

supernatant was separated and collected and immediately frozen in liquid nitrogen. Cells were gathered into three types of analysis:

- Assessment of neuropeptide stimulation on cytotoxicity and apoptosis: stimulated cells were assessed for live/death quantification by a real-time assay (IF) and by apoptosis annexin assay (FCA)
- Evaluation of eosinophil activation in response to neuropeptides: cell activation was evaluated by analyzing the expression of pro-inflammatory mediators and secretory activity:
    - Eosinophil pro-inflammatory activation was assessed by analyzing the expression of genes encoding for eosinophil cationic proteins by qPCR.
    - Secretory activity was assessed by analyzing the subcellular localization of vesicular transport-related proteins by IF.
- Analysis of CRF expression and release in response to neuropeptides: CRF localization and expression was assessed by IF and by FCA analysis. CRF release was evaluated by measuring concentration in the supernatant by ELISA. CRF gene expression was also evaluated by qPCR.

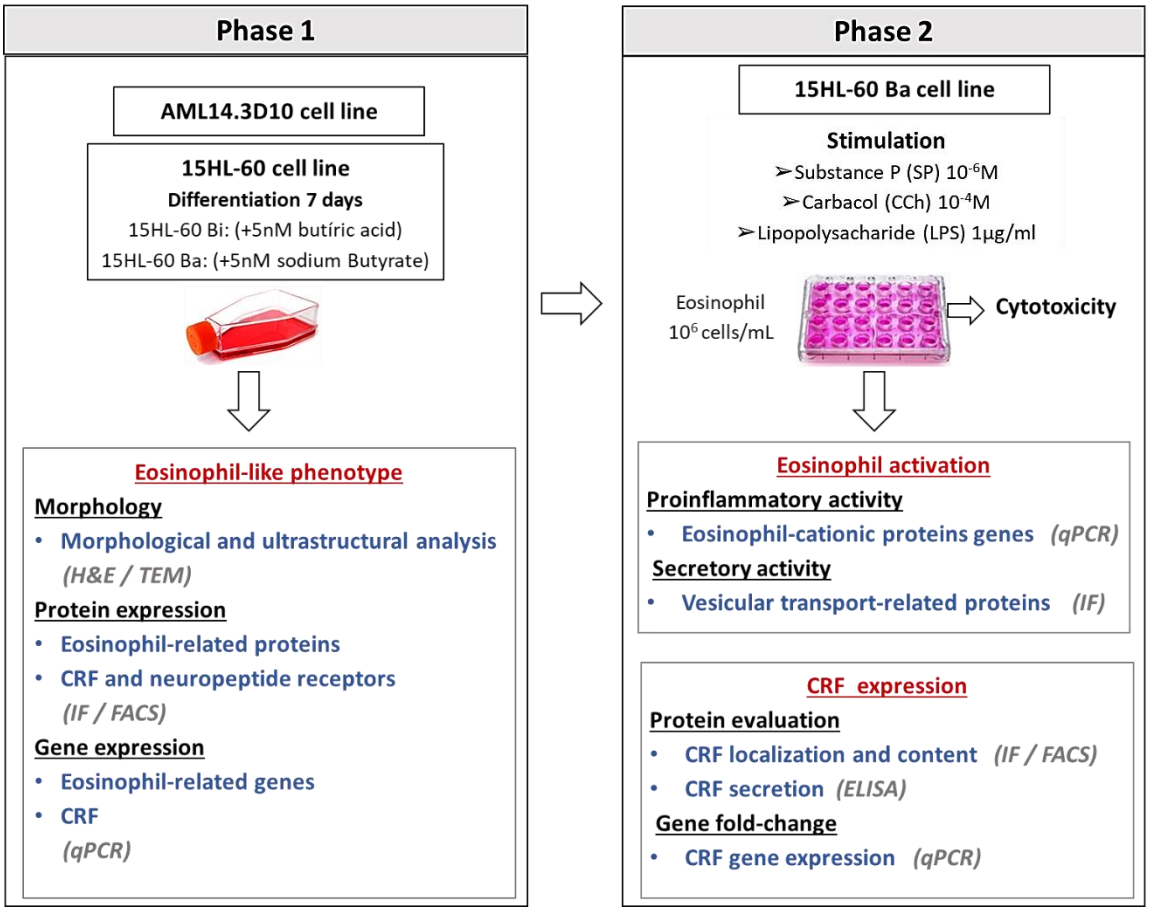


Figure 1. Experimental design for *in vitro* experiments with different eosinophil-like cell lines used. 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60Ba: 15HL-60 cells differentiated with sodium butyrate. H&E: hematoxylin eosin histology; TEM: transmission electron microscopy; CRF: corticotropin-releasing factor; IF: immunofluorescence; FCA: flow cytometry; qPCR: quantitative PCR

Material and methods

Hematoxylin-eosin (H&E) staining

Cell pellets ($2x10^5$ cells) were resuspended in 200 µL of PBS, and 100 µL of suspension were added to a cytofunnel apparatus attached to a glass slide and centrifuged in a cytocentrifuge at 200xg for 6 minutes. Cytospin preparations were fixed with methanol. Cells were rehydrated in distilled water and stained with hematoxylin for 7 minutes. Then, coverslips were washed with tap water and covered with acid solution for 1 minute. The coverslips were placed on tap water with 5 drops of ammoniac and then stained with eosin for 30 seconds. Finally, slides were washed with tap water and covered with DPX mounting media.

Transmission electron microscopy

Cell pellets (6-8 x 10^6 cells) were immersed in a fixative solution of 2.5% (v/v) glutaraldehyde + 2% paraformaldehyde (w/v) (PFA) in 0.2M phosphate buffer (PB) for at least 48h at 41C. Cell pellets were then post-fixed, dehydrated and infiltrated in Eponate 12^{TM} resin (Ted Pella, Inc.), polymerized and contrasted, as previously described in Chapter 1 methods section. Ultrathin sections were examined using a Hitachi H-1,400 microscope at 75 kV equipped with a MegaView III camera (Soft Imaging System).

Flow cytometry immunostaining

Cell pellets (2x10^5 cells) were washed in cold phosphate buffered saline (PBS) (Gibco) supplemented with 2% fetal bovine serum (FBS) and centrifuged at 300 xg 7 min at 4ºC in round-bottom polystyrene tubes. When permeabilization was required (for cytoplasmic proteins, table 2), cell pellets were pre-treated with BD Cytofix/Cytoperm reagent (BD Biosciences) for 20 minutes at 4ºC and washed twice with Perm/Wash buffer (BD wash) (BD Biosciences). Cell pellets were incubated with primary antibody (table 1) diluted in either PBS + 2% FBS buffer (non-permeabilized samples) or with BD wash buffer (for permeabilized samples) for 1 hour on ice. After incubation, cells were washed twice and incubated for 30min at 4ºC in the dark with the appropriate secondary antibody (Anti-rabbit/mouse/goat IgG Alexa Fluor 488 conjugated). Cells were washed twice in either BD wash or PBS + 2% FBS and resuspended in PBS. Immunostained cells were collected and analyzed on a FacsCalibur Flow Cytometer (BD Biosciences) or LRSFortessa Flow Cytometer (BD Biosciences). In non-permeabilized cells, the analysis was performed just after the staining and 10 µL of a viability reagent (7-AAD) (BD Biosciences) were added to a viability control tube (without primary and secondary antibody) in a 200µL of final volume. In permeabilized and fixed cells, samples were kept at 4ºC and light protected until their analysis (maximum one day after staining).

Immunofluorescence immunostaining

For immunofluorescence analysis, immunostaining was performed in suspension in round-bottom polystyrene tubes as described before for FCA staining (table 2). Then, 1x10^5 cells were collected in

coverslips by cytospin (as described above for H&E staining), incubated for 10 min with DAPI (10ng/mL), washed twice with PBS and mounted with Prolong gold antifade mountant media (Invitrogen) and kept at 4ºC light protected until analysis.

Antibody (Ab)	**Ab Host and trading house**	**Protein name**	**Permeabilization**	**Ab dilution**	**Ab diluent**
Eosinophil-related proteins					
Anti-ITGB7	**Rat anti-human PE conjugated, BD**	**Integrin beta-7**	**No**	**1/10** -	**PBS+2%FBS**
Anti-MBP	Mouse anti-human, AbD serotec	Major basic protein	Yes	1/25	BD wash
Secretory activity proteins					
Anti-SNAP23	**Rabbit anti-human, Abcam**	**Synaptosome associated protein 23**	**Yes**	**1/50**	**BD wash**
Anti-VAMP2	Rabbit anti-human, Abcam	Vesicle-associated membrane protein 2	Yes	1/50	BD wash
Neruopeptide and LPS receptors					
Anti-ChRM1	**Rabbit anti-human, Bioss**	**Cholinergic receptor muscarinic 1**	**No**	**1/100**	**PBS+2%FBS**
Anti-ChRM2	Rabbit anti-human, Bioss	Cholinergic receptor muscarinic 2	No	1/100	
Anti-ChRM3	Rabbit anti-human, Bioss	Cholinergic receptor muscarinic 3	No	1/100	
Abti-CHRNA7	Rabbit anti-human, Bioss	Cholinergic receptor nicotinic alfa 7	No	1/100	
Anti-TLR4	Rabbit anti-human, Abcam	Toll-like receptor 4	No	1/100	
CRF					
Anti-CRF	Goat anti-human, Sta Cruz Biotec	Corticotropin-releasing factor	Yes	1/50	BD wash

Table 1. Antibodies and dilutions used for immunofluorescence or FCA analysis of cells.

RNA isolation

Cell pellets (3-5x10^6 cells) were resuspended in 350 µL of RLT lysis buffer from the RNAeasy Mini Kit (Qiagen) and homogenized through a 20G (0.9 mm) needle at least 5-10 times. After cell disruption, RNA was isolated according to the manufacturer's instructions. On-column DNAse treatment (Qiagen) was performed to complete genomic DNA removal. RNA was eluted in 30µL of RNase-free water and stored at -80ºC. An aliquot (2µL) was separated for RNA quantification in nanodrop.

cDNA synthesis and quantitative PCR (qPCR)

cDNA synthesis was performed using 1 µg of total RNA with the High Capacity Reverse Transcription Reagents Kit (Thermo Fisher Scientific), following manufacturer instructions. Transcriptional analysis of eosinophil-related genes (table 2) and CRF was assessed by qPCR using validated TaqMan Gene Expression Assays (Thermo Fisher Scientific) on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems). Human cyclophilin A (PPIA) gene was used as the endogenous control for data normalization. Quantification of gene expression was performed in a minimum of three different passages of each cell line (i.e three biological replicates) and each sample, including distilled water as negative control, was run in triplicate (i.e. three technical replicates). Data was analyzed either by the $2^{-\Delta Ct}$ method[23] for cell lines comparison, or by the $2^{-\Delta\Delta Ct}$ method (using basal condition as reference sample), as previously described[23], for treatment effect comparison.

Gene assay	Gene name	Specie	TaqMan assay
Eosinophil cationic proteins			
***EPX* (EPO)**	Eosinophil peroxidase	Homo sapiens	*Hs00946094_m1*
***PRG2* (MBP)**	Proteoglycan 2, eosinophil major basic protein	Homo sapiens	*Hs00794928_m1*
***RNASE2* (EDN)**	Ribonuclease A family member 2	Homo sapiens	*Hs00795553_s1*
***RNASE3* (ECP)**	Ribonuclease A family member 3	Homo sapiens	*Hs01923184_s1*
***CRH* (CRF)**	Corticotropin releasing factor	Homo sapiens	*Hs01921237_s1*
PPIA	Cyclophilin A	Homo sapiens	*Hs99999904_m1*

Table 2. **Gene expression assay probes used for qPCR analysis in AML14.3D10 and 15HL-60 cells**

Cell stimulation

For eosinophil stimulation, $1x10^6$ cells/mL were collected and stimulated in a 24-well plate, with SP (Sigma), with a stable analog of ACh, CCh (Sigma), with LPS (Sigma), as a control of non-neuropeptide stimulation, or with vehicle (table 3) for 30, 90, 180 minutes and 24 hours. Respective antagonists were added 30 minutes prior to stimulation. The basis for selecting appropriate concentrations of the different mediators is described in the supplementary material.

Mediator	**Vehicle of dilution**	**Concentration of stimulation**	**Receptors analyzed by IF**	**Antagonist used**
Substance P (SP)	Water, 1% BSA, 0.05M acetic acid	10^{-6}M	Neurokinin 1 receptor ***(NK1R)*** Neurokinin 2 receptor ***(NK2R)***	CP-96345 (10^{-6}M)
Carbachol (CCh)	PBS	10^{-4}M	Cholinergic Receptor Muscarinic 1 ***(CHRM1)*** Cholinergic Receptor Muscarinic 2 ***(CHRM2)*** Cholinergic Receptor Muscarinic 3 ***(CHRM3)*** Cholinergic Receptor Nicotinic Alpha 7 Subunit ***(CHRNA7)***	Atropine Sulfate (10^{-6}M)
Lipopolysaccharide (LPS)	PBS	1µg/ml	Toll like receptor 4 ***(TLR4)***	

BSA: bovine serum albumin

Table 3. Mediators and antagonists used for stimulation and their receptors, analyzed in AML14.3D10 and 15HL-60 cells

Apoptosis assay

Apoptosis assay was performed with the FITC-annexin V apoptosis detection kit (BD), following manufacture instructions. Briefly, $2x10^5$ cells were washed in cold PBS and resuspended in 1X Binding Buffer at a final concentration of 10^6 cells/ml. Next, 5 µl of FITC Annexin V and 5 µl of

propidium iodide was added and samples incubated for 15 min at room temperature in the dark. After two washes with PBS and centrifugation cells were resuspended in 400 µl of 1X Binding Buffer and analyzed in a FacsCalibur Flow Cytometer within 1 hour.

Cytotoxicity assay

Cytotoxicity assay was performed with the IncuCyte® cytotoxicity assay methodology (Essen Bioscience) in the Incucyte® platform. Cytotoxicity reagent (EssenBioscience) (1/1,000 dilution) was added to medium containing cells at 2x104 cells/mL. Cells (100 µL per well) were immediately seeded in a 96-well plate, pre-coated with 5 µg/mL of fibronectin (Sigma) diluted in 0.1% Bovine serum albumin (BSA). Immediately, SP and CCh treatments were added at a 2X and the plate was placed into the IncuCyte to perform live-cell analysis. Images were taken every hour within 48h. Analysis was performed on the image set containing all the images collected within 48h in triplicate and normalized with the confluency of cells.

Statistical analysis

Statistical analyses were performed with Graphpad Prism 6. Data are expressed as median (range) or mean $\pm$ standard deviation. Data distribution was tested by the D'Agostino and Pearson omnibus normality test. Normally distributed data were compared by the one-way ANOVA followed by Bonferroni multiple comparisons post-hoc test. Non-parametric distribution was compared by the non-parametric ANOVA, Kruskal-Wallis test, followed by Dunn's multiple comparison post-hoc test. Statistical analysis of time-course experiments was performed by two-way ANOVA followed by Bonferroni multiple comparison pot-hoc test. *P* values of ≤ 0.05 were considered significant.

RESULTS

RESULTS FROM PHASE 1: eosinophilic phenotype evaluation in eosinophil-like cell lines

Eosinophil-like cell lines were first evaluated in order to identify the cell model that phenotypical and functionally better resembles a mature eosinophil to stablish the best experimental tool to be used in the second phase of the study.

Differentiation with butyric acid decreases 15HL-60 cell viability

As the differentiation process of 15HL-60 cells involves a period of 7 days without changing the media, we first assessed whether cell viability may get compromised in this cell line. Both AML14.3D10 and 15HL-60 Und cells showed similar percentage of viable cells, while the differentiation process of 15HL-60 cells induced a significant cell death but only when the butyric acid protocol was applied (figure 2).

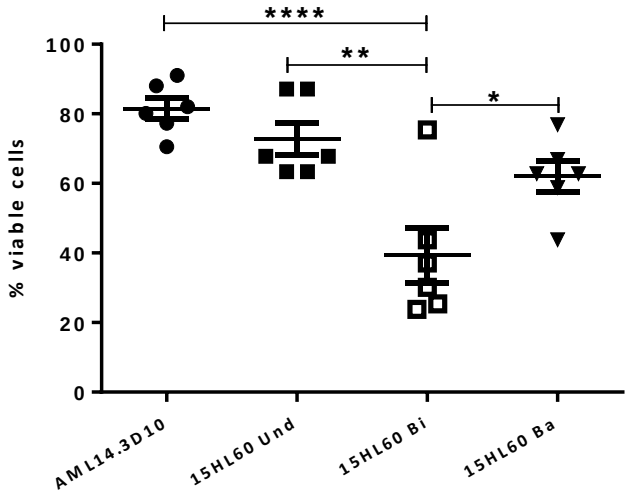


Figure 2. Cell viability of AML14.3D10 and 15HL-60 cells. Percentage of viable cells measured with tripan blue staining. Data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni multiple comparison post-hoc test. *P<0.05 **P<0.01 ****P<0.0001. 15HL-60 Und: undifferentiated 15HL-60 cells; 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells differentiated with sodium butyrate.

15HL-60 cells differentiated with butyrate display eosinophil-like morphological features

Morphological features of AML14.3D10 and 15HL-60 cells were evaluated and compared to terminally differentiated eosinophils from blood and from the jejunal mucosa of healthy subjects. Both, blood and intestinal eosinophils showed the typical morphologic features of mature eosinophils: high granular density in the cytoplasm with intense eosin coloration, a characteristic bilobed nucleus and a low nucleus to cytoplasm ratio. Cell lines showed less characteristics of mature cells. AML14.3D10 cells were composed of a heterogeneous population with a variable morphology and size. They appeared as little granulocytes with a multilobed nucleus, generally with kidney-shape form and larger than the cytoplasm. Cytoplasmic granules were also few in numbers and weakly stained with eosin (figure 3). 15HL-60 cells were also composed of a heterogeneous population before and after differentiation. However, after the differentiation procedure some cells showed a decreased nuclear to cytoplasm ratio. In addition, although with and without differentiation some cells presented little granulation and are basophil-like, there were also cells showing numerous strongly eosin stained eosinophilic granules (arrow), being qualitatively more prevalent in 15HL-60 Ba cells (figure 3).

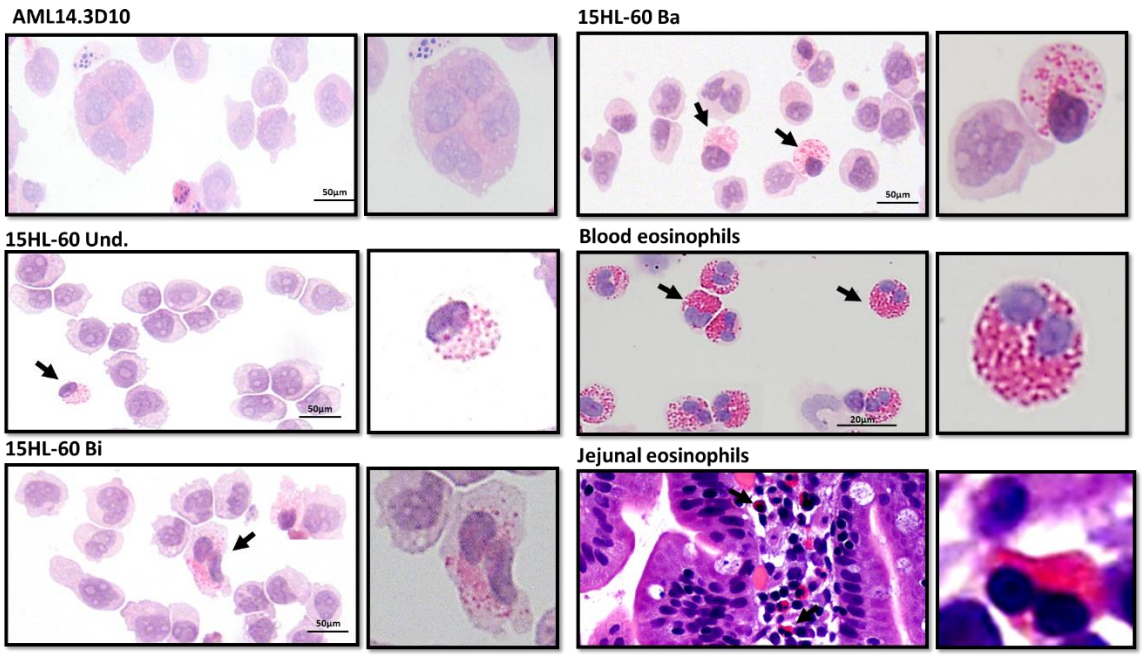


Figure 3. Hematoxylin and eosin staining of AML14.3D10 and 15HL-60 cell lines, and blood and jejunal eosinophils. Representative images of hematoxylin/eosin staining. The insert to the side of image represent an enlargement of one or two cells. Black arrows indicate cells with cytoplasmic eosinophil granules stained pink with eosin. 15HL-60 Und: undifferentiated 15HL-60 cells; 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells differentiated with sodium butyrate. A typical eosinophil from blood and the jejunal mucosa is also shown for reference.

The observed morphological differences between both cell lines were confirmed by ultrastructural analysis (figure 4). Indeed, AML14.3D10 cells showed a large multilobed nucleus with several lobes and a few small electrodense granules in the cytoplasm. On the other hand, 15HL-60 Und cells presented also a large nucleus, and some electron-dense although rather small granules in the cytoplasm (figure 4A). The observed granules were crystalloid-free, not fully formed (figure 4B). After differentiation with butyric acid, in 15HL-60 cells, the nucleus to cytoplasm ratio was reduced together with a slight increase in granule numbers that were still small and without electrodense crystal structure core. On the contrary, after differentiation with sodium butyrate the nucleus to cytoplasm ratio was reduced, granules were higher and spherical and highly electrodense also showing a crystalline lattice core, similar to those observed in blood and jejunal eosinophils (figure 4B).

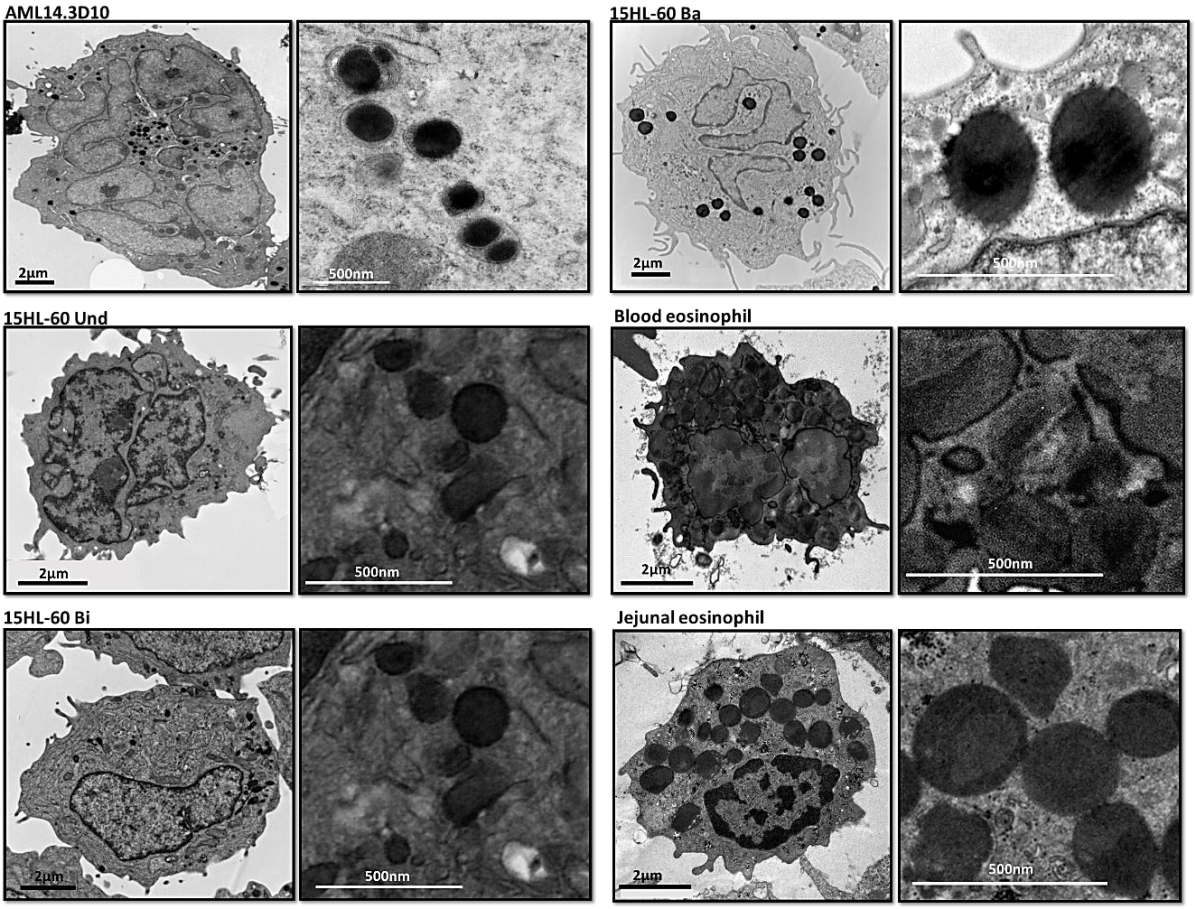


Figure 4. Ultrastructural analysis of AML14.3D10 and 15HL-60 cells. Representative images (left, at 15.000-20.000 x)) of the different cell lines, and an example of the previous image at higher magnification (50.000 x) (right) with detail of granules. Bar size indicates 2µm and 500nm, respectively. 15HL-60 Und: undifferentiated 15HL-60 cells; 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells differentiated with sodium butyrate. Blood and mucosal jejunal eosinophils obtained from healthy donors are shown for reference.

AML14.3D10 and 15HL-60 Ba cells express higher amounts of eosinophil-related proteins

Major basic protein and integrin beta 7 are described to be expressed in mature eosinophils[24,25]. Both cell lines showed cytoplasmic expression of MBP, irrespective of the differentiation protocol used for 15HL-60 cells (figure 5A), despite less intensity was observed in AML14.3D10 and 15HL.60 Und cells. AML14. 3D10 cells showed higher numbers of MBP positive cells than 15HL-60 Bi cells, but not than 15HL-60 Und and 15HL-60 Ba (figure 5B left). No differences in mean fluorescence intensity (MFI) were observed (figure 5B right).

On the other hand, positive cells for the membrane marker integrin beta-7 were higher in AML14.3D10 cells than in 15HL-60 Und cells, while the differentiation protocol showed a trend towards increase positive cells without reaching statistical significance (figure 5C left). MFI was also higher in AML14.3D10 compared to 15HL-60 Bi and 15HL-60 Und cells (figure 5C right), suggesting less expression of this protein in the cell membrane.

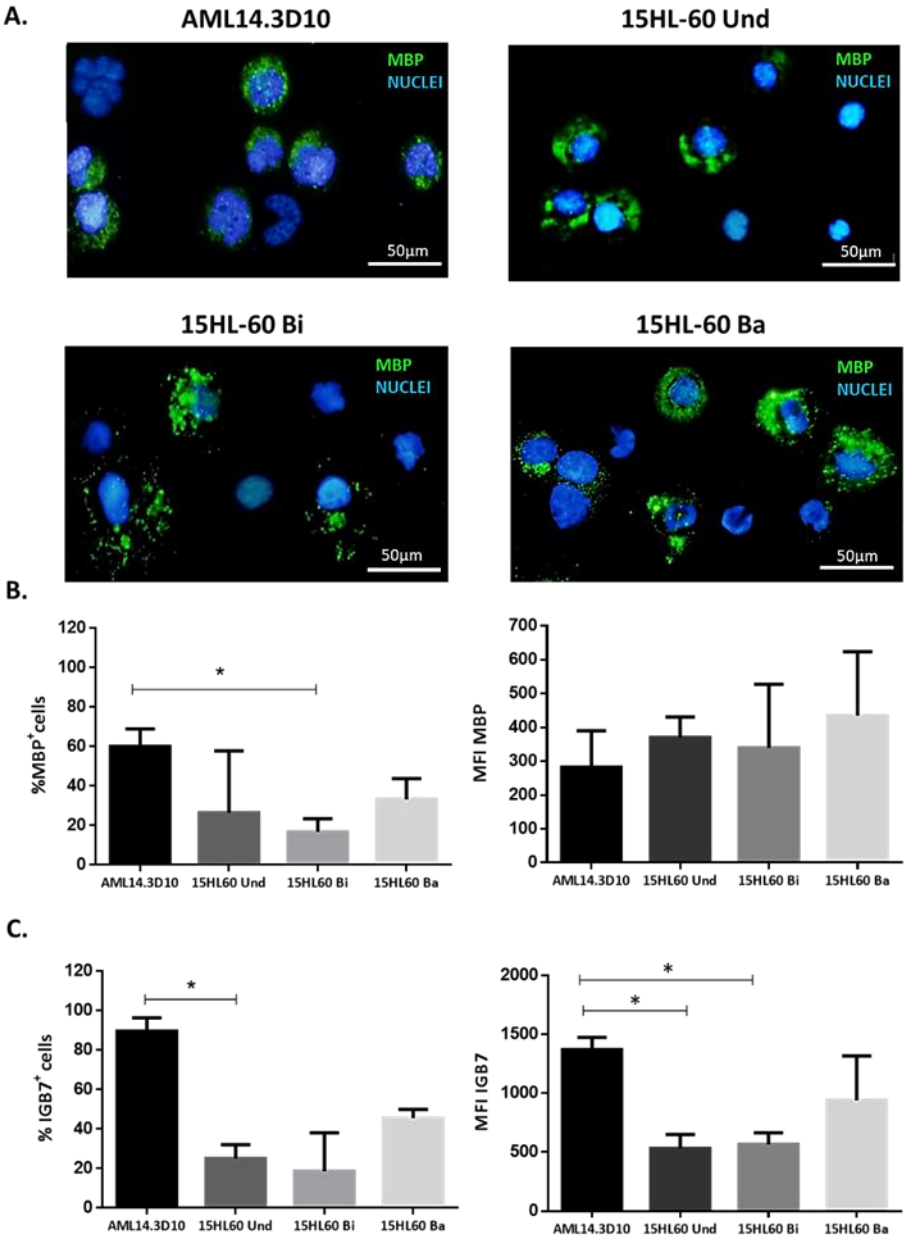


Figure 5. Eosinophil-related protein expression in AML14.3D10 and 15HL-60 cells. **A.** MBP staining in AML and 15HL-60 cells. Nuclei were stained with DAPI for reference. **B.** FCA analysis of MBP and **C.** ITGB7 expression in AML14.3D10 and 15HL-60 cells percentage of positive cells (left graph) and mean fluorescence intensity (MFI) in viable cells (right graph). Data are expressed as median ± range. Graphs represent data from 3 independent

experiments. Statistical analysis was performed using the non-parametric ANOVA (Kruskal-Wallis test) and post-hoc Dunn's test. * *P*< 0.05. 15HL-60 Und: undifferentiated 15HL-60 cells; 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells differentiated with sodium butyrate. MBP: major basic protein; ITGB7: integrin beta-7.

AML14.3D10 cells show higher expression of eosinophil-specific genes than 15HL-60 cells

Based on previous studies, eosinophil-specific genes during maturation are either upregulated such as CCR3 and CLC, or downregulated, such as cationic proteins, as they are subjected to different regulatory process.[26,27] The analysis of cationic protein genes showed higher levels of mRNA levels in AML14.3D10 cells of the cationic proteins major basic protein (proteoglycan-2, *PRG2*), eosinophil peroxidase (*EPX)* and eosinophil-derived neurotoxin (*RNASE2)* compared to 15HL-60 Und cells (figure 6) also showed higher expression of EPX than 15HL-60 Bi cells. However, AML14.3D10 cells also displayed higher expression of *CCR3* and *CLC* than 15HL-60 cells, which is controversial in relation to cationic protein mRNA levels, suggesting an anomalous gene expression. These results confirm different stages of maturation between both cell lines, suggesting less mature state in AML14.3D10 cells. Differentiation of 15HL-60 cells did not modify gene expression.

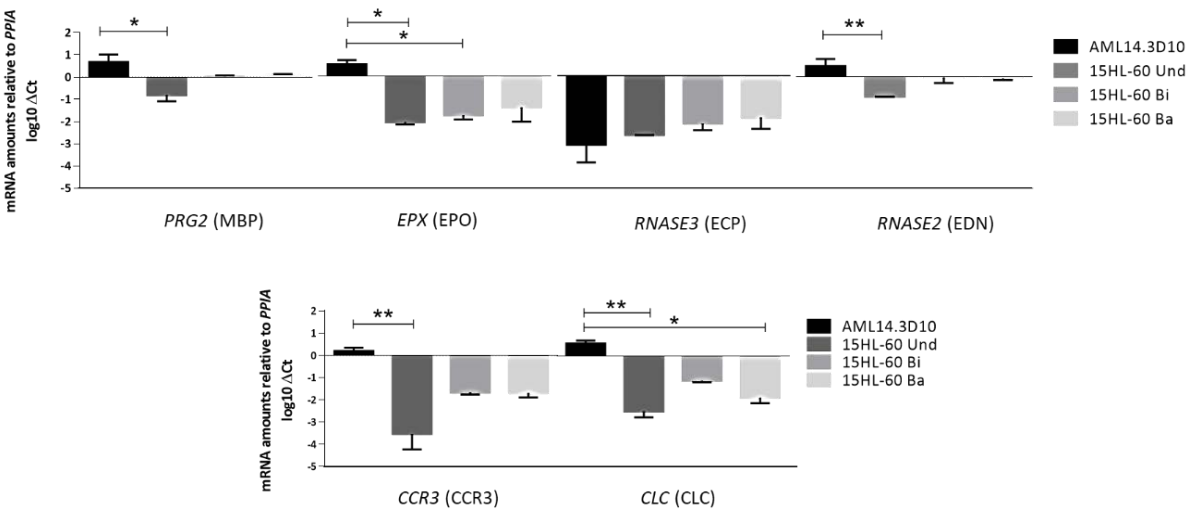


Figure 6. Eosinophil-related genes expression in AML14.3D10 and 15HL-60 cells. Relative mRNA amounts expression by qPCR of eosinophil-related proteins represented as log10 ΔCt values normalized to PPIA. Data are expressed as median ± range. Graphs represents data from 3-6 for independent experiments. Statistical analysis was performed using the non-parametric ANOVA (Kruskal-Wallis test) with Dunn's multiple comparisons post-hoc test. *P<0.05 ** P<0.01. 15HL-60 Und: undifferentiated 15HL-60 cells; 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells

differentiated with sodium butyrate. *PRG2:* Proteoglycan 2; *EPX:* eosinophil peroxidase; *RNASE3:* ribonuclease A family member 3; *RNASE2:* ribonuclease A family member 2, *CLC:* Charcot-Leyden crystal galectin; *CCR3*: C-C chemokine receptor 3.

Only the 15HL-60 cell line show CRF expression

To ultimately determine the utility of eosinophil-like cell lines as an *in vitro* model to study the mechanisms involved in stress-mediated eosinophil activation, CRF expression was analyzed in both cell lines.

CRF expression analysis showed that AML14.3D10 cells did not express the CRF protein (figure 7 A). 15HL-60 Und cells, however, showed, visually, some CRF staining in the cytoplasm of some cells. Results were validated by FCA using 3 different CRF antibodies (supplementary material chapter 2, figures S1 and S2). After differentiation, cytoplasmic CRF protein expression was increased in both 15HL-60 Bi and 15HL-60 Ba cells, showing higher intensity in 15HL60 Ba cells (figure 7 A). The analysis of gene expression by qPCR also showed expression only in 15HL-60 cells, and differences after differentiation were not significant (figure 7 B)

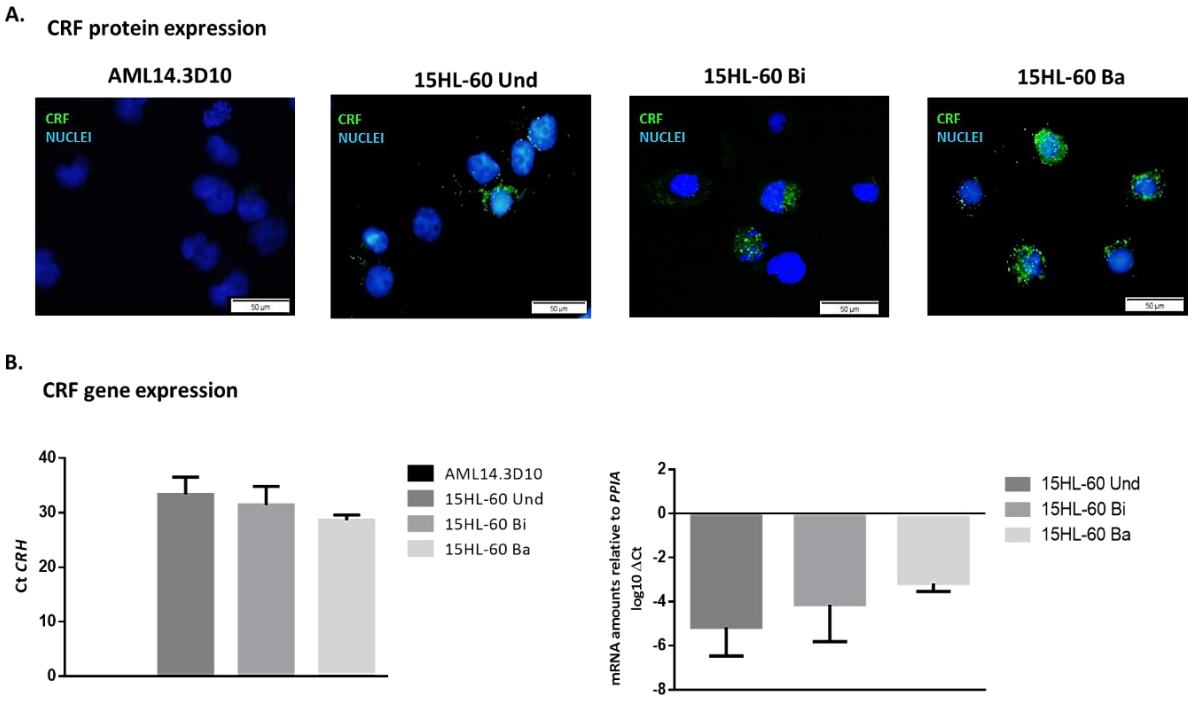


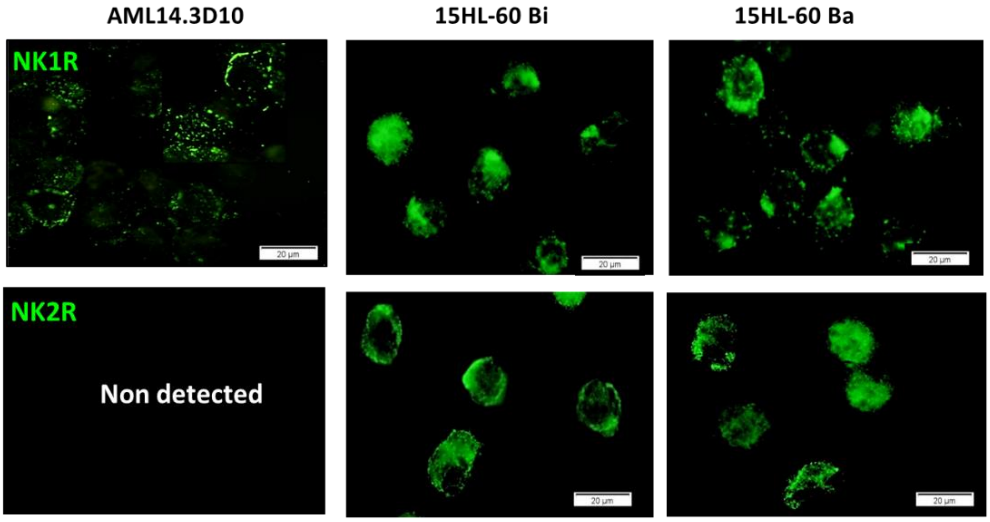
Figure 7. CRF expression in AML14.3D10 and 15Hl-60 cell lines. A. CRF staining in AML14.3D10 and 15HL-60 cells. Nuclei were stained with DAPI for reference. Representative images from 3 independent experiments. **B**. *CRH* mRNA expression is represented as cycle threshold (Ct) (left) or as log10 ΔCt values normalized to PPIA in 15HL-60 cells (right). Data are expressed as median ± range. Graphs represent data from 3-6 for independent

experiments. Statistical analysis was performed using the non-parametric ANOVA (Kruskal-Wallis test) with Dunn's post-hoc test. Und.15HL-60: undifferentiated 15HL-60 cells; 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells differentiated with sodium butyrate; *CRH*: corticotropin-releasing hormone

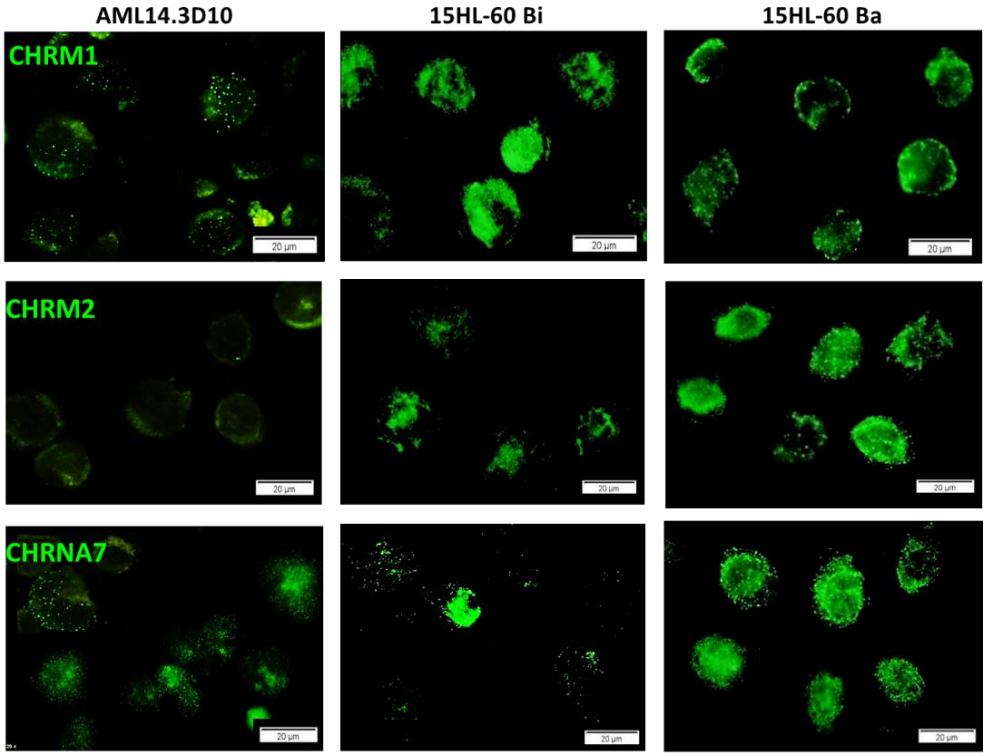
AML14.3D10 and 15HL-60 cells express neuropeptide and lipopolysaccharide receptors

To analyze eosinophil activation in response to neuropeptide stimulation we first confirmed receptor expression for SP, Ach and LPS in AML14.3D10 and differentiated 15HL-60 cells. SP receptors (NK1R and NK2R) were expressed in differentiated 15HL-60 cells, whereas in AML14.3D10 cells only NK1R was detectable (figure 8 A). On the other hand, all cells showed marked staining of ACh muscarinic receptors (CHRM1/M2 and M3) and nicotinic receptor (CHRNA7) (figure 8B) and the LPS receptor TLR4 (figure 8 C).

A. SP receptors



B. ACh receptors



C. LPS receptor

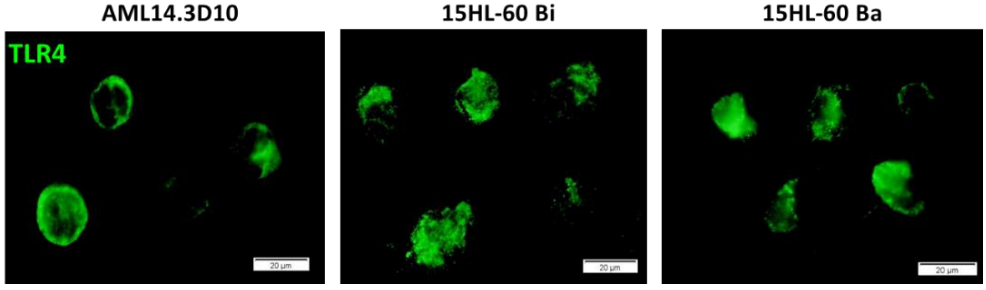


Figure 8. Receptor expression of mediators used for eosinophil stimulation. Representative images of receptor staining in eosinophil cell lines**. A.** SP receptors **B.** ACh receptors and **C.** LPS receptors. Negative control (only secondary antibody) was used for threshold to set the positive signal. Bars indicate 20µm. 15HL-60 Bi: 15HL-60 cells differentiated with butyric acid; 15HL-60 Ba: 15HL-60 cells differentiated with sodium butyrate. SP: substance P; NK1R/NK1R2: tachykinin receptor 1/2; ACh: acetylcholine; CHRM1/2/3: cholinergic receptor muscarinic 1/2/3; CHRNA7: cholinergic receptor nicotinic alpha 7; LPS: lipopolysaccharide; TLR4: toll-like receptor 4.

RESULTS FROM PHASE 2: evaluation of 15HL-50 Ba cells activation in response to *in vitro* stimulation with stress-like factors

The results of Phase 1 showed that 15HL-60 Ba cells display a more terminally differentiated eosinophil-like phenotype and expresses CRF together with neuropeptide and LPS receptors. This section demonstrates that 15HL-60 Ba cells constitute the best model to study eosinophil activation by neuromediators, as compared with the response of AML14.3D10 and 15HL-60 Bi cells (results are shown in supplementary material chapter 2; figures S3-S8).

Stimulation with SP and CCh does not trigger apoptosis and cytotoxicity

LPS are known to enhance eosinophil survival[28], however neuropeptide effects are not well known. To exclude potential apoptotic or cytotoxic effects of treatment, cell death rates were evaluated after SP and CCh treatment. Both, SP and CCh induced low rate of apoptotosis (figure 9A) and cytotoxicity (figure 9B).

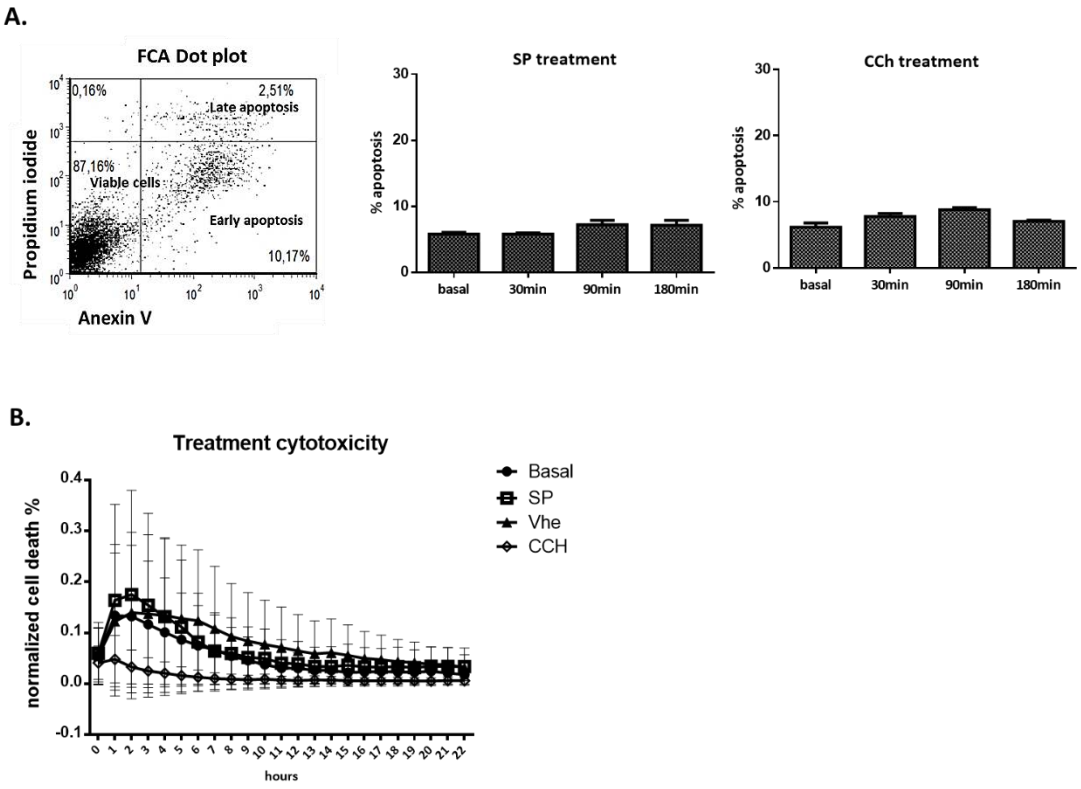


Figure 9. Evaluation of apoptosis and cytotoxicity after stimulation with SP and CCh. **A.** (left) Representative dot plot of FACS analysis for annexin V (FITC) and propidium iodide showing the percentage of cells in different quadrant established with negative control (only cells). Percentage of the sum of cells in early and late apoptosis (right). **B.** Analysis of the percentage of death cells normalized to the confluency of the plate in basal conditions and after stimulation with SP and CCh. Graphs represent results from 3 replicates. Data are expressed as mean ± SEM. Statistical analysis was performed using the non-parametric ANOVA (Kruskal-Wallis test) with Dunn's post-hoc test. CCh: carbachol; SP: substance P; Vhe: vehicle.

15HL-60 Ba cells display a low pro-inflammatory gene expression profile after stimulation with SP and CCh

To characterize pro-inflammatory activation in 15HL-60 Ba cells after neuropeptide stimulation, gene expression of eosinophil cationic proteins was evaluated in a time-course analysis. No differences on cationic proteins gene expression was detected after SP and CCh treatment (figure 10A and B). In contrast, LPS treatment induced the up-regulation of MBP (*PRG2*), MBP EDN (*RNASE2*) and ECP (*RNASE3*) (figure 10 C).

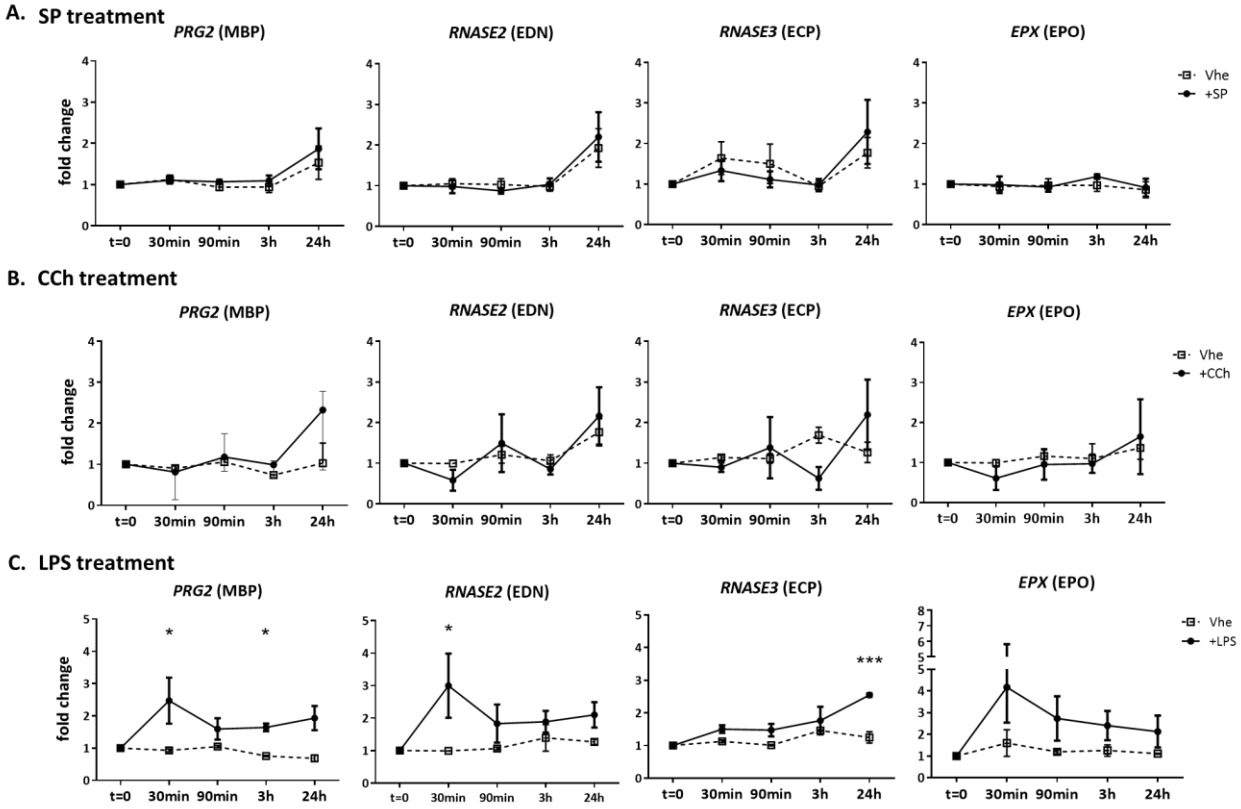
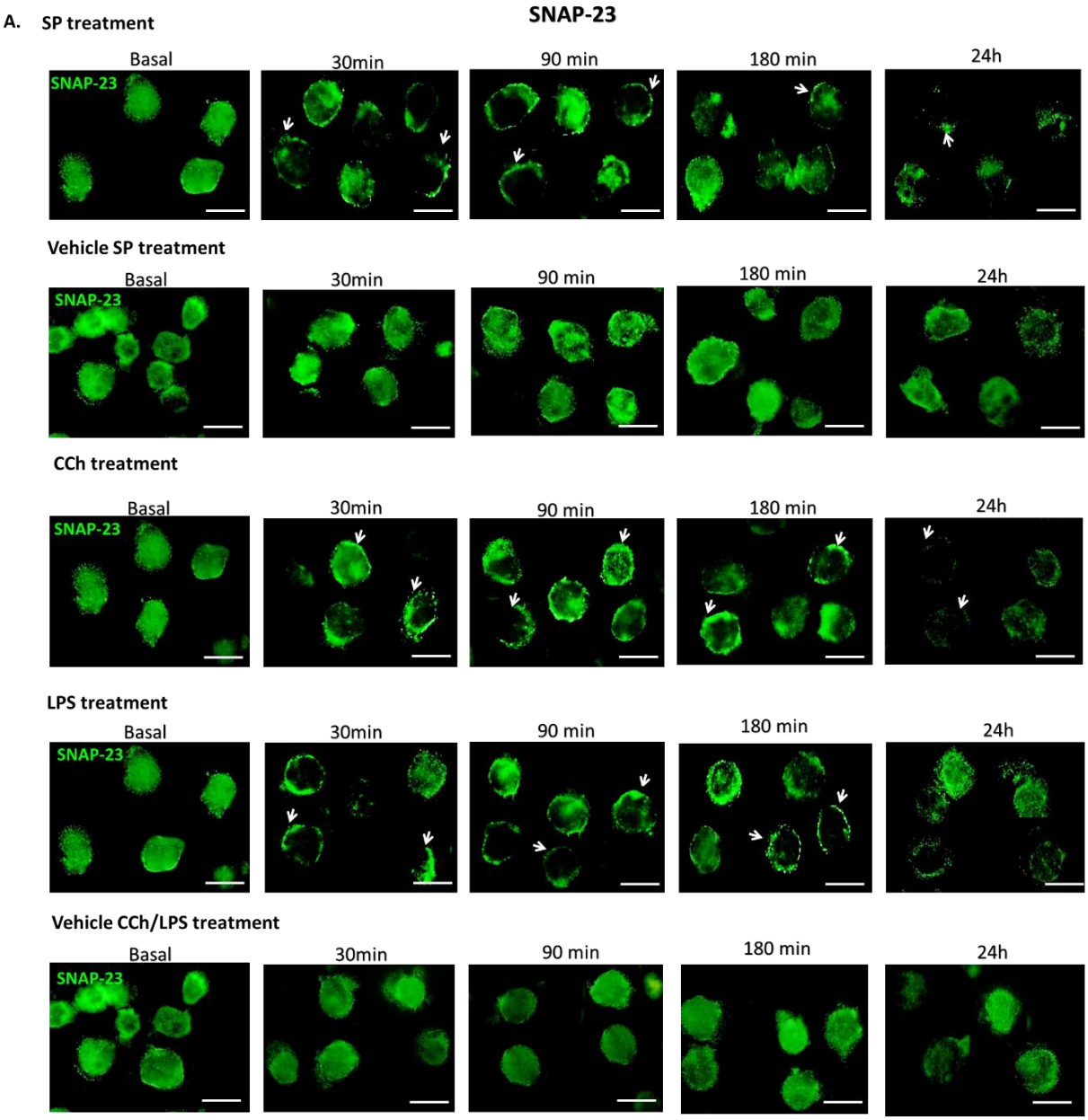


Figure 10. Time-course analysis of gene expression of eosinophil cationic proteins after stimulation with SP, CCh and LPS. Cells were treated with SP, CCh or LPS and vehicle control on the indicated time points and recollected, and gene expression analyzed by qPCR. Fold-change was calculated by normalizing the data with the endogenous control gene (*PPIA*) and comparing each time point to untreated cells (t=0). Graphs represent results from 3 independent experiments. Data are expressed as mean ± standard error. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparison post-hoc test * $P<0.05$ *** $P<0.001$ *vs.* vehicle. CCh: carbachol; LPS: lipopolysaccharide; SP: substance P;Vhe: vehicle; *PRG2:* Proteoglycan 2; *RNASE2:* ribonuclease A family member 2; *RNASE3*: ribonuclease A family member 3: *EPX*: eosinophil peroxidase

Secretory activity of 15HL-60 Ba cells is enhanced after stimulation with SP and CCH, and involves VAMP-2 and SNAP-23

To determine whether stimulation with neuropeptides trigger eosinophil degranulation, the expression of two proteins involved in vesicular transport and membrane docking and fusion, SNAP23 and VAMP2, was analyzed. Time-course analysis showed intensification and mobilization of both proteins from the cytoplasm to the membrane after 30 minutes of SP and CCh stimulation that was maintained until 24h of treatment (figure 11 A and B). LPS treatment only triggered

redistribution of SNAP23 to the plasma membrane, suggesting that different secretory mechanisms operate in response to neuropeptide and pro-inflammatory signals (Figure 11 A and B).



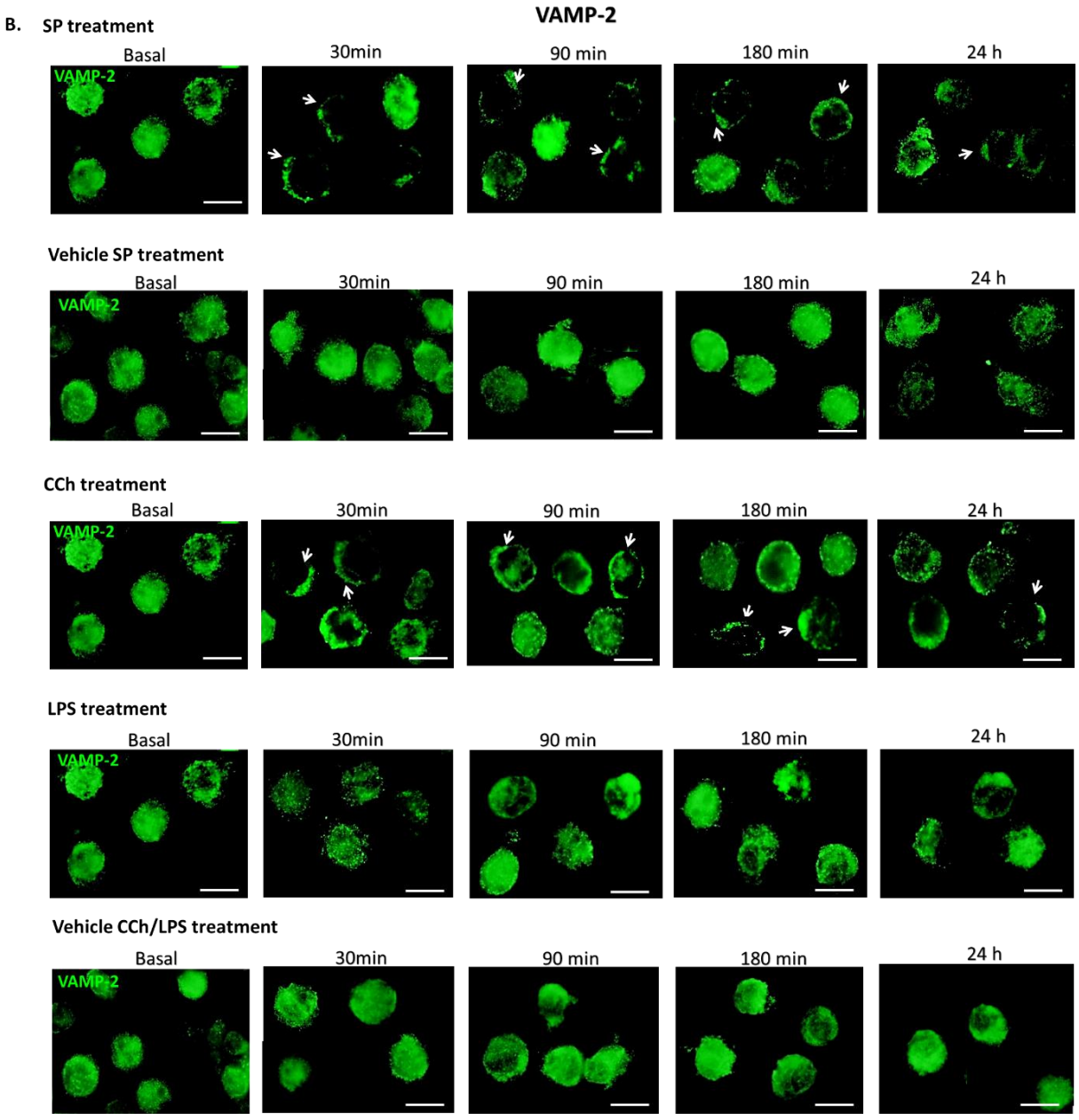
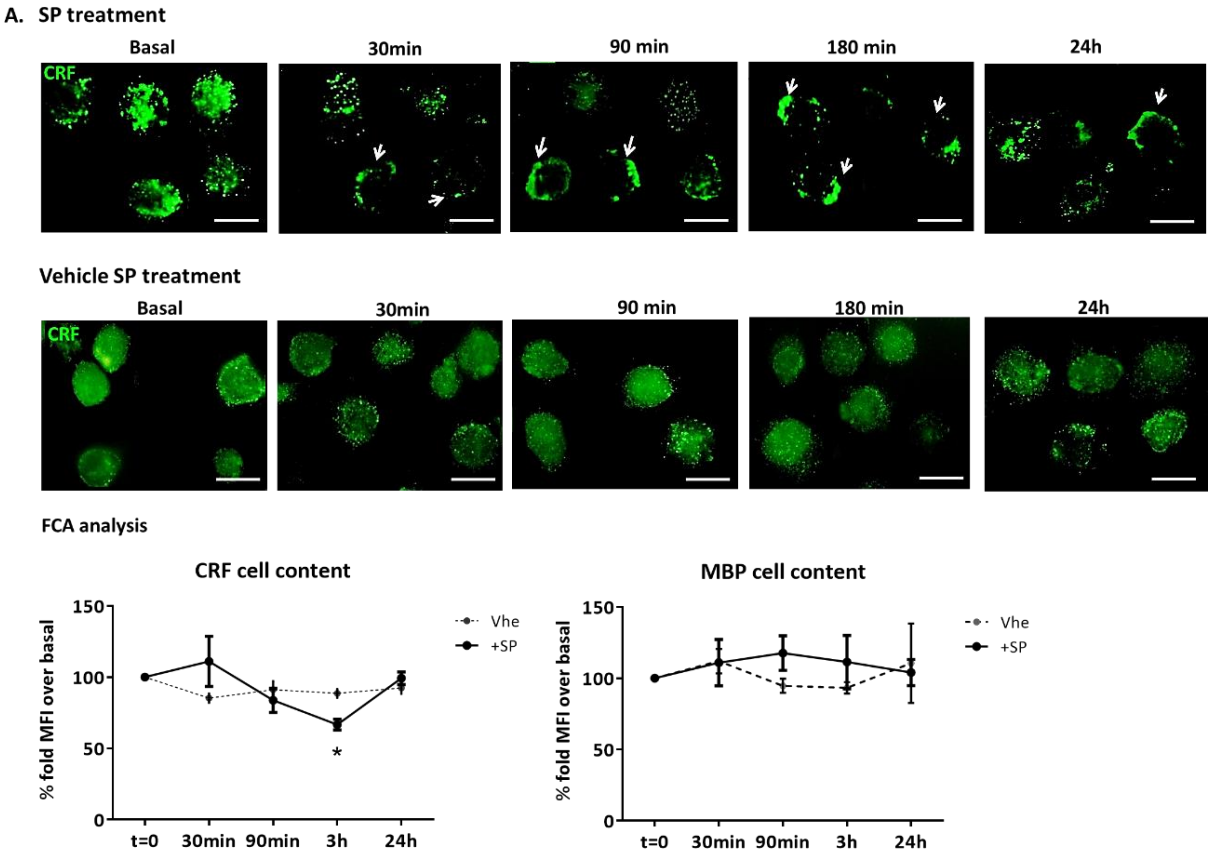


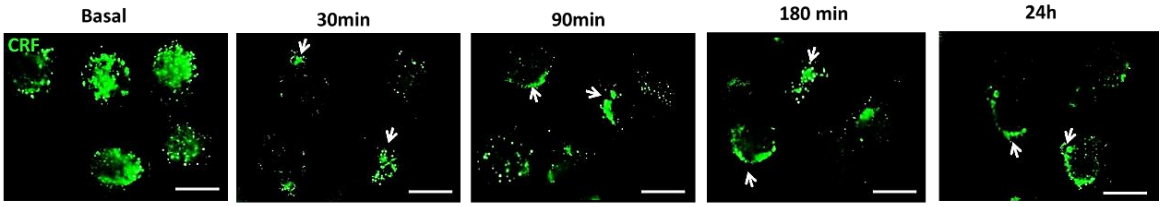
Figure 11. Secretory activity of 15HL-60 Ba cells after stimulation with SP, CCh and LPS. Representative images of a time-course experiment of **A**: SNAP-23 and **B.** VAMP-2 protein location evaluated by IF after SP, CCh, LPS and vehicle stimulation. Negative control (only secondary antibody) was used for threshold for setting the positive signal. White arrows showed relocation of SNAP-23 and VAMP-2 from cytoplasm to plasma membrane of cells after stimulation. Images are representative of 3 independent experiments. Bars indicate 10 µm. CCh: carbachol; LPS: lipopolysaccharide; SP: substance P; SNAP23: Synaptosomal-associated protein 23; VAMP-2: Vesicle-associated membrane protein 2

15HL-60 Ba cells mobilizes and releases CRF after stimulation with SP and CCh but not after LPS.

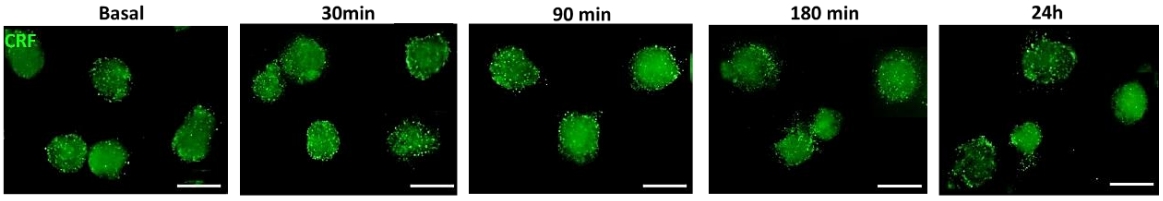
We next sought to identify CRF cytoplasmic location before and after stimulation to identify CRF-vesicular mobilization to plasma membrane by IF. Visual analysis showed CRF mobilization from cytoplasm to the plasma membrane and a quantitative decrease in CRF content in response to the SP and CCh (figure 11A and B), but not after LPS (figure 11C). Analysis of MBP content analyzed under the same conditions, revealed no changes after stimulation with SP and CCh (figure 11A and B), indicating specific CRF mobilization. On the contrary, LPS stimulation induced an increase cytoplasmic MBP protein expression (figure 11C), suggesting a replenishment of cytoplasmic granules.



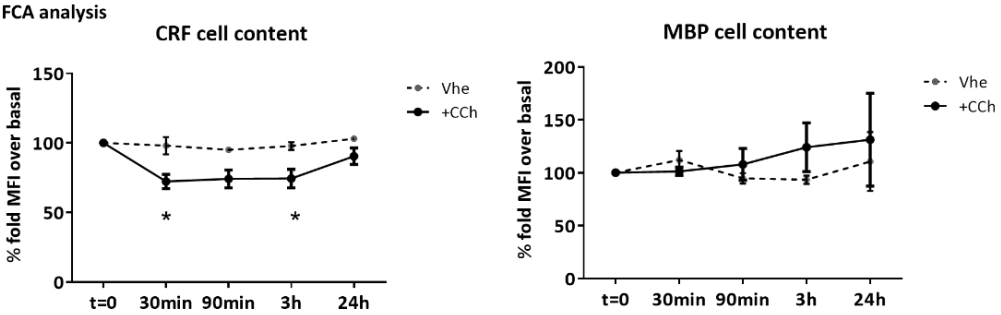
B. CCh treatment



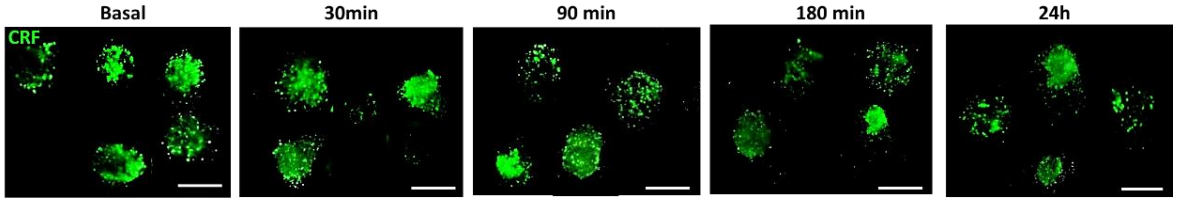
Vehicle CCh treatment



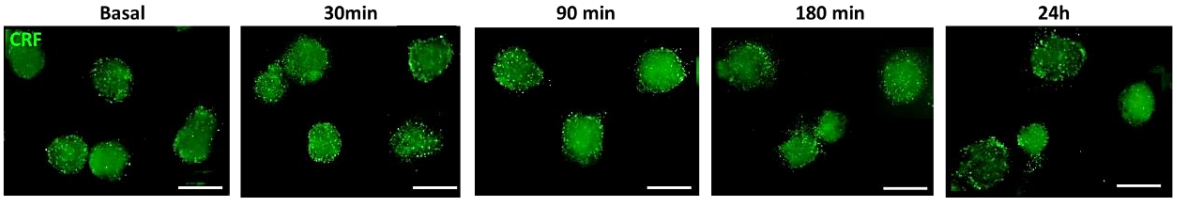
FCA analysis



C. LPS treatment



Vehicle LPS treatment



FCA analysis

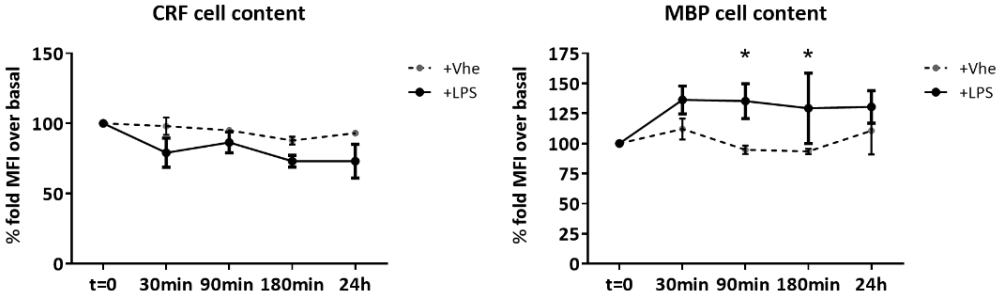


Figure 11. CRF localization and content in 15HL-60 Ba cells after stimulation with SP, CCh and LPS. Representative images of a time-course analysis of CRF protein localization evaluated by IF, and CRF and MBP total protein content evaluated by FCA after **A.** SP stimulation; **B.** CCh stimulation and. **C.** LPS stimulation and their respective vehicles. Negative control (only secondary antibody) was used for threshold for setting the positive signal. White arrows in IF images indicate relocation of CRF from the cytoplasm to the plasma membrane. Images are representative of 3 independent experiments. Bars indicate 10µm. FCA data are expressed as % of fold mean fluorescence intensity (MFI) change over basal condition of the same experiment. Data are expressed as mean ± standard error. N=8 for SP and CCh in CRF evaluation. N= 4 for LPS for CRF evaluation. N=4 for MBP evaluation. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparison post-hoc test * *P*<0.05 *vs*. vehicle. SP: substance P; CCh: carbachol; LPS: lipopolysaccharide; Vhe: vehicle; CRF: corticotropin-releasing factor; MBP: major basic protein; FCA: flow cytometry analysis.

Increased *CRH* gene expression in 15HL-60 Ba cells stimulated with SP is mediated by NK1R receptor

Finally, CRF gene expression after stimulation was evaluated in eosinophils, together with antagonist pre-treatment to elucidate mechanisms of activation. *CRH* mRNA expression levels were up-regulated after 30 minutes of treatment with SP and rescued in cells pre-treated with the NK1R antagonist, aNK1. No statistical differences after CCh treatment were observed.

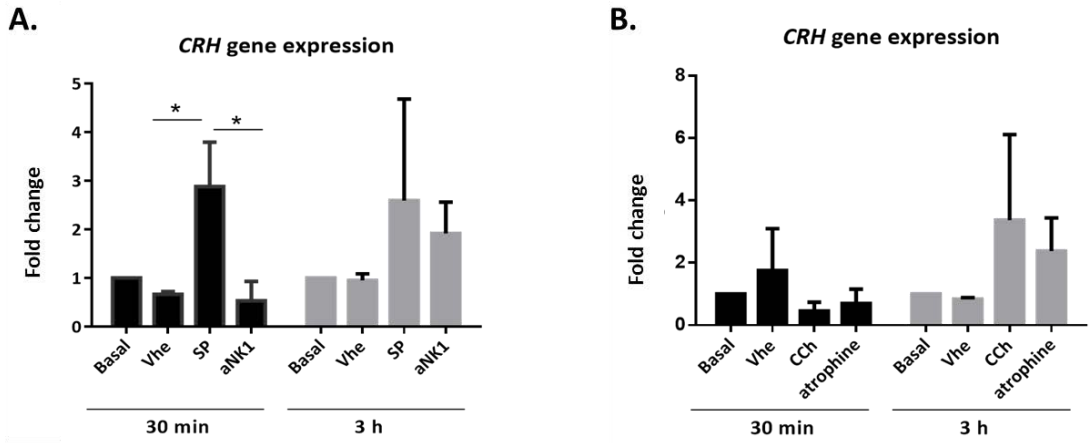


Figure 13. *CRH* gene expression assay after SP and CCh stimulation and pre-treated with antagonists. Cells were treated with **A.** SP and **B** CCh, with vehicle control and pretreated with antagonist on the indicated time points, and gene expression analyzed by qPCR. Fold-change was calculated by normalizing the data with the endogenous control gene (*PPIA*) and comparing each time point to untreated cells (t=0). Graphs represent results from 4 independent experiments Data are presented as mean ± standard error. Statistical analysis was performed by one-way non-parametric ANOVA (Kruskal-Wallis test) followed by Dunn's multiple comparisons post-hoc test. * *P*<0.05**;** SP: substance P; NK1R: neurokinin receptor 1; CCh: carbachol; Vhe: vehicle.

DISCUSSION

The present study provides first evidence on phenotypic differences between eosinophil-like *in vitro* models, determining that the cell line 15HL-60 differentiated with sodium butyrate better represents eosinophil-like features. Moreover, *in vitro* eosinophil stimulation with SP and CCh showed eosinophil activation with a low pro-inflammatory profile and piecemeal degranulation, suggesting an alternative pathway activation different from LPS stimulation leading to CRF release.

Emerging research has identified eosinophils also as key players in the neuro-immune circuitry leading to intestinal dysfunction, as they can produce and respond to neuromediators. Brain-gut axis dysfunction and immune activation has been reported in patients with functional gastrointestinal disorders, where the interaction between psychosocial factors, including stress, has a profound impact on gut physiology and symptoms. Eosinophil activation by neuro-hormonal stimuli provides an interesting biological substrate to study possible mechanisms of intestinal dysfunction related to stress. However, functional studies of human gastrointestinal eosinophils are scarce possibly due to the technical difficulties and low yields of eosinophil isolation protocols from human biopsies. To overcome this limitation, eosinophil-like stable cell lines represent a valuable tool for functional studies. Although, stable cell lines, unlike the primary culture, provide an unlimited source, fairly homogeneous and easy to use model, they are genetically manipulated or derive from malignant origin not representing neither healthy nor particular intestinal disease states. Therefore their phenotype, native functions and their responsiveness to stimuli may not reflect those of the primary cells, which clearly limits the translational capacity of such model systems.[29]

In this regard, two main eosinophil cell lines have been described and widely used. The AML14.3D10 and the 15HL-60 cell line. AML14.3D10 cells are derived from acute myeloid leukemia and have been reported to maintain an advanced state of differentiation and a high rate of proliferation in the absence of cytokine supplementation.[18] Our results showed that, despite AML14.3D10 cells express eosinophil-specific proteins, they also showed high expression of eosinophil cationic proteins,

described to be reduced in mature eosinophils[27,30], suggesting less differentiation state. In addition, morphological features showed more characteristics of promyelocyte cells[31], confirming an immature state. Moreover no CRF expression was detected, probably due to cytogenetic abnormalities of this cell line[18], which make these cells a not suitable tool for the proposed objectives of this study. As an alternative, the HL-60 clone 15 (15-HL60) was also selected as previous studies showed that, similar to mucosal eosinophils, these cells expressed CRF already in basal conditions,[13] which was confirmed in our study. 15-HL60 cells were established through long-term culture under alkaline conditions of HL-60 cells, derived from promyelocytic leukemia, where they acquired features of eosinophilic progenitors.[19,32] Nevertheless, these cells must be induced with butyric acid at 0.5mM for 7 days to terminally mature and differentiate into eosinophils.[19,33] However, reviewing the literature, a discrepancy was found in the use of butyric acid or sodium butyrate.[13,3435,21] Butyric acid is a short-chain fatty acid presented as oily liquid and appears mainly in its deprotonated form in solutions with a pH>pKa (=4.82), contributing to a slight acidity in a typical pH of culture medium (pH 7.2-7.8).[36] The sodium salt of butyric, i.e. sodium butyrate, is a more stable molecule than butyric acid and it easily dissociates to butyrate in an aqueous solution. The possible acidification of the culture medium of butyric acid treatment or its bioavailability, since it is not as soluble as the sodium butyrate salt, could explain the differences observed between the treatments. The existence of different protocols for the differentiation of these cells can determine significant differences, as some add interleukins and other factors besides butyrate[25,35]. Moreover, the markers for monitoring cell differentiation and maturation are not well clarified either. Our results showed no changes in protein and gene expression before and after differentiation, in contrast to what has been reported in other studies.[22,25] However, we observed discrepancies in MBP protein basal expression of cells without differentiating[22], perhaps due to changes in the maintenance protocol of the cell line. Also, the reported increase in integrin beta 7 observed during maturation is not very pronounced [25], and therefore may are not optimal markers. Gene expression profile confirmed also an immature state of these eosinophils, although in a more advanced stage than AML.14.3D10 cells. Morphological and

ultrastructural characteristics confirmed the differences in the maturation stage, however, these cells are composed of a heterogenous population which should be taken into account for interpretation of results.

Neuromediators such as SP and ACh and their receptors play an important role in the modulation of stress responses in the central nervous system as well as in stress-induced intestinal inflammation[37–40] and, recently, have been reported to induce the release of CRF from eosinophils in animal and *in vitro* models, where used 15HL-60 cells, and other inducible eosinophilic cell line, the EoL-1.[17,13] The present study extends existing knowledge by revealing that stress factors induce eosinophil activation by piecemeal degranulation and CRF release, in a non-pro-inflammatory pathway, similar to what we have described in mucosal eosinophils from IBS-D patients (chapter 1). Pro-inflammatory eosinophil activation has been reported in several inflammatory conditions and in response to LPS challenge, where eosinophils show upregulation and degranulation, releasing cationic proteins.[41–43] Here we reported that only LPS stimulation, and not neuromediators, induced an increase in cationic protein expression. However, many other proteins such as cytokines[34] have been described to be upregulated in eosinophils during inflammatory states, so signal transduction pathway assay would be the best first approximation to neuromediator activation effect to understand which pathways are active in inflammatory conditions. On the other hand, eosinophils can elicit inflammatory actions through degranulation, as they contain pre-formed mediators ready to rapidly release. Piecemeal degranulation are specifically mediated by the participation of a vesicular anchored soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE), the vesicle-associated membrane protein 2 (VAMP2), that binds to 2 target membrane SNAREs, syntaxin and synaptosome-associated protein of 23KDa (SNAP23).[34] We showed piecemeal degranulation after SP and CCh stimulation by the peripheral redistribution of VAMP2 and SNAP23 immunofluorescence, accompanied by a depletion of signal from the cytoplasm.[44,45] SNAP23 is described to be a plasma membrane protein, so cytoplasm localization and redistribution may reflect the presence of newly synthesized proteins destined for the plasma membrane. By contrast, LPS only showed a

redistribution of SNAP23 and not VAMP2, suggesting a different secretory profile, maybe exocytosis, where other VAMP proteins are associated.[46] The similar pattern observed in VAMP2 and CRF mobilization together with a depletion of CRF in cells after stress factor stimulations, indicates a piecemeal mechanism of CRF release in response to stress. In line with other studies, SP induced eosinophil CRF expression via NK1R.[17] However, it would be necessary to confirm whether piecemeal degranulation is mediated via NK2R in response to SP, and via muscarinic receptors in response to CCh, as described in other studies.[13,17]

In conclusion, we demonstrate phenotypic differences among two eosinophil-like cell lines and differentiation protocols, concluding that the sodium butyrate-differentiated 15HL-60 cell line better resembles the phenotype identified in the jejunal mucosa of IBS-D patients. Using this cell model, we provided functional evidence of a non-classical pro-inflammatory mechanism of eosinophil activation in response to neuropeptides that involves piecemeal degranulation and CRF release. Thus, eosinophil activation via neuropeptides derived from nerve or immune cells could be an important contributor in the pathophysiology in IBS-D that warrants further research in this field.

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DISCUSIÓN GENERAL

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El Síndrome del Intestino Irritable es un trastorno digestivo de curso crónico, con una elevada prevalencia en los países occidentales (10-20% población)[267] e impacto en la calidad de vida y que conlleva grandes repercusiones socioeconómicas. Esto se debe fundamentalmente al desconocimiento que aún existe sobre su fisiopatología y, consecuentemente, a la inexistencia de biomarcadores diagnósticos útiles y de estrategias terapéuticas eficaces. Sin embargo, aunque no se conoce en profundidad el origen de este síndrome, el estrés crónico y ciertos trastornos psicológicos como la ansiedad o la depresión se han asociado tanto al desarrollo como a la perpetuación de las manifestaciones clínicas. De hecho, alrededor del 50% de los pacientes con SII, y de otros trastornos funcionales digestivos como la dispepsia funcional, presentan comorbilidad con trastornos psiquiátricos y sufren más estrés crónico que la población sana.[124,126,127,268,269] Además, el inicio, la gravedad y la persistencia de ciertos subtipos de SII, sobre todo subtipo diarrea (SII-D), se han vinculado a acontecimientos vitales estresantes y/o traumáticos.[122–125] Así, determinados estados psicológicos podrían incidir en la magnitud y percepción de los síntomas, configurando un paradigma de modelo biopsicosocial de la enfermedad.

El origen de los síntomas en el SII, principalmente el subtipo diarrea, apunta, contrariamente a lo que se ha establecido clásicamente, al intestino delgado como un elemento significativo[270–272], debido en parte a las variadas funciones individualizadas que desempeña y a la contribución del sistema nerviosos entérico (SNE) en la regulación de procesos fisiológicos como la respuesta inmunitaria, la motilidad intestinal, la función de la barrera intestinal y la secreción epitelial.[273] Aunque su fisiopatología no está establecida, sus características cardinales, la alteración de la motilidad gastrointestinal y la hipersensibilidad visceral, podrían ser el resultado de una respuesta neuro-inmunológica inadecuada frente al estrés psicológico crónico y las infecciones gastrointestinales.[96].

Hallazgos comunes destacan una alteración de la función barrera intestinal en asociación con la presencia de una activación de bajo grado del sistema inmunitario intestinal, además de una alteración del eje HPA y de la respuesta al estrés, en el que el CRF juega un papel clave.[153,167,274] En este marco de modelo biopsicosocial del SII, el eosinófilo, como célula residente intestinal, principalmente en el intestino delgado, con un papel clave en otras enfermedades gastrointestinales y asociada directamente con el sistema nervioso, el estrés y la activación del mastocito, cumple los requisitos como célula candidata a participar en la fisiopatología del SII-D. Por todo ello, la hipótesis de trabajo que plantea este estudio es que la activación del eosinófilo de la mucosa intestinal en el yeyuno, en respuesta al estrés contribuye a la disfunción intestinal en el SII y participa en la exacerbación de los síntomas a través de la liberación de CRF.

Como primera aproximación para evaluar nuestra hipótesis, y gracias al perfil transcriptómico entre pacientes con SII-D y sujetos controles que previamente se realizó en el grupo[151,275], el cual mostró la existencia de vías directa e indirectamente relacionadas con el reclutamiento y activación del eosinófilo como vías relevantes en el SII-D, nos planteamos evaluar, mediante un abordaje celular y molecular, el reclutamiento, infiltrado y el estado de activación y contenido de CRF del eosinófilo en la mucosa yeyunal de estos pacientes.

La vía de señalización eotaxina-CCR3 es la vía principal de reclutamiento del eosinófilo al intestino y es también una vía implicada en procesos inflamatorios, en los que un aumento en su expresión se relaciona con un mayor infiltrado y activación pro-inflamatoria del eosinófilo en trastornos gastrointestinales[19–2] como ocurre en la enfermedad inflamatoria intestinal[276–281] y en otras patologías inflamatorias como el asma y la alergia.[277,282]

El infiltrado eosinofílico yeyunal no mostró diferencias entre pacientes y controles, similar a lo observado en otros estudios en colon[283], a pesar de que sí se había descrito, junto a una mayor degranulación, en el duodeno de pacientes con dispepsia funcional[284,285], otro trastorno funcional digestivo también presente en algunos de los pacientes con SII. Las distintas regiones del intestino presentan una heterogeneidad inmunológica y funcional a lo largo del TGI intrínseca a la anatomía

diferencial del intestino[273] que podría explicar las diferencias observadas. Además, también observamos una regulación negativa en el yeyuno de los pacientes de la expresión de eotaxinas CCL24 y CCL26 (eotaxinas 2 y 3, respectivamente) y del receptor de eotaxina CCR3, junto con una disminución de la expresión de las proteínas catiónicas citotóxicas del eosinófilo EPX (EPO), RNASE2 (EDN) y RNASE3 (ECP), comparado con el grupo control. Estos resultados, a diferencia de lo que se observa en enfermedades inflamatorias, sugiere la presencia de condiciones limitantes o restrictivas en el yeyuno de pacientes con SII-D para el reclutamiento y la activación de eosinófilos, que pueden explicar la presencia de un infiltrado semejante al de sujetos sanos. Estos hallazgos pueden indicar la existencia de vías homeostáticas reguladoras, en parte a través de la vía eotaxina-CCR3, que limitan la actividad inflamatoria mediada por eosinófilos en la mucosa, lo cual se desconoce y podría ser una vía prometedora para el estudio de mecanismos de control de la inflamación intestinal.

Aunque habitualmente los eosinófilos se han considerado en el contexto de la inflamación alérgica y en las enfermedades parasitarias, éstas células participan en una amplia variedad de procesos fisiológicos y patológicos[242,253], y pueden modular respuestas inflamatorias a través de la liberación extracelular de una amplia variedad de otros productos derivados de gránulos.[286]

Nuestros resultados muestran que, a pesar del perfil proinflamatorio bajo de los eosinófilos de la mucosa yeyunal, éstos muestran mayor prevalencia y grado de degranulación tipo *piecemeal* en los pacientes que el grupo control sano, analizado por cambios ultraestructurales de los gránulos citoplasmáticos. Por otro lado, el análisis del número y el área de los gránulos también se ha descrito como un evento relevante en el estado de activación de eosinófilos. Los eosinófilos hipodensos (que contienen menos gránulos o son más pequeños) son particularmente importantes en la fisiopatología de diversas enfermedades inflamatorias, como el síndrome hipereosinofílico (HES)[287] o la colitis ulcerosa[288], y se describen como más activados con una actividad citotóxica.[287,289] En este trabajo hemos descrito que los eosinófilos de los pacientes con SII-D muestran un aumento en el área de los gránulos, pero con un número total de gránulos similar a los sujetos control, que no cumplen, por lo

tanto, las características para ser denominados hipodensos. En concordancia con los datos de expresión génica y el fenotipo morfológico, estos resultados refuerzan que los eosinófilos del SII-D se encuentran activados, pero no promoviendo una actividad proinflamatoria como se observa en otras enfermedades inflamatorias gastrointestinales. Esto sugiriere que los eosinófilos pueden adquirir diferentes estados de activación que no necesariamente se reflejan en la expresión de proteínas inflamatorias o en un mayor infiltrado.

Cada vez son más numerosos los estudios que apuntan al eosinófilo como célula clave en el control de la función intestinal. Esta función la ejerce gracias a la interacción neuro-inmunológica que establece con el entorno, ya que pueden responder, almacenar y liberar una amplia variedad de neuropéptidos y CRF[182,185,225,228] y expresan receptores para numerosos neurotransmisores liberados por neuronas.[184,192,238,258,290–292] Tanto el estrés agudo como el estrés crónico aumentan la permeabilidad epitelial a moléculas de pequeño y gran tamaño en el yeyuno y en el colon de roedores y humanos[92,147,293], con la participación de los mastocitos y del CRF.[89,294,295] El principal mediador de la respuesta al estrés es el CRF, cuyos efectos sobre la función del TGI han sido demostrados tanto a nivel del SNC como periférico, con efectos tanto en la motilidad y sensibilidad visceral, como en la permeabilidad intestinal.[75] Aunque se ha descrito que la fuente de CRF a nivel periférico se encuentra en los nervios entéricos y en las células inmunitarias como los eosinófilos[258]y las células dendríticas[296], su origen en la mucosa yeyunal en humanos no se había descrito hasta el momento. Este trabajo proporciona la primera evidencia de la presencia de CRF exclusivamente en los eosinófilos de la mucosa yeyunal, concretamente en los gránulos citoplasmáticos. Además, de forma relevante, la cuantificación de partículas de CRF demuestra mayor cantidad por gránulo en eosinófilos de pacientes con SII-D en comparación con el grupo control. Además, la cantidad de CRF en los eosinófilos correlaciona tanto con los factores psicológicos, como el estrés o depresión, como con la severidad del SII medido por la puntuación global que recoge varias variables sintomáticas, así como por separado con el dolor abdominal y la distensión, y el número y consistencia de las deposiciones. La cantidad de CRF también correlaciona con el grado de degranulación en los

eosinófilos, lo que podría sugerir una mayor secreción de CRF en el grupo de pacientes, aunque habría que examinarlo con mayor profundidad.

Las implicaciones fisiopatológicas a nivel local aún no se han estudiado, pero se asume que los receptores de CRF presentes en el intestino serán los responsables de mediar los efectos subsiguientes. De hecho, los receptores de CRF (CRFR1 y CRFR2) están ampliamente distribuidos en el tracto gastrointestinal[93], principalmente en las neuronas entéricas y en las células de lá*mina propia*, predominantemente en los mastocitos de la mucosa colónica.[297] Nuestro estudio ha identificado la disminución del receptor CRFR1 sin cambios en el CRFR2. A pesar de que no se conoce el papel de los receptores de CRF en el yeyuno todavía, un candidato en mediar los efectos derivados del CRF es el mastocito, el cual, como se ha descrito anteriormente, está ampliamente implicado en el control de la función de la barrera intestinal. Además, el aumento de la permeabilidad también se ha correlacionado con el dolor o molestia abdominal y con el hábito intestinal en el SII[153,156,298], lo que coloca al eosinófilo y al CRF como un vínculo importante, previamente desconocido, en la secuencia de eventos de señalización en el control de la función barrera en el SII-D. En este sentido, se ha demostrado que una activación constante de los mastocitos mediante la estimulación con CRF, acaba disminuyendo la expresión del receptor CRFR1 debido a una sobreestimulación, lo que podría explicar la menor expresión de CRFR1 en los pacientes de nuestro estudio.

El incremento de CRF en los eosinófilos yeyunales podría deberse al mayor estrés basal y depresión registrados en estos pacientes. Existen evidencias en animales de experimentación donde el aumento de expresión de CRF en los eosinófilos es paralelo al período de estrés, disminuyendo gradualmente tras la eliminación del estímulo. Los eosinófilos expresan una amplia variedad de receptores de neuropéptidos y pueden interactuar directamente con los nervios tanto en homeostasis como en enfermedad[184,192,238,258,290,299], por lo que la activación del eosinófilo por mecanismos neuro-hormonales proporciona un marco interesante para estudiar posibles mecanismos de activación relacionados con el estrés y el SII-D. Así, como último objetivo de esta

tesis, planteamos analizar el estado de activación y contenido de CRF en los eosinófilos en respuesta a estímulos relacionados con el estrés.

Debido a la dificultad de aislar un número adecuado de células de la mucosa yeyunal con una biopsia por cápsula de Watson o de sangre, se evaluó la respuesta en un modelo celular. Las líneas celulares eosinofílicas estables representan una herramienta valiosa para los estudios funcionales, aunque su origen maligno y las anormalidades genéticas que presentan no representan un eosinófilo diferenciado normal, lo que limita la capacidad de traslación. Por ello, primero evaluamos dos líneas celulares diferentes para establecer el modelo más adecuado y más parecido, dentro de las propias limitaciones, a los eosinófilos diferenciados de la mucosa. A pesar de que se han descrito y utilizado en varios estudios funcionales dos líneas celulares eosinofílicas, como la AML14.3D10 y 15HL-60, no se ha evaluado en profundidad sus características morfológicas y fenotípicas en relación a su funcionalidad en estudios traslacionales, sino más bien aplicado al estudio de la biología de esta célula.[258,300–304]

La línea celular AML14.3D10, derivada de una leucemia mieloide aguda, mantiene un avanzado estado eosinofílico en ausencia de suplementación con citoquinas[305]. Nuestro análisis, sin embargo, a pesar de que hemos observado que presentan un elevado número de células positivas para proteínas expresadas en células maduras, como la proteína básica principal (*major basic protein*, MBP) o la integrina beta-7[303,306], ha revelado que sus características morfológicas y la expresión de elevados niveles de mRNA de proteínas catiónicas y otras específicas del eosinófilo, indican un estado de diferenciación poco avanzado, más característico de pro-mielocitos.[307–309] Además, tampoco expresan CRF, quizá por las anormalidades citogenéticas que presentan, por lo que concluimos que no es un buen modelo para el objetivo propuesto.

Como alternativa se escogió la línea celular 15HL-60 la cual, a pesar de que se ha de inducir la diferenciación durante 7 días con 0,5M de ácido butírico[310,311], se ha demostrado que expresan CRF[258], corroborándolo nosotros después. Esta línea celular es un subclon derivado del cultivo, en condiciones alcalinas, de la línea HL-60 derivada de una leucemia promielocítica.[310,312] Debido a la

inespecificidad en la referencia a ácido butírico o butirato de sodio como compuesto para inducir la diferenciación[258,313304,314], y debido a sus diferencias químicas en solución acuosa, decidimos evaluar la maduración del eosinófilo mediante dos protocolos distintos referidos en la literatura. Lo que observamos fue que el protocolo de diferenciación con ácido butírico disminuye la viabilidad tras los 7 días, quizá debido al efecto del pH por ser un ácido[315], por lo que dificulta posteriores análisis funcionales. También, a pesar de que no observamos diferencias en los marcadores de genes y proteínas para evaluar la maduración tras la diferenciación, sí observamos diferencias morfológicas y ultraestructurales, siendo la línea diferenciada con butirato fenotípicamente más parecida a un eosinófilo maduro. Esto podría explicar la respuesta analizada a neuromediadores parecida, aunque más evidente en la diferenciada con butirato, entre las dos diferenciaciones, y también sus diferencias con la línea más inmadura AML14.3D10. Además, comprobamos que expresan receptores para neuromediadores como SP y ACh. Estos neuromediadores y sus receptores juegan un papel importante en la modulación de las respuestas de estrés en el sistema nervioso central, así como en la inflamación intestinal[316–319] y, recientemente, también se ha relacionado con la degranulación de CRF en los eosinófilos.[192,258]

Nuestros resultados amplían el conocimiento existente al mostrar, por primera vez, que la estimulación por neuromediadores, a diferencia de la estimulación con lipopolisacárido de origen bacteriano, induce la activación por degranulación selectiva tipo *piecemeal*, mediante movilización de vesículas y liberación de CRF, con un perfil proinflamatorio bajo en la expresión de proteínas citotóxicas. Con este último trabajo hemos querido obtener una mayor comprensión del mecanismo de activación de los eosinófilos de la mucosa secundario a una condición de estrés crónico, mediante la comparación con un modelo *in vitro*. Asimismo, hemos demostrado que las características de activación del modelo *in vitro* en respuesta al estrés son similares a las características de activación observadas en los eosinófilos de la mucosa de pacientes con SII-D. En este estudio, sin embargo, no realizamos un ensayo con antagonistas de los receptores de SP y ACh para evaluar el mecanismo de activación de la secreción de CRF, aunque hemos evaluado la expresión, determinando, tal y como ya

se había publicado anteriormente, que la SP induce su expresión a través del receptor NK1R.[192] Futuros estudios funcionales en la línea celular deberían incluir los antagonistas así como evaluar con mayor profundidad las vías de activación en los eosinófilos.

Asimismo, tampoco hemos evaluado la expresión de receptores de SP y ACh en los pacientes con SII-D. A pesar de que se ha descrito un aumento de fibras inmunorreactivas de SP y mayor concentración de SP en el colon de estos pacientes[140,320,321], así como mayor actividad colinérgica[322], que apuntan a mayor liberación de neuropéptidos a nivel local, sería interesante averiguar su localización en el yeyuno y si existe alguna correlación con la activación de los eosinófilos.

Estos resultados refuerzan, en la línea de nuestro grupo, que el SII-D se extiende más allá del colon afectando también al intestino delgado. La activación de los eosinófilos por neuropéptidos secundario a un estrés psicológico o local y la liberación de CRF, puede ser un factor relevante en la fisiopatología del SII-D y en la inflamación intestinal y disfunción de la barrera asociada al estrés.

CONCLUSIONES

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Los resultados obtenidos en esta tesis doctoral han dado lugar a las siguientes conclusiones:

1. La mucosa del yeyuno de los pacientes con SII-D posee un perfil transcripcional característico asociado, entre otros, a alteraciones en la vía de señalización de reclutamiento del eosinófilo al intestino.

2. La mucosa del yeyuno de los pacientes con SII-D presenta una disminución en la expresión génica de proteínas catiónicas citotóxicas del eosinófilo, así como un incremento de la actividad secretora de estos eosinófilos.

3. En la *lámina propia* de la mucosa yeyunal, los eosinófilos son las únicas células que almacenan CRF, la cual se localiza en los gránulos citoplasmáticos.

4. Los eosinófilos de la mucosa yeyunal de los pacientes con SII-D presentan mayor grado de activación por degranulación tipo *piecemeal* y mayor contenido de CRF en sus gránulos.

5. El contenido de CRF en los eosinófilos se asociado a la severidad clínica del SII-D, incluyendo dolor, frecuencia y consistencia de las deposiciones, así como el nivel de estrés crónico y de depresión.

6. La estimulación de eosinófilos con sustancia P y carbacol (y no con lipopolisacárido) muestra un perfil de activación por degranulación tipo *piecemeal* con liberación de CRF, sin aumentar la expresión y degranulación de proteínas citotóxicas. Este perfil es similar al identificado en los eosinófilos del yeyuno de pacientes con SII-D, por lo que podría ser una nueva herramienta para el estudio de mecanismos de activación del eosinófilo intestinal en trastornos funcionales digestivos como el SII.

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ANEXO

ANEXO 1: SUPPLEMENTARY MATERIAL FROM CHAPTER 1

Participants

Clinical and demographic characteristics of participants and experimental procedures

Inclusion criteria	**HC**	**IBS-D**
Age: 18-60 years	yes	yes
Rome III criteria for IBS-D	no	yes
Naïve (newly-diagnosed)	no	yes
Acceptance of the study protocol	yes	yes
Exclusion criteria		
Clinical history of food allergy	yes	yes
Positivity to SPT to foodstuff	yes	yes
Infectious gastroenteritis	yes	yes
Gastrointestinal comorbidities	yes	yes
Pregnancy	yes	yes
Major psychiatric disorders	yes	yes
Use of medication (steroids, immunosuppressive drugs, anti-histaminic and mast cell stabilizers)	yes	yes

Table S1. Inclusion and exclusion criteria for study subjects.

	Gender	**Age (years)**	**Intensity of abdominal pain (0-100)**	**Bowel Movements**	**Stool consistency (Bristol, 1-7)**	**Dyspepsia**	**Atopy**	**Holmes -Rahe**	**Cohen**	**Beck's**
H1	F	28,6	0			N/A	0	85	13	2
H2	F	28,0	0			N/A		29	14	2
H3	F	29,0	0	1,0	3,2	N/A	0	110	19	4
H4	F	24,0	0	1,0	2,3	N/A		55	13	0
H5	M	29,0	0	1,5	3,0	N/A	1	151	11	3
H6	F	27,0	0	2	3	N/A	1	64	14	2
H7	M	35,0	0	2	4	N/A	0	64	13	1
H8	M	31,0	0			N/A	1	179	15	1
H9	M	32,7	0			N/A		90	17	3
H10	F	41,0	0			N/A	0	75		22
H11	F	28,0	0	1	3,1	N/A		95	25	4
H12	F	34,0	0			N/A	0	183	13	1
H14	M	42,0	0			N/A	1	75	24	2
H15	M	21,0	0	1,0	3,9	N/A	1	154	22	0
H16	M		0			N/A	0	69	20	1

H17	F	21,5	0	1	3	N/A	0	112	4	0
H18	F	48,5	0			N/A	0	63	12	0
H19	M	45,6	0	1	3	N/A	0	127	12	5
H20	F	28,7	0	1	4	N/A	0	189	18	2
H21	M	53,3	0	1	2	N/A		121	8	4
H22	M	29,1	0			N/A	0	125	10	1
H23	M	41,4	0		5,0	N/A	0	267	24	6
H24	M	47,4	0	1,0	4,0	N/A	0	85	20	4
H25	M	63,8	0	1,0	4,0	N/A	0	229	28	9
H26	M	22,5	0		3,0	N/A	1	108	15	0
IBS1	F	68,0	62	3,0	5,0	0		44	25	21
IBS2	F	33,0	22	5,0	5,1	0	1	63	18	1
IBS3	F	55,0	22	5,0	5,3	0				
IBS4	F	28,0	33	8,0	5,5	0	0	303	22	6
IBS5	M	41,0	30	12	7	0	0	99	13	2
IBS6	M	34,0	25	2	5,5	1	0	54	36	14
IBS7	F	50,0	65	3	6	1	0	227	25	7
IBS8	F	36,0	39	3	6	0	1	73	35	14
IBS9	M	27,0	60	1	5	1	1	87	24	2
IBS10	F	35,0	60	5	6	1	0	350	32	23
IBS11	M	22,0	43	3,5	6,5	0	0	308	20	8
IBS12	F	21,0	80	7	6,5	1	1	274	20	10
IBS13	F	39,0	100	10	6,5	1	0	64	18	13
IBS14	F	24,0	17	7	6,5	1	1	25	30	13
IBS15	F	62,0	76	4	6	1	1	889	39	24
IBS16	M	44,0	31	3	6,5	0	0	113	26	5
IBS17	F	27,0	71	2,0	5,1	1		242	12	2
IBS18	M	27,0	42	2,0	5,2	0		157	17	5
IBS19	F	21,0	51	4,0	4,9	0		138	20	3
IBS20	F	39,0	51	3,0	4,9	0		44	22	6
IBS21	M	43,0	73	4,0	5,0	0	1	399	38	26
IBS22	F	45,0	70	5,0	4,5	1	0	85	16	5
IBS24	F	57,00	35	7,0	3,4	0	1	203	15	13
IBS23	M	36,0	15	5,0	4,8	1	1	104	20	5
IBS25	F	34,0	62	6,0	4,7	1	1	57	24	5
IBS26	F	31,0	80	9,0	4,3	0	1	178	46	36
IBS27	M	53,0	70	7,0	6,3			237	30	28
IBS28	F	59,0	69	6,0	5,1	1		96	9	6
IBS29	F	38,0	34	5,0	6,9	0		89	24	5
IBS30	F	30,0	21	3,0	3,8	0	1	112	18	3
IBS31	F	37,0	87	6	6,5	1	0	397	37	31
IBS32	F	38,0	45	4	6,1	1	0	142	27	19
IBS33	F	36,0	15	4	5,4	1	1	71	32	6
IBS34	F	29,0	16	6	6,5	1	0	186	26	17
IBS35	M	38,0	50	8,0	5,8	0	1	25	13	2
IBS36	F	59,0	67	3	6	1	0	246	38	24

IBS37	M	32,2	36	3	6		1	157	29	7
IBS38	F	36,0	40	3	6	1	0	177	24	18
IBS39	F	20,6	73	3	6	1	1	457	36	19
IBS40	M	21,1	6	2,5	6	1	0	25	27	0
IBS41	M	42,1	71,5	1,5		1	0	164	22	14
IBS42	F	37,3	71	1,5	2,0	1	1	25	9	1
IBS43	F	37,9	18	1	5,5	0		65	24	3

Table S2. Clinical and demographical characteristics of participants

Participant	Experimental procedure	Participant	Experimental procedure	Participant	Experimental procedure
H1	1+2+3	IBS1	1+2+3	IBS26	2+3
H2	2	IBS2	2	IBS27	2+3
H3	1+2+3	IBS3	2+3	IBS28	2+3
H4	1+2+3	IBS4	1+2+3	IBS29	2+3
H5	2 + 3+ 4 + 5	IBS5	4 + 5	IBS30	1+2+3
H6	2+3	IBS6	4 + 5	IBS31	4 + 5
H7	2 + 3+4 + 5	IBS7	4	IBS32	4 + 5
H8	1+2 + 3+4 + 5	IBS8	4 + 5	IBS33	4 + 5
H9	4 + 5	IBS9	4	IBS34	4 + 5
H10	2+3	IBS10	4 + 5	IBS35	5
H11	2 + 3+ 4 + 5	IBS11	4	IBS36	4
H12	2 + 3+ 4 + 5	IBS12	4 + 5	IBS37	2
H13	2 + 4 + 5	IBS13	4	IBS38	2
H14	2+3	IBS14	4 + 5	IBS39	2
H15	4 + 5	IBS15	4	IBS40	2
H16	4 + 5	IBS16	4	IBS41	2
H17	4 + 5	IBS17	2+3	IBS42	2
H18	4 + 5	IBS18	1+2+3	IBS43	2
H19	4 + 5	IBS19	2+3		
H20	4 + 5	IBS20	1+2+3		
H21	2	IBS21	2+3		
H22	2	IBS22	2		
H23	2	IBS24	2+3		
H24	2	IBS23	2+3		
H25	2	IBS25	2+3		

Table S3. Experimental procedures and clinical and demographic characteristics of participants 1: gene expression by microarray; 2: gene expression by qPCR; 3: Eosinophil quantification by MBP Immunohistochemistry; 4: CRF quantification by Immuno-gold in TEM; 5: eosinophil granule area and degranulated area quantification by ImageJ on TEM images.

Antibodies and conditions used

Primary antibody	Supplier	Dilution
Monoclonal mouse anti-human CD3 (clone PS1)	Leica Novocastra	1/100
Polyclonal rabbit anti-*human* CD117	Dako	1/50
Polyclonal rabbit anti-*human* MBP	Abcam	1/50

Table S4. Antibodies used for immunohistochemistry

Antibody	Supplier	Primary Ab dilution	pH Incubation	Secondary Ab dilution	Particle size secondary Ab (nm)
Monoclonal mouse anti-human CRF	AbCam	1/50	7.4	1/50	10
Monoclonal goat anti-human CRF	Sta Cruz Biotech.	1/50	8.5	1/50	15
Secondary antibodies	British BioCell International.				

Table S5. Antibodies. pH and gold nanoparticle size for CRF immuno-gold

CRF nano-immunolabeling validation

CRF antibody specificity

Mouse anti-human CRF was tested in dot blot assays using rat-brain tissue extract (Sigma) as positive control. cell lysate (AML14.3D10 cells). as negative control and primary antibody absorbed as a control of reaction (figure S1). Dot blot was performed in nitrocellulose membranes with pre-absorbed lysates (5µL) and primary antibody (2µL) as control of signal. for 1 h followed by blocking with 5% milk in Tris-Tween-Buffer-Saline (TTBS buffer) for 1h. Primary antibody (1/1000) was added diluted in blocking solution for 1h at room temperature (RT). followed by 3 washes in TTBS. Secondary antibody (1/2500) in blocking solution was incubated 1h RT. washed and detected with Western Glo Chemiluminescent detection reagent.

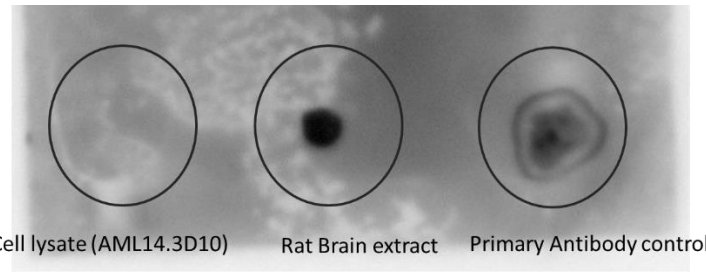
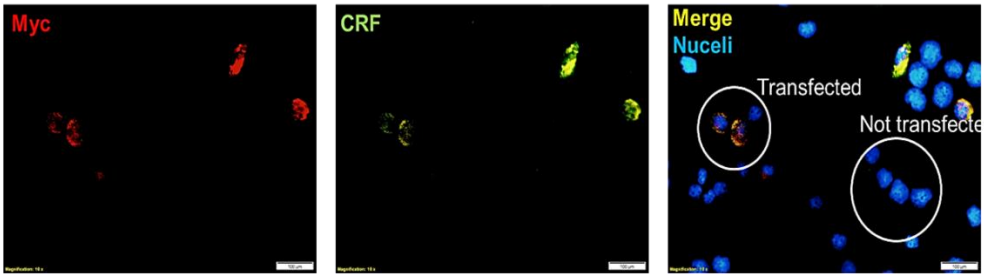


Figure S1. Dot Blot of mouse anti-human CRF in cell lysate (AML14.3D10 cells) and rat brain extract.

Goat anti-human CRF was tested in HEK-293 cell line (embryonic kidney). that presumably lack CRF expression. transfected with a vector containing CRF and the flag epitope Myc-tag (CRH (Myc-DDK-tagged)-Human corticotropin releasing hormone (CRH). Origene). Transfection of HEK cells were performed with lipofectamine 3000 (Thermo Fisher Scientific) following manufacturer's instructions. CRF and Myc in transfected cells were detected by IF (protocol as previously described in methods chapter 2). Anti-CRF and anti-Myc were incubated separately or sequentially in HEK cells. Interestingly. not transfected cells did not show CRF positivity and Myc expression whereas transfected cells showed CRF and Myc signal (figure S2 A). Signal was similar between CRF and Flag when incubated separately. and colocalizing when incubated altogether in HEK cells (figure S2 B).

A.



B.

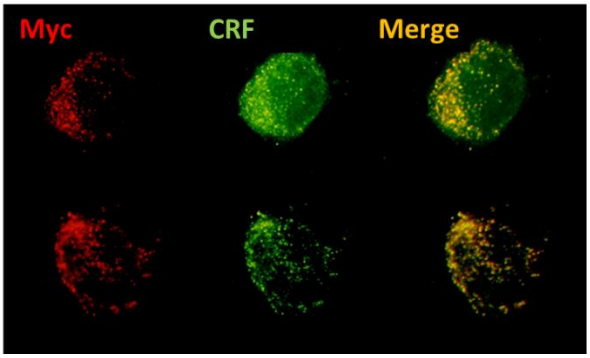


Figure S2. Anti-Myc and anti-CRF (goat anti-human CRF) label in transfected HEK cells. **A.** Myc and CRF expression in transfected and not transfected HEK cells**.** Bars indicate 100µm**. B**. Myc and CRF colocalization in transfected HEK cells with sequentially incubation of antibodies. Similar patterns were observed when antibodies were incubated separately.

Negative control and background assay in TEM

Incubation of ultrathin sections with the secondary antibody (negative control assay) was performed in order to identify unspecific signal. Unspecific signal was observed in mitochondrial enterocytes, blood vessels (elements presumably lacking the antigen) as well as epithelial cells (figure S3 left) at 1/5 secondary antibody dilution and pH 7.2. To avoid unspecific staining, two conditions were modified: dilution of secondary antibody and pH of the incubation buffer. Background signal was reduced by decreasing dilution to 1/30 and changing pH to 8.5 (figure S3 right)

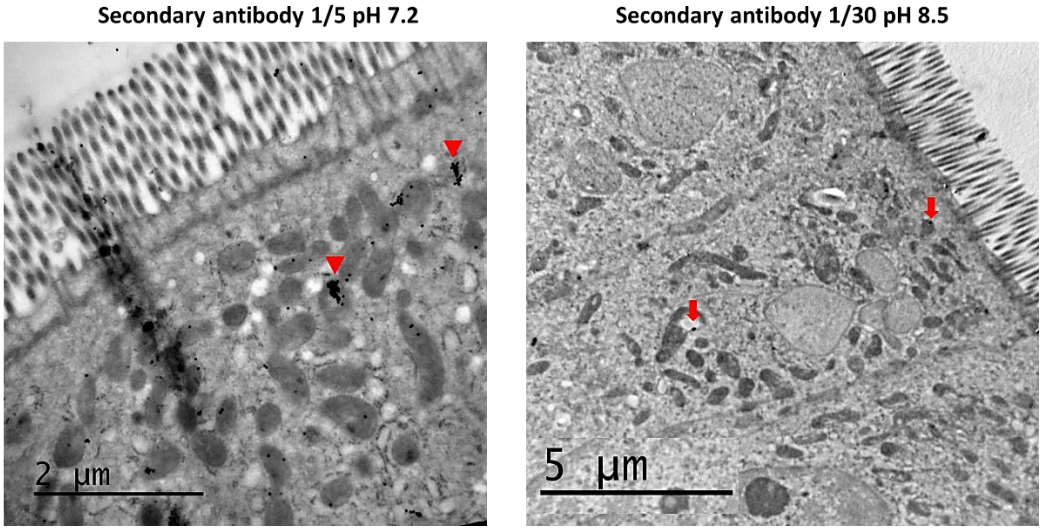


Figure S3. Negative control of CRF immunolabelling (only secondary antibody). Representative images of the jejunal epithelium. before and after pH optimization. Left: arrowhead indicate agglomeration of nano-immunogold particles. Right: arrow indicate nano-immunogold particle

Negative control with secondary antibody did not show labeling in mucosal immune cells. including eosinophils (figure S4).

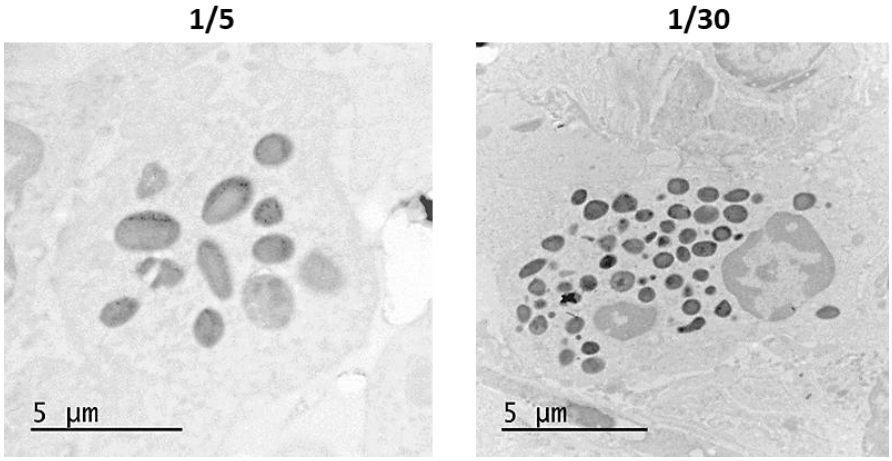


Figure S4. Representative micrographs showing no labelling in eosinophil granules after incubation with secondary antibody at 1/5 or 1/30 dilution (negative control) at pH 8.5.

Incubation of ultrathin sections after secondary antibody and pH optimization with the primary antibody was performed in order to identify specific versus unspecific signal.

Signal was observed in eosinophil cytoplasm and granules, and in the cytoplasm of other mucosal cells at a dilution of primary antibody 1/5. To optimize unspecific staining of primary antibody due to concentration an antibody titration was performed. Primary antibody dilution was tested at 1/5, 1/20, and 1/50, and a reduction in the signal in eosinophils was identified, mostly in the cytoplasm but not in granules (figure S5), confirming granule specificity (table S6).

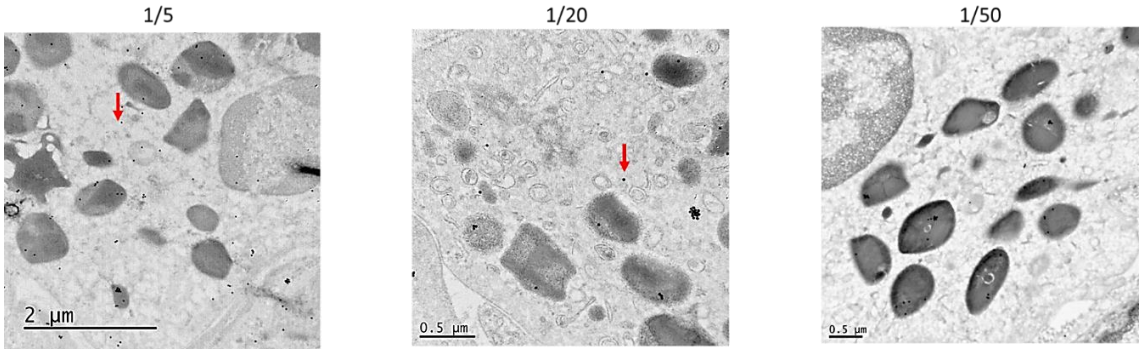


Figure S5. Eosinophil cytoplasm images of CRF labeling at different primary antibody concentrations. Arrow indicate CRF nano-immunogold particle in eosinophil cytoplasm. 1/5. 1/20 and 1/50 are dilutions of primary antibody at pH8.5 and 1/30 secondary antibody.

DILUTION OF PRIMARY ANTIBODY	NUMBER OF GRANULES COUNTED	CRF/GRANULE AVERAGE	CRF IN CYTOPLASM
1/5	85	3.87	yes
1/20	51	3.23	Yes. but weak signal
1/50	79	2.83	no

Table S6. Experimental conditions to test primary antibody signal. Different dilutions of the primary antibody translated into specific and unspecific CRF signal. The number of eosinophils counted and the location of CRF labeling are indicated.

Moreover, primary antibody at a dilution 1/5 also showed signal in mucosal cell cytoplasm, as for example lymphocytes, together with signal in mitochondria and nucleus (figure S6 left).

Decreasing dilution of primary antibody also reduced unspecific signal in mucosal cells. Conditions tested were 1/20 and 1/50 (primary antibody) and 1/30 (secondary antibody) at pH8.5. At 1/50, only some cells showed positivity in mitochondria and nucleus (figure S6 right),

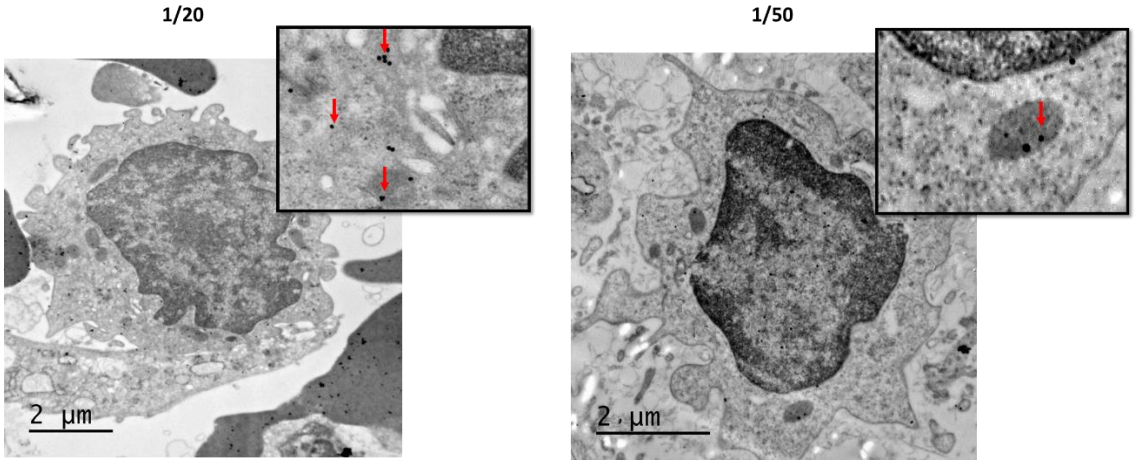


Figure S6. Representative micrograph of unspecific signal in mucosal cells. A lymphocyte is shown after incubation with different primary antibody concentrations (1/20 dilution left, 1/50 dilution right). Arrows indicate nano-immunogold particle in lymphocyte cytoplasm and mitochondria (left), and only in mitochondria (right).

After primary and secondary antibody, and pH optimization from 7.2 to 8.5, background signaling in mitochondria, blood vessels and nucleus was still observed, although optimized until reach low particles (figure S7), then considered as un-specificity inherent to the tissue nature.

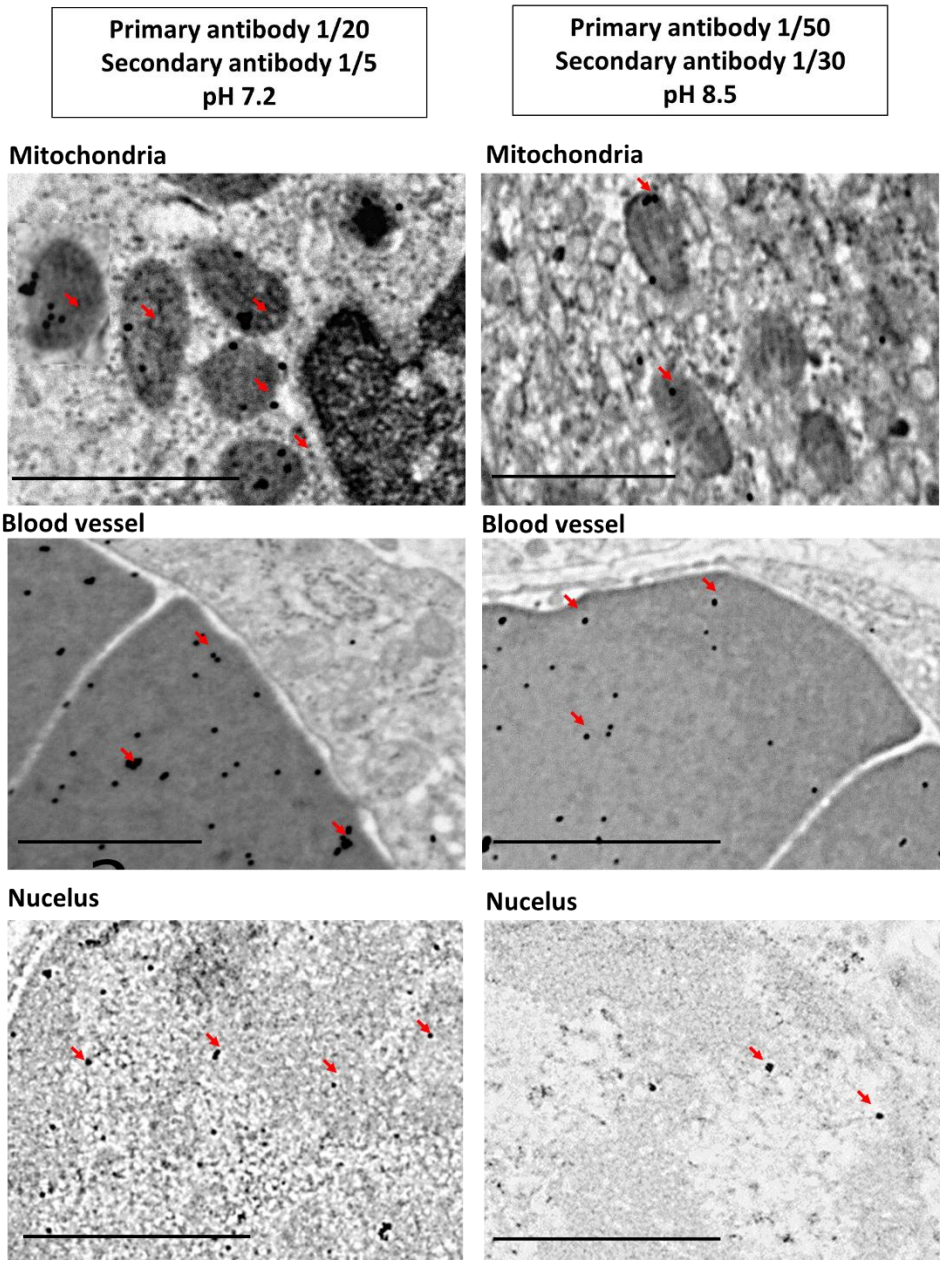


Figure S7. Representative micrographs showing unspecific labeling of the jejunal epithelium. nucleus from mucosal cell. blood vessel and mitochondria after pH and primary and secondary antibody optimization**.** Arrow indicate nano-immunogold particle

CRF antibody reproducibility

An important criterion for validation and standardization is antibody reproducibility. In this case, we compared particle counts with two different primary antibodies, previously validated (section 1.3.1) and optimized, to same target samples to expect to yield similar results. Measure of CRF nano-

immunogold particle counts in eosinophils was performed first with one antibody in random samples from HC and IBS-D. Samples with higher, medium and lower CRF labeling were selected from HC and IBS-D and counted and compared. A different count between antibodies was observed in the same samples. However, the same signal proportion was maintained between samples and groups (table S7).

SAMPLE	CRF/GRANULE AB 1 (MOUSE)	CRF/GRANULE AB 2 (GOAT)
IBS7	**9.23 ± 3.28**	**4.59 ± 2.01**
IBS35	**7.31 ± 2.20**	**3.50 ± 1.87**
IBS10	**7.4 ± 2.11**	**2.75 ± 0.21**
IBS16	**4.45 ± 1.78**	**2.76± 0.42**
HC19	**1.73 ± 0.50**	**2.14 ±0.8**
HC6	**2.92 ± 0.8 1**	**2.14 ±0.55**
HC15	**4.1 ± 0.85**	**2.00 ±0.1**

Table S7. CRF counts between two different primary antibodies in HC and IBS-D samples and factor correction

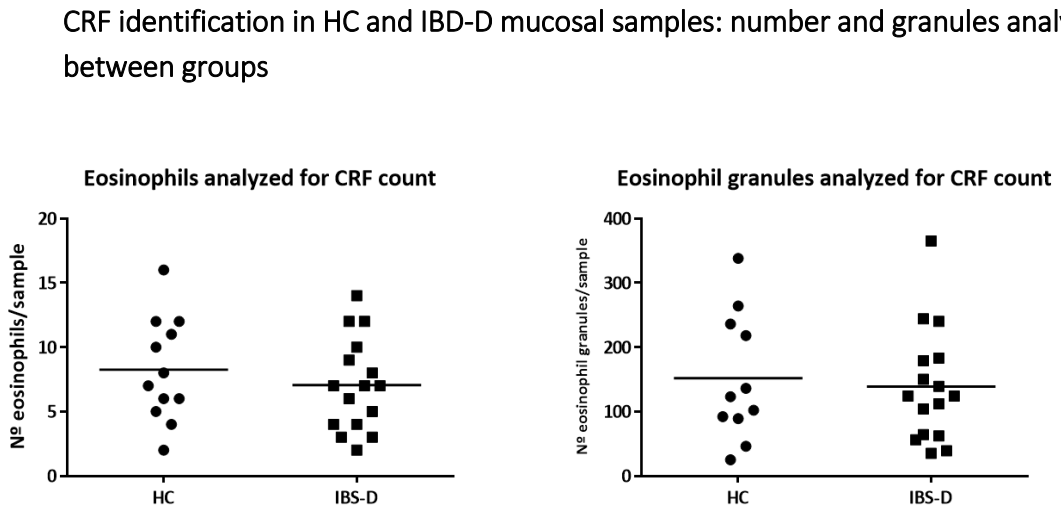


Figure S8. Number of eosinophils and granules analyzed in HC and IBS-D patients for CRF counting

ANEXO 2: SUPPLEMENTARY MATERIAL FROM CHAPTER 2

CRF protein expression validation in eosinophil-like cells by FACS

CRF expression in eosinophils has been identified in mice but has not been determined in human eosinophils yet. Therefore, to evaluate CRF expression a validation assay was performed with 3 different antibodies in 15HL-60 cells (table S1). Two of the antibodies were additionally tested in rat brain homogenates and in HEK cells with and without transfection of CRF (supplementary data chapter 1).

Moreover, to confirm the results obtained with the FCA protocol described in methods (Cytoperm kit, BD Biosciences), one additional protocol with two different permeabilization reagents were compared together with the isotype control (only in undifferentiated 15HL-60 cell line) with the same dilution as the primary antibody.

The alternative protocol used is as follows: $2x10^5$ cells were collected as previously described and fixed in 4% paraformaldehyde for 10 minutes at 4ºC. After two washes with PBS, cells were permeabilized in buffer (PBS + 0.2% bovine serum albumin (BSA) + 0.05% sodium azide) with 0.1% saponin or 0,1% triton for 10 minutes at room temperature. After centrifugation and washes, cells were incubated in 1mL blocking buffer (PBS+ 5% FBS + 2% BSA) for 1 hour at room temperature. Cells were centrifuged and washed and incubated overnight with CRF or isotype control antibody in blocking buffer at 4ºC. After two washes, cells were incubated with the appropriate secondary antibody diluted in PBS for 30 minutes at 4ºC, washed and resuspended in PBS to a final concentration of $1x10^6$cels/mL and analyzed by FACS.

The analysis of CRF labelling showed CRF positive label (red) separated from negative control (black), following a similar profile with the 3 different antibodies tested (figure S1).

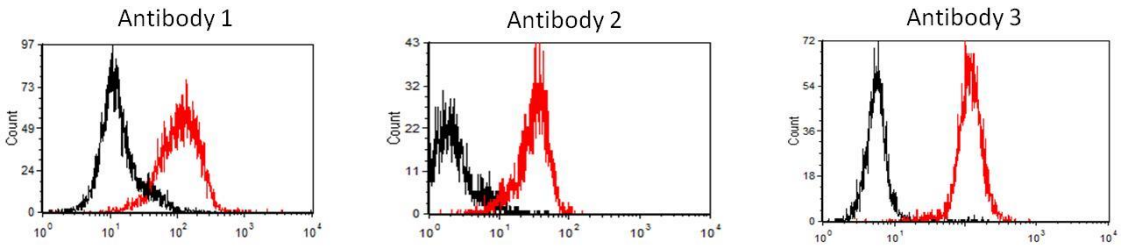


Figure S1. CRF protein expression validation with 3 antibodies in 15HL-60 cells. FACS histograms showing CRF expression (MFI; x-axis) in undifferentiated 15HL-60 cells with 3 different CRF-specific antibodies (red curve) or with secondary antibody only (black curve).

	Antibody	Dilution	Clone	Trading house
Antibody 1	Mouse anti-human CRF	1/50	5D5	AbD serotec
Antibody 2	Goat anti-human CRF	1/50	C-20	Sta Cruz Biotechnology
Antibody 3	Mouse anti-human CRF	1/50	2B11	Sta Cruz Biotechnology
Goat IgG isotype control		1/1250		Abcam

Table S1. Human anti-CRF antibodies used for CRF validation

The analysis of CRF signal (red), compared to negative and isotype control, between 3 different protocols of permeabilization and labeling showed separation of CRF histogram in all protocols, although it seems that with saponin there is less signal, indicating less permeabilization.

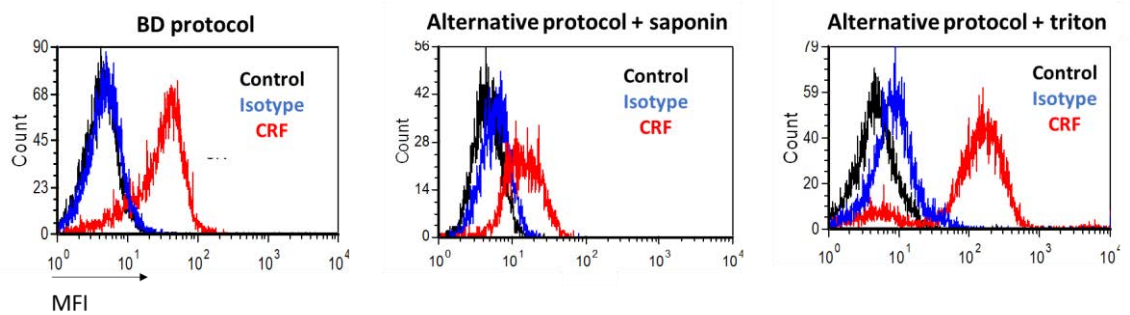


Figure S2. CRF and isotype control FACS histogram, with 3 different FACS protocols in 15HL-60 cells. FACS histograms showing CRF expression (MFI; x-axis) analyzed with specific goat anti-human CRF antibody (red curve) or isotype control (blue curve) or without primary antibody (black curve) comparing 3 different FACS protocols (BD protocol, alternative protocol permeabilized with saponin or alternative protocol permeabilized with triton).

Selection of treatment concentrations

The concentration of SP used to stimulate cells was selected based on the literature, where at 10^{-6}M a greater effect in the production and release of CRF in eosinophils[1] and in degranulation in mast cell line[2] were observed. CCh concentration was selected after testing three different concentrations (10^{-2}M, 10^{-4}M, 10^{-6}M) on AML14.3D10 cells and analyzing the gene expression of eosinophil-related proteins and ACh receptors (data not shown). SP receptor antagonist NK1R was CP-96345 (Sigma) was used at 10^{-6}M, a concentration selected based on previous literature[1]. The Ach muscarinic receptors antagonist was atropine sulfate (Sigma) used at 10^{-6}M, also based on the literature.[3]

The time points for the time-course analysis (30 min, 90min, 180min and 24h) were selected according to the literature, as follows. Previous studies described a CRF peak release between 30 min and 4h after SP stimulation of EoL-1 eosinophil cell line.[1] In another study, CRF was quantified in culture media of 15HL-60 eosinophil cell line 6 and 24h after stimulation with CCh.[3] Also, RANTES release, another eosinophil cytokine, was detected between 5 and 240 minutes, with a maximum at 60 minutes. EPO and β-hexosaminidase release at maximum concentration was detected between 60 and 120 minutes[4]. Based on these previous observations, 30 min and 24h were selected for the present study.

Analysis of activation and CRF location and content after SP, CCh and LPS stimulation in AML14.3D10 and 15HL-60 Bi cells

Apoptosis assay

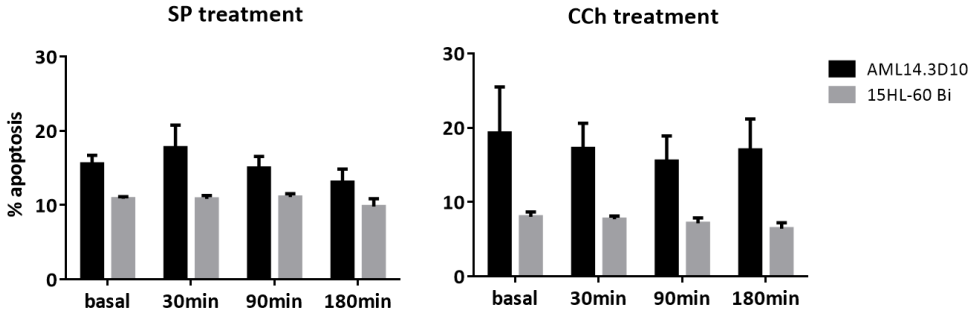
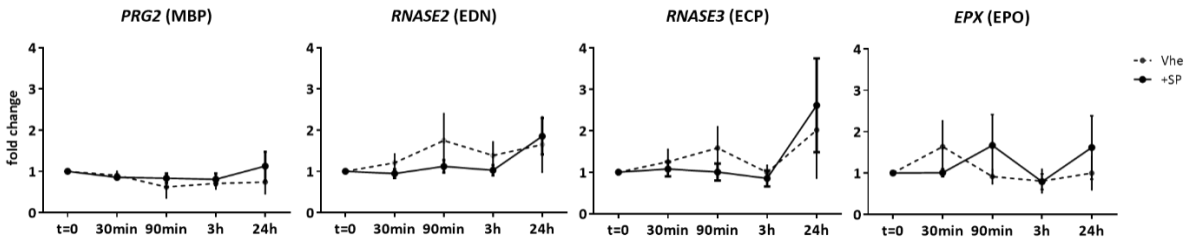


Figure S3. Apoptosis evaluation after stimulation with SP and CCh the AML14.3D10 and 15HL-60 Bi cell lines. Representative graphs of FACS analysis of annexin V (FITC) and propidium iodide, showing the percentage of cells in different quadrant established with negative control. Data are presented as mean ± standard error. Graphs represents results from 2 independent experiments. SP: substance P; CCh: carbachol; 15 HL-60 Bi: 15HL-60 cells differentiated with butyric acid

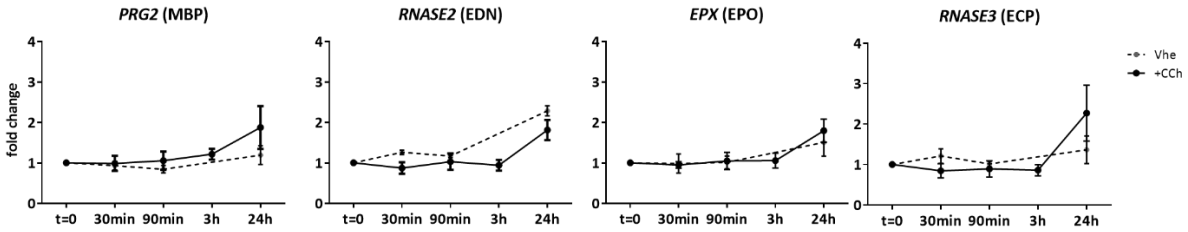
Gene expression assay of eosinophil cationic proteins in AML14.3D10 and 15HL-60 Bi cells after stimulation

AML14.3D10 CELL LINE

SP treatment



CCh treatment



LPS treatment

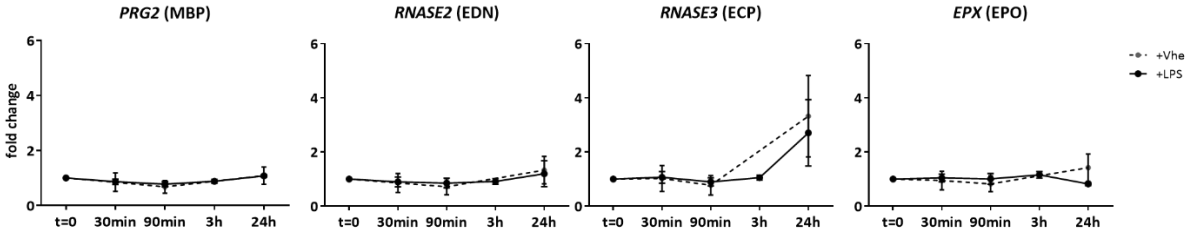
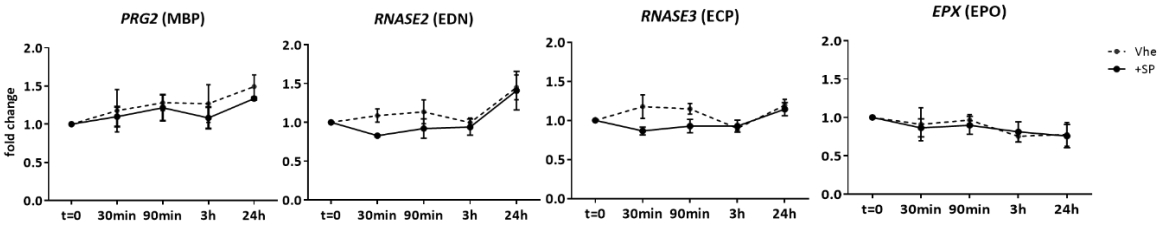


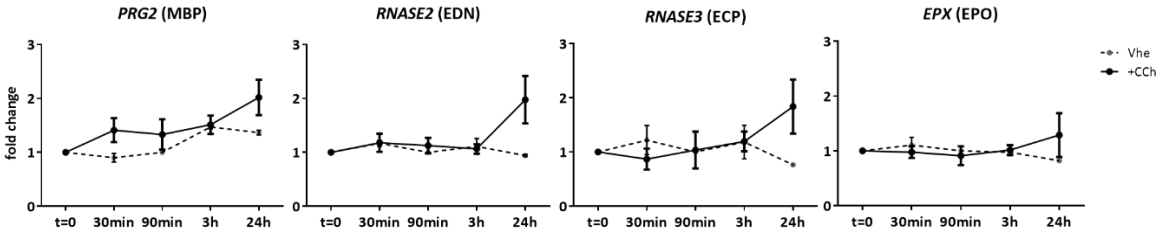
Figure S4. Time-course analysis of gene expression of eosinophil cationic proteins after stimulation with SP, CCh and LPS in AML14.3D10 cells. Cells were treated with SP, CCh or LPS and vehicle control on the indicated time points and recollected, and gene expression analyzed by qPCR. Fold-change was calculated by normalizing the data with the endogenous control gene (*PPIA*) and comparing each time point to untreated cells (t=0). Graphs represent results from 3 independent experiments. Data are expressed as mean ± standard error. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparison post-hoc test * *P*<0.05 *** *P*<0.001 *vs.* vehicle. CCh: carbachol; LPS: lipopolysaccharide; SP: substance P;Vhe: vehicle; *PRG2:* Proteoglycan 2; *RNASE2:* ribonuclease A family member 2; *RNASE3*: ribonuclease A family member 3: *EPX*: eosinophil peroxidase

15HL-60 Bi CELL LINE

SP treatment:



CCh treatment



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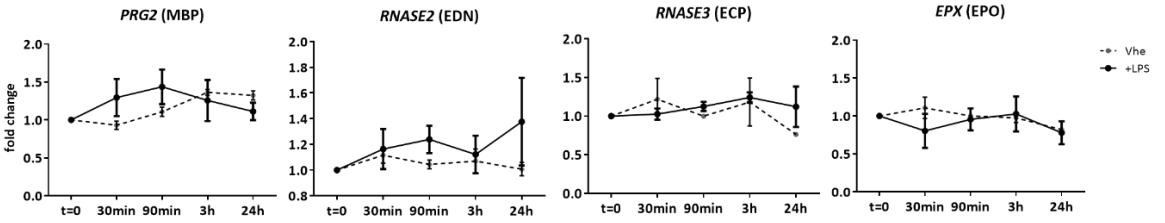
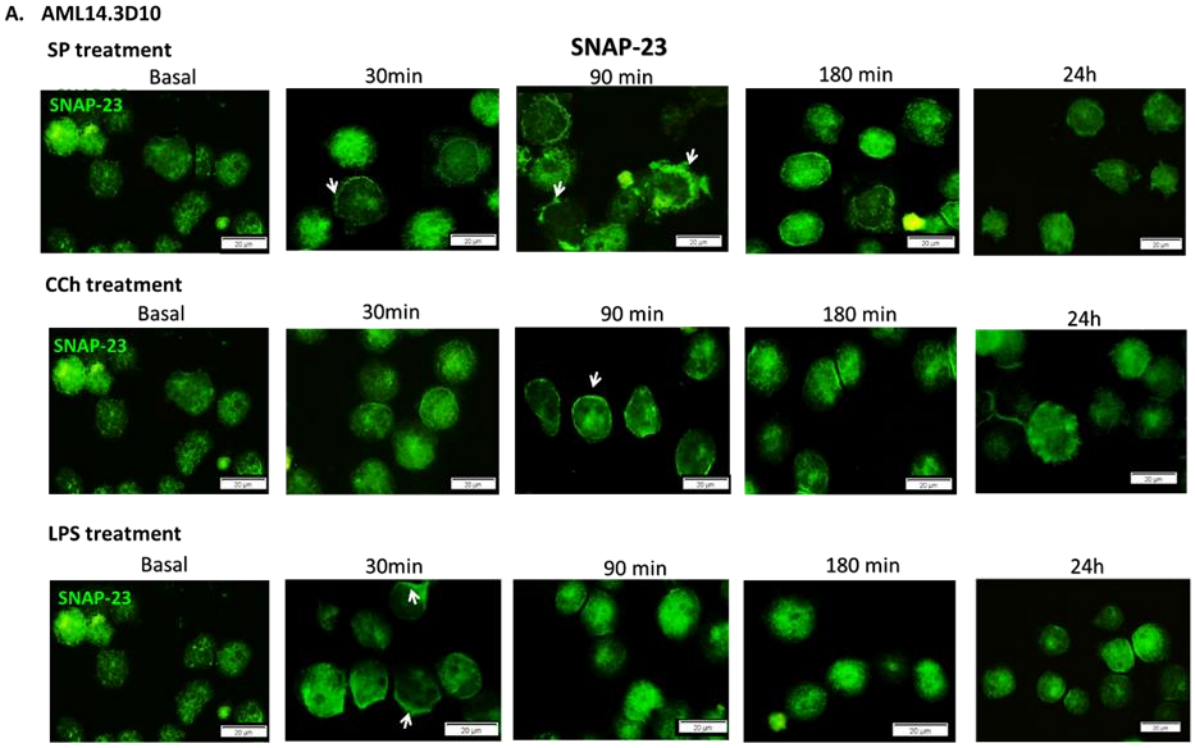


Figure S5. Time-course analysis of gene expression of eosinophil cationic proteins after stimulation with SP, CCh and LPS in 15HL-60 Bi cells. Cells were treated with SP, CCh or LPS and vehicle control on the indicated time points and recollected, and gene expression analyzed by qPCR. Fold-change was calculated by normalizing the data with the endogenous control gene (*PPIA*) and comparing each time point to untreated cells (t=0). Graphs represent results from 3 independent experiments. Data are expressed as mean ± standard error. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparison post-hoc test * *P*<0.05 *** *P*<0.001 *vs.* vehicle. CCh: carbachol; LPS: lipopolysaccharide; SP: substance P;Vhe: vehicle; *PRG2:* Proteoglycan 2; *RNASE2:* ribonuclease A family member 2; *RNASE3*: ribonuclease A family member 3: *EPX*: eosinophil peroxidase

AML14.3D10 and 15HL-60 Bi cells secretory activity after SP, CCh and LPS stimulation

AML14.3D10 cell line



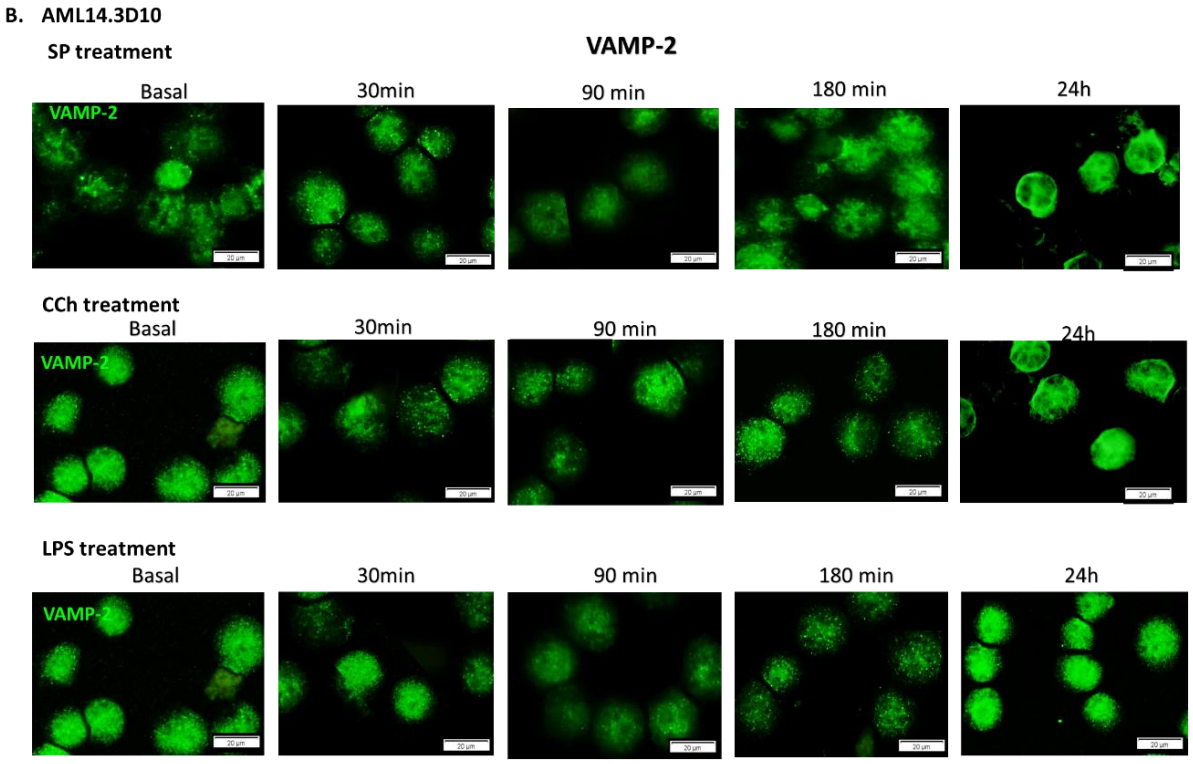


Figure S6. Secretory activity of AML14.3D10 cells after stimulation with SP, CCh and LPS. Representative images of a time course (basal, 30, 90, 180 minutes and 24h) of **A**: SNAP-23 and **B.** VAMP-2 protein location evaluated by IF after SP; CCh, LPS and vehicle of SP and CCh/LPS stimulation. Negative control (only secondary antibody) was used to determine the threshold for setting the positive signal. White arrows showed relocation of SNAP-23 and VAMP-2 from cytoplasm to plasma membrane of cells after stimulation. Images are representative of 3 independent experiments. Bars indicate 10 µm. CCh: carbachol; LPS: lipopolysaccharide; SP: substance P; SNAP23: Synaptosomal-associated protein 23; VAMP-2: Vesicle-associated membrane protein 2.

15HL60 Bi cell line

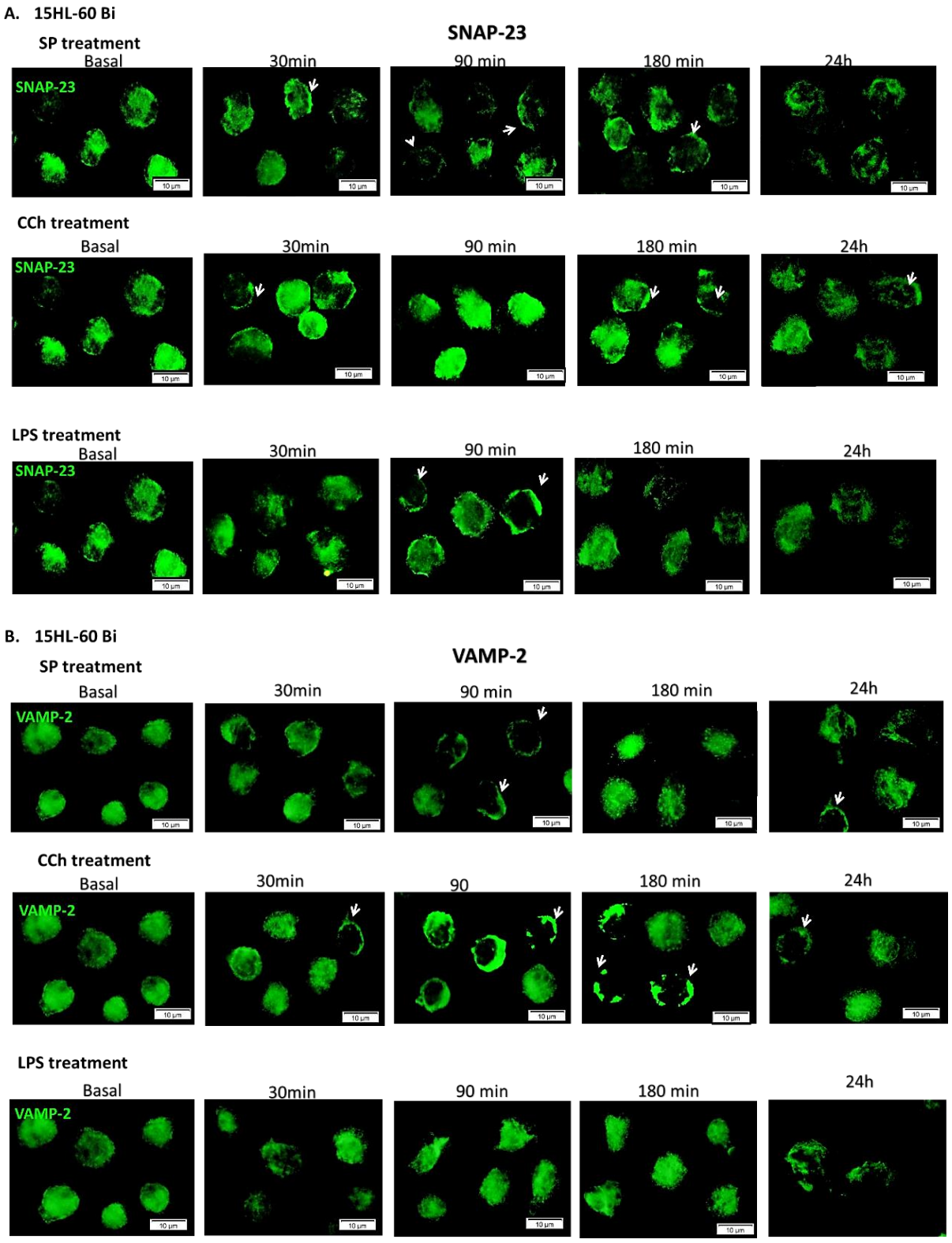
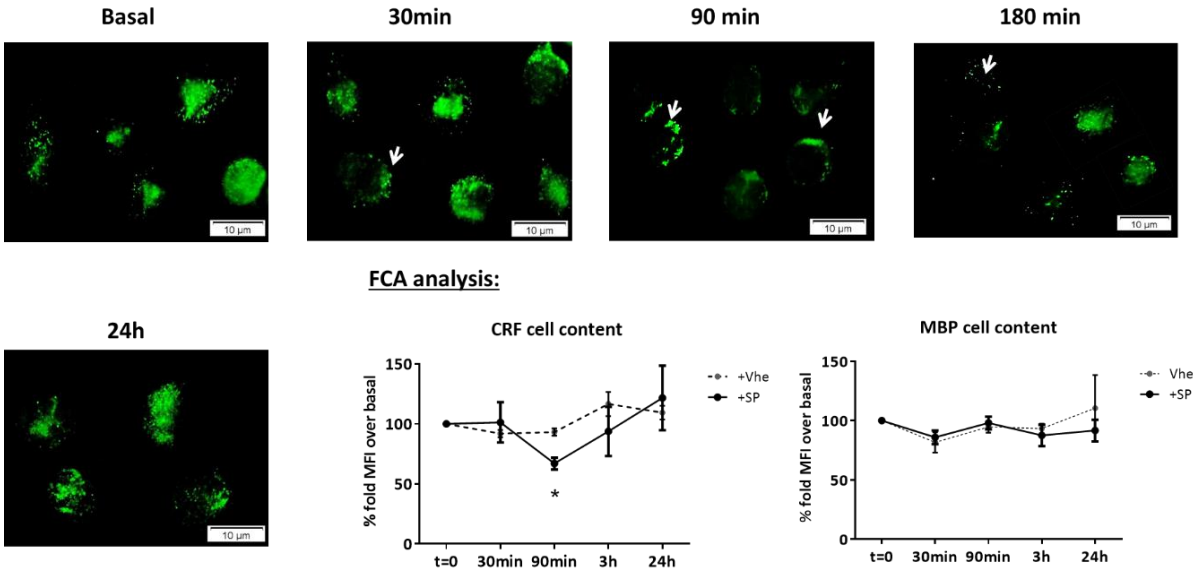


Figure S7. Secretory activity of 15HL-60 Bi cells after stimulation with SP, CCh and LPS. Representative images of a time course (basal, 30, 90, 180 minutes and 24h) of **A**: SNAP-23 and **B.** VAMP-2 protein location evaluated by IF after SP; CCh, LPS and vehicle of SP and CCh/LPS stimulation. Negative control (only secondary antibody) was used to determine the threshold for setting the positive signal. White arrows showed relocation of SNAP-23 and VAMP-2 from cytoplasm to plasma membrane of cells after stimulation. Images are representative of 3

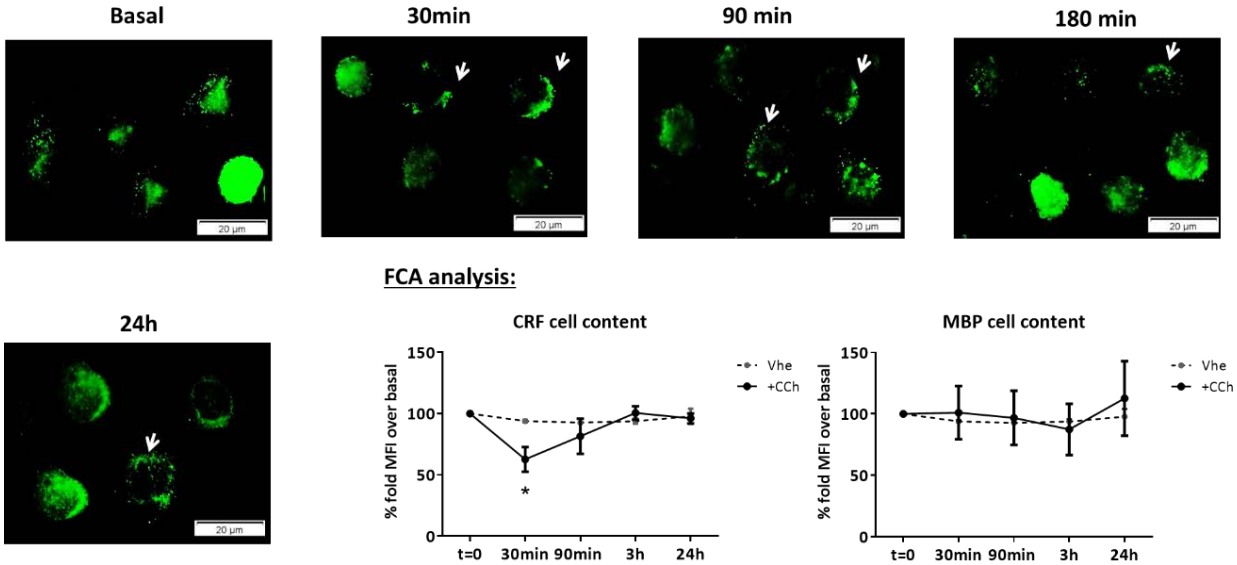
independent experiments. Bars indicate 10 µm. CCh: carbachol; LPS: lipopolysaccharide; SP: substance P; SNAP23: Synaptosomal-associated protein 23; VAMP-2: Vesicle-associated membrane protein 2.

CRF location and content in 15HL-60 Bi cells after stimulation

SP treatment



CCh treatment:



LPS treatment

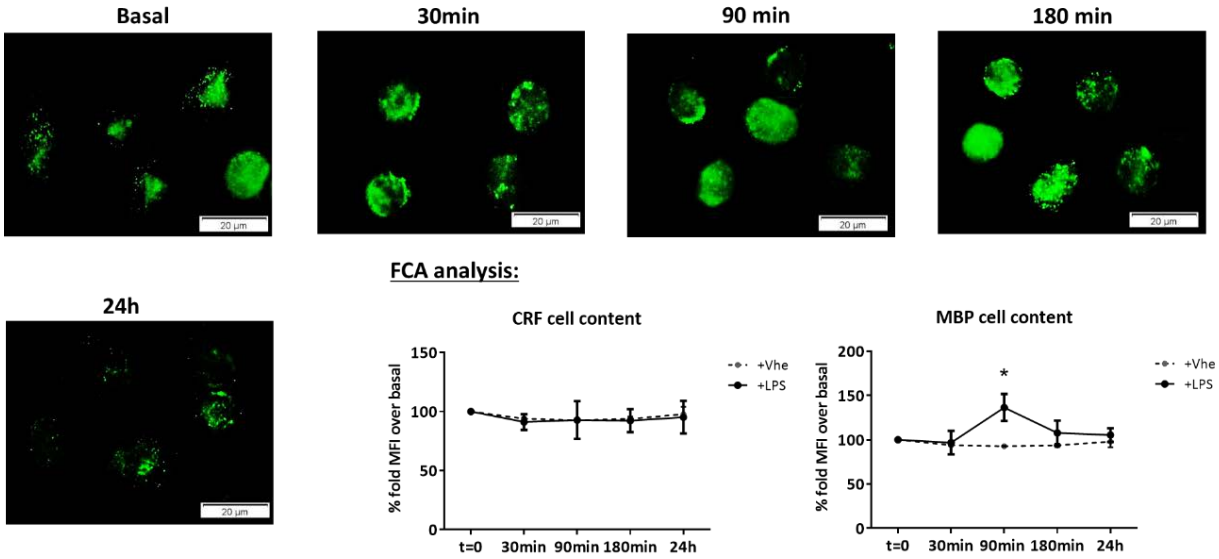


Figure S8. CRF location and content after stimulation in 15HL-60 Bi cells. Representative images of a time-course analysis of CRF protein localization evaluated by IF, and CRF and MBP total protein content evaluated by FCA after **A.** SP stimulation; **B.** CCh stimulation and. **C.** LPS stimulation and their respective vehicles. Negative control (only secondary antibody) was used to determine the threshold for setting the positive signal. White arrows in IF images indicate relocation of CRF from the cytoplasm to the plasma membrane. Images are representative of 3 independent experiments. Bars indicate 10µm. FCA data are expressed as % of MFI change over basal condition of the same experiment. Data are expressed as mean ± standard error. N=8 for SP and CCh in CRF evaluation. N= 4 for LPS for CRF evaluation. N=4 for MBP evaluation. Statistical analysis was performed by two-way ANOVA followed by Bonferroni multiple comparison post-hoc test * $P<0.05$ *vs*. vehicle. MFI: mean fluorescence intensity; SP: substance P; CCh: carbachol; LPS: lipopolysaccharide; Vhe: vehicle; CRF: corticotropin releasing factor; MBP: major basic protein; FCA: flow cytometry analysis.

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