Tesis Doctoral

EFECTO DE NEUROPÉPTIDOS EN EL RESTABLECIMIENTO DE LA HOMEOSTASIS EN INFLAMACIÓN Y AUTOINMUNIDAD

Memoria presentada por el licenciado Alejo Chorny para optar al grado de Doctor por la Universidad de Granada.

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LISTA DE ABREVIATURAS

AM	adrenomedulina
AMPc	adenosín monofosfato cíclico
APC	antigen presenting cell (célula presentadora de antígeno)
AR	artritis reumatoide
BBB	blood brain barrier (barrera hematoencefálica)
BCR	B cell receptor (receptor de linfocito B)
CIA	collagen induced arthritis (artritis inducida por colágeno)
CLP	cecal ligation and puncture (ligación cecal y punción)
CNS	central nervous system (sistema nervioso central)
CST	cortistatina
DC	cendritic cell (célula dendrítica)
DCvip	célula dendrítica diferenciada con VIP
DOM	disfunción orgánica múltiple
DTH	delayed type hypersensitivity (reacción de hipersensibilidad retardada)
EAE	encefalomielitis autoinmune experimental
GHR	grelina
HMGB1	high mobility group box-1
LPS	lipopolisacárido
EM	esclerosis múltiple
NO	nitric oxide (oxido nítrico)
PAMP	pathogen-associated molecular pattern (patrón molecular asociado a patógeno)
PKA	proteina kinasa A
PRR	pattern-recognition receptor (receptor de reconocimiento de patrones)

RNS	especie reactiva del nitrógeno
ROS	especie reactiva del oxígeno
SAA	Serum amyloid A (A amiloide sérica)
TCR	T cell receptor (receptor de linfocito T)
Th	T helper
TLR	toll like receptor (receptor de tipo toll)
Treg	regulatory T cell (célula T reguladora)
UCN	urocortina
VIP	vasoactive intestinal peptide (peptido intestinal vasoactivo)

INTRODUCCIÓN

1. SISTEMA INMUNE. CARACTERÍSTICAS GENERALES

Todos los seres vivos, desde las bacterias hasta los humanos, han desarrollado estrategias para combatir infecciones parasitarias (1). Mientras que la gran mayoría de las especies animales se enfrenta a los ataques microbianos basándose exclusivamente en el sistema inmune innato, los mamíferos han desarrollado como segunda línea de defensa el sistema inmune adaptativo (2). Así, el sistema inmune de los mamíferos consiste en dos brazos interrelacionados, el evolutivamente más antiguo sistema inmune innato y el altamente específico sistema inmune adaptativo. La combinación entre la inmunidad innata y adaptativa permite al sistema inmune de los mamíferos reconocer y eliminar patógenos invasores con máxima eficacia y mínimo daño al propio organismo, al mismo tiempo que provee protección contra re-infecciones por el mismo patógeno (2). El sistema inmune innato actúa como una primera línea de defensa reconociendo conjuntos de estructuras moleculares específicas de los microbios y que se encuentran altamente conservados (*pathogen-associated molecular patterns* o PAMPs). Este reconocimiento lo realiza a través de un limitado grupo de receptores codificados en la línea germinal denominados PRR (pattern-recognition receptor) (3). Además, el sistema inmune innato es capaz de detectar la presencia de daño tisular mediante el reconocimiento de moléculas o células que normalmente se encuentran compartimentalizadas en células o tejidos intactos, pero que son liberadas después de producirse una lesión (4).

El sistema inmune adaptativo utiliza un conjunto diverso de receptores reordenados somáticamente (receptores de células T (TCR) y receptores de células B (BCR)), que poseen la capacidad de reconocer un amplio espectro de antígenos. Sin embargo, aún cuando los linfocitos T y B expresan receptores con una diversidad enorme, la activación de estas células depende de señales derivadas del sistema inmune innato (5).

1.1 Inflamación

La supervivencia resulta imposible sin una vigilante defensa contra ataques y daños. El sistema inmune innato patrulla continuamente el cuerpo en busca de invasores y cuando encuentra un ataque, involuntariamente pone en marcha una respuesta inflamatoria discreta y localizada con el fin de desbaratar las amenazas patogénicas (6).

A un nivel básico, la respuesta inflamatoria aguda provocada por infección o daño tisular involucra el reclutamiento de componentes sanguíneos como plasma y leucocitos al sitio de

infección o daño. En el caso de las infecciones, la respuesta es desencadenada por PRR del sistema inmune innato, tales como los Toll-like receptors (TLR). Este reconocimiento inicial de la infección es mediado por macrófagos y mastocitos residentes en los tejidos, originando la producción de una batería de mediadores inflamatorios que incluye citoquinas, quimioquinas, aminas vasoactivas, eicosanoides y productos de cascadas proteolíticas (7). El principal y más inmediato efecto de estos mediadores es la obtención de un exudado inflamatorio local: proteínas plasmáticas y leucocitos (principalmente neutrófilos) que están normalmente restringidos a los vasos sanguíneos ahora ganan acceso al tejido extravascular en el sitio de infección (7). El endotelio activado de los vasos sanguíneos permite la extravasación de los neutrófilos, los cuales se activan una vez que alcanzan el tejido afectado, ya sea por contacto directo con los patógenos o bien a través de la acción de las citoquinas secretadas por las células residentes en los tejidos. Los neutrófilos activados intentan matar los agentes invasores liberando los contenidos tóxicos de sus gránulos, los cuales incluyen especies reactivas de oxígeno (ROS) y de nitrógeno (RNS), proteinasa 3, catepsina G y elastasa (8). Estos potentes efectores no discriminan entre blancos microbianos y huésped, por lo que el daño colateral a los tejidos del huésped es inevitable (6).

Las citoquinas TNFα e IL-1β, dos de los principales mediadores de la respuesta inflamatoria, combinados con otros mediadores como IFNγ, son capaces de exacerbar el daño en las células endoteliales disparando la muerte celular de las mismas por apoptosis. A su vez, el daño y la muerte de las células endoteliales favorece el proceso de trombosis. De esta forma, las células endoteliales apoptóticas pierden su capacidad anticoagulante y adquieren funciones pro-coagulantes (9). TNFα e IL-1β también inducen la síntesis del factor tisular, el principal iniciador de la coagulación. Si bien este factor se encuentra normalmente secuestrado dentro de las células endoteliales y es incapaz de iniciar la cascada de coagulación, en las células apoptóticas el factor tisular es expuesto a la sangre, iniciando de esta forma la cascada de coagulación. La formación de trombos, los cuales son un agregado a las plaquetas y fibrina intravascular, es un componente importante de la inflamación aguda, ya que supone una barrera física en los tejidos infectados al limitar la diseminación de los microbios (9).

Una respuesta inflamatoria aguda exitosa resulta en la eliminación de los agentes infecciosos seguida por una fase de resolución y reparación, la cual es mediada principalmente por macrófagos reclutados y residentes (10). Si la respuesta inflamatoria aguda falla en eliminar al patógeno, el proceso inflamatorio persiste, adquiere nuevas características y el infiltrado de neutrófilos es reemplazado por macrófagos y células T. Si aún los efectos combinados de estas células resultan insuficientes, se desarrolla un estado de inflamación crónico (7).

Si bien la inflamación preserva la vida, como puede inferirse a partir del alto riesgo de contraer infecciones graves en personas que poseen alguna deficiencia genética en alguno de los principales componentes de proceso inflamatorio (11, 12), el control de la magnitud de la respuesta inflamatoria es crucial. Respuestas insuficientes resultan en inmunodeficiencias, las cuales a su vez pueden derivar en infecciones y cáncer; por otro lado, respuestas excesivas pueden causar morbilidad y mortalidad en enfermedades como artritis reumatoide, enfermedad de Crohn, ateroesclerosis, diabetes, Alzheimer, esclerosis múltiple e isquemia cerebral y de miocardio (13). Es más, si la inflamación se expande al torrente sanguíneo, como ocurre en los síndromes de sepsis y shock séptico, las consecuencias pueden ser catastróficas, resultando la respuesta inflamatoria más peligrosa aún que el estímulo inicial (13).

1.2 Sepsis y shock séptico.

La sepsis está actualmente considerada como una compleja desregulación de la inflamación que surge cuando el huésped es incapaz de contener exitosamente una infección. Esta desregulación del proceso inflamatorio afecta en última instancia múltiples órganos mediante efectos en el endotelio, epitelio y células inmunes, originando daños irreversibles (14). La definición de sepsis ha estado siempre basada en características clínicas de la enfermedad y hasta el presente, no existe un panel de biomarcadores que puedan ser usados para definir la sepsis (15). Así, la sepsis está definida por signos de una respuesta inflamatoria sistémica a una infección. La constelación de signos y síntomas que se utilizan para diagnosticar la sepsis incluyen anormalidades en la temperatura corporal, ritmo cardíaco, ritmo respiratorio y número de leucocitos en sangre. La sepsis se define como severa cuando estos signos se encuentran asociados con signos de disfunción de órganos, tales como hipoxemia, oliguria, acidosis láctica, altos niveles de enzimas hepáticas, o función cerebral alterada. Shock séptico, a su vez, describe una sepsis severa junto con hipotensión a pesar de resucitación mediante fluido (15). La tasa de mortalidad en humanos con sepsis, varía entre el 30% y 70%, dependiendo de la calidad de cuidados médicos (16). A medida que la sepsis progresa en shock séptico, el riesgo de muerte incrementa substancialmente. Mientras que una sepsis temprana es habitualmente reversible, los pacientes que padecen shock séptico, sucumben a pesar de terapias hospitalarias agresivas (14). El desarrollo del shock séptico puede ocurrir de manera abrupta, manifestándose con coagulación intravascular diseminada, necrosis extendida en órganos críticos, y muerte dentro las 24-48hs. La tasa de mortalidad del shock séptico puede alcanzar el 80%, aún cuando se trate de

pacientes admitidos en cuidados intensivos, se los trate con antibióticos y se les provea los mejores cuidados paliativos disponibles (14, 17).

1.2.1. Inicio

Como se mencionó anteriormente, las células del sistema inmune reconocen la presencia de daño tisular o infecciones microbianas a través de los PRR, provocando una rápida respuesta de defensa. Durante la sepsis, ocurre una excesiva estimulación de las células inmunes debido a las grandes cantidades de inductores endógenos y exógenos de la inflamación que se liberan tanto de los microorganismos invasores como de los tejidos dañados, originando, de esta forma, una activación sistémica de la respuesta inflamatoria. Como resultado, la sepsis es acompañada por una desregulada respuesta de citoquinas (denominada *"cytokine storm"*), convirtiendo respuestas que normalmente son beneficiosas en la lucha contra las infecciones en una inflamación excesiva y dañina (18).

El reconocimiento del lipopolisacárido (LPS) mediante el TLR4 se cree que es un importante desencadenante de la respuesta inflamatoria durante la sepsis (18). En las bacterias Gram-negativas el LPS posee un papel dominante respecto al reconocimiento y activación del sistema inmune. En esta interacción inicial entre el huésped y los microbios, los macrófagos juegan un papel esencial, liberando no sólo las clásicas citoquinas proinflamatorias IL-1β, IL-6 y TNFα, sino también otras como IL-12, IL-15 e IL-18 (14). En particular, TNFα e IL-1 β son unos de los principales mediadores de las características inmunopatológicas en el shock inducido por LPS (19). Estas citoquinas son liberadas en las primeras horas desde la estimulación con LPS y subsecuentemente activan un segundo nivel de cascadas proinflamatorias que incluyen citoquinas, quimioquinas, mediadores lipídicos, ROS y RNS, al mismo tiempo que incrementan la expresión de las moléculas de adhesión, iniciando así la migración de células inflamatorias a los tejidos (14). La producción sostenida de estos mediadores junto con su entrada a circulación sistémica provoca una progresiva disfunción endotelial, lesiones microvasculares y activación de la cascada de coagulación (18). Así, las etapas tempranas de la sepsis derivan en una desregulación severa de varios sistemas del organismo como resultado de un ambiente proinflamatorio.

1.2.2. Mecanismos de muerte

La causa última de la muerte en pacientes con sepsis es una disfunción orgánica múltiple (DOM). Si bien la patogénesis de DOM es multifactorial y no se comprende completamente, se sabe que la deposición de fibrina que causa oclusiones microvasculares, los exudados tisulares que comprometen la adecuada oxigenación y los desórdenes de la homeostasis microvascular traen como consecuencia la hipoperfusión e hipoxia de los tejidos, resultando en factores dominantes en la desarrollo de DOM (14). Además, mientras que los infiltrados celulares, principalmente neutrófilos, dañan los tejidos directamente, citoquinas como TNFα incrementan la producción de óxido nítrico (NO), aumentando la inestabilidad vascular y contribuyendo a la depresión miocárdica típica de la sepsis (20). Llamativamente, autopsias que se llevaron a cabo en pacientes que murieron de sepsis no muestran correlación entre los análisis histológicos y el grado de disfunción en los órganos. La escasa muerte celular observada en órganos como el corazón, hígado, pulmones y riñones no refleja las evidencias clínicas de un fallo severo en el funcionamiento de estos órganos (21). Por lo tanto, se ha propuesto que la DOM es consecuencia de un proceso denominado "hibernación celular" (21, 22). Dado que durante la sepsis los tejidos se encuentran hipoperfundidos, existe una reducción en la disponibilidad energética que parece provocar, como mecanismo de adaptación, la reducción de los procesos metabólicos celulares, previniendo de esta forma una muerte celular masiva, pero desencadenando la disfunción del órgano si finalmente no se regula.

1.2.3. Modelos animales

Teniendo en cuenta la variabilidad y dificultades para clasificar la sepsis humana, se han establecido distintos modelos animales con el fin de abordar un estudio más reproducible de la patofisiología de la sepsis (23, 24). De hecho, estos modelos han servido como base preliminar para ensayar agentes terapéuticos antes de los ensayos clínicos humanos (25, 26) y han contribuido al entendimiento de los mecanismos de defensa durante infecciones (27). Considerando el agente iniciador, los modelos de sepsis pueden ser divididos en tres categorías: *(1)* administración exógena de una toxina (como por ejemplo LPS o zimosan), *(2)* administración exógena de un patógeno viable (como una bacteria), y *(3)* alteración de barreras protectoras endógenas del animal.

(1) Administración de LPS

Dada la alta frecuencia de infecciones Gram-negativas en los pacientes con sepsis (16), se asumió que grandes cantidades de LPS deberían estar presentes, por lo que se establecieron modelos animales de sepsis en los cuales se administran altas dosis de esta endotoxina bacteriana. La administración de LPS, ya sea i.v. o i.p, causa signos similares a la sepsis, tales como alteraciones hematológicas e incremento en los niveles de citoquinas proinflamatorias en suero (27). En este modelo, TNFα e IL-1β han emergido como mediadores cruciales en el desarrollo de las respuestas in vivo al LPS, ya que anticuerpos que bloquean la unión a sus respectivos receptores aumentan la supervivencia de ratones con endotoxemia, y la inyección de TNFα o IL-1β es capaz de recapitular muchas de las características causadas por el LPS o la misma sepsis. De hecho, estas dos citoquinas se convirtieron en blancos de intervenciones terapéuticas en la sepsis humana (27).

Sin embargo, el modelo experimental de LPS y la sepsis humana difieren en varios puntos importantes, especialmente en los niveles sistémicos de citoquinas y en la cinética de secreción de las mismas. Después de la administración de LPS, los niveles de TNFα, por ejemplo, alcanzan su pico mucho antes y a niveles superiores comparado con la sepsis humana. De hecho, tanto en humanos como en otros modelos de sepsis, el bloqueo de TNFα con anticuerpos no mejoró la supervivencia o incluso la disminuyó (28). Estos resultados sugirieron que el modelo de LPS podría no reflejar fielmente la sepsis humana. Actualmente, existe un acuerdo general entre los investigadores que considera que la inyección de LPS representa un modelo de shock séptico o inflamación sistémica, pero no de sepsis (26).

(2) Administración de bacterias vivas

La inoculación de animales con bacterias ha sido una herramienta común para estudiar los mecanismos de la sepsis. No obstante, las altas dosis de bacterias comúnmente administradas habitualmente no colonizan ni se replican en el huésped, debido a una rápida lisis mediada por el complemento (29). Esto sugiere que la inyección de bacterias puras, al igual que la administración de LPS, representa un modelo de shock por endotoxinas y no un verdadero modelo de infección (30).

(3) Ligación cecal y punción

El modelo de ligación cecal y punción (cecal ligation and puncture-CLP-) es considerado uno de los modelos de excelencia en la investigación en sepsis. Este modelo mimetiza la enfermedad humana de peritonitis causada por apendicitis o diverticulitis perforante. La técnica consiste en una apertura quirúrgica de la cavidad abdominal, exteriorización y ligación del ciego, y punción de la porción ligada del ciego. Este proceso crea una perforación intestinal con escape del contenido fecal al peritoneo, lo cual establece una infección polimicrobiana y provee una fuente inflamatoria de tejido necrótico. El modelo de CLP recrea no sólo la respuesta inmune del huésped, sino también las fases hemodinámicas y metabólicas de las sepsis humana (27).

No obstante, cabe recalcar que no existe un único modelo ideal de sepsis ni shock séptico. Ambas enfermedades son síndromes complejos que involucran distintos sistemas de órganos y la evaluación de nuevas estrategias terapéuticas precisa del uso de un panel de distintos modelos, cada uno utilizado con el fin de contestar distintos interrogantes (30).

1.2.4. Tratamiento

Actualmente, las terapias disponibles para la sepsis se limitan ala siguientes intervenciones clínicas: *(1)* antibióticos de amplio espectro con el fin de erradicar la infección (31), *(2)* drogas esteroideas antiinflamatorias (32), *(3)* proteína C activada (33), *(4)* terapias dirigidas a restablecer los niveles de oxígeno en los tejidos (34), *(5)* terapia intensiva de insulina para normalizar la glucemia (35). Sin embargo, estas terapias no sólo mostraron un éxito limitado, sino que además existen importantes contraindicaciones que rodean la administración de dichas terapias en el manejo de la sepsis (36). Por lo tanto, el desarrollo de nuevos enfoques terapéuticos efectivos para la sepsis resulta una prioridad emergente en el campo de la medicina.

1.2.5. Una nueva diana terapéutica: High Mobility Group Box-1

La búsqueda de mediadores de la sepsis capaces de servir como blancos de terapias contra la enfermedad condujo a la identificación de la proteína high mobility group box-1 (HMGB1) como una citoquina inflamatoria de cinética tardía que contribuye a la progresión de la patología de la sepsis y otros desórdenes inflamatorios (37). HMGB1 fue originalmente identificada como una proteína de unión al DNA que funciona como cofactor estructural crítico en la regulación de la transcripción (38). Sin embargo, estudios posteriores mostraron que HMGB1 puede ser liberada al medio extracelular por células inmunes activadas, donde funciona

como una citoquina proinflamatoria (37). Originalmente se describió que HMGB1 puede ser liberada por dos mecanismos distintos, o bien pasivamente después de muerte celular por necrosis (y no por apoptosis) (39) o bien secretada activamente por células de la inmunidad innata (40). Una vez en el medio extracelular, es reconocida por receptores del sistema inmune como un marcador de necrosis (41). Si bien niveles extracelulares fisiológicos de HMGB1 juegan un papel importante en la resolución de infecciones o lesiones, niveles anormalmente altos de HMGB1 pueden resultar letales (42). De esta forma, y al igual que otras citoquinas proinflamatorias, cantidades moderadas de HMGB1 inducen una respuesta inmune beneficiosa para confinar la infección, restringir el daño tisular y promover la cicatrización de heridas y regeneración del tejido. Pero cantidades excesivas de HMGB1 extracelular pueden llevar al daño tisular, la disfunción de órganos y la muerte (37, 43).

HMGB1 induce la quimiotaxis y activación de células involucradas en la inflamación, tales como neutrófilos y macrófagos, y aumenta la expresión de moléculas de adhesión en las células endoteliales, lo cual promueve la adhesión, activación y subsecuente extravasación de los leucocitos al tejido (44, 45). Aún cuando estos procesos son críticos para una fisiológica resolución de la inflamación, niveles excesivos de HMGB1 producen trastornos en las funciones de la barrera epitelial, provocando fuga vascular e hipoperfusión de tejidos (44, 45). De hecho, la administración de HMGB1 exógena es capaz de recapitular muchos de los signos asociados a la sepsis; en particular, perturba la función del epitelio intestinal e induce lesiones pulmonares agudas (46, 47). En modelos murinos de endotoxemia HMGB1 aparece en circulación 8h después de la administración de una LD_{50} de LPS, incrementando sus niveles séricos hasta alcanzar un *plateau* entre las 16 y 32h (48). En el caso de modelos de sepsis como CLP, sus niveles circulantes son detectables a partir de las 18h de la inducción de peritonitis y se mantienen elevados al menos por 72 h (49). Esta aparición tardía de HMGB1 circulante, por un lado se correlaciona con la muerte por endotoxemia o sepsis, mientras que por otro, la distingue de los demás mediadores tempranos de las respuestas inflamatorias sistémicas descritos previamente (50). Citoquinas con cinéticas tempranas, como el TNFα, son producidas en los primeros minutos de la estimulación inmune y sus niveles séricos retornan a niveles cercanos a los basales en las primeras horas durante el progreso de la sepsis (51). De hecho, en muchos de los escenarios de enfermedades inflamatorias progresivas como la sepsis, el incremento sistémico transitorio de estas citoquinas tempranas se resuelve incluso antes de que el desorden sea diagnosticado (52). Por lo tanto, la inhibición específica de citoquinas tempranas como el TNFα sólo proporciona una angosta ventana para la intervención clínica, pero que podría ser significativamente ampliada con blancos terapéuticos como HMGB1. En este sentido, se ha

observado que estrategias dirigidas a bloquear la función citoquina de HMGB1, i.e. administración de anticuerpos bloqueantes o antagonistas específicos, son capaces de prevenir la DOM y rescatar animales en modelos experimentales de sepsis severa aún cuando sean aplicadas después de la aparición de la enfermedad (49).

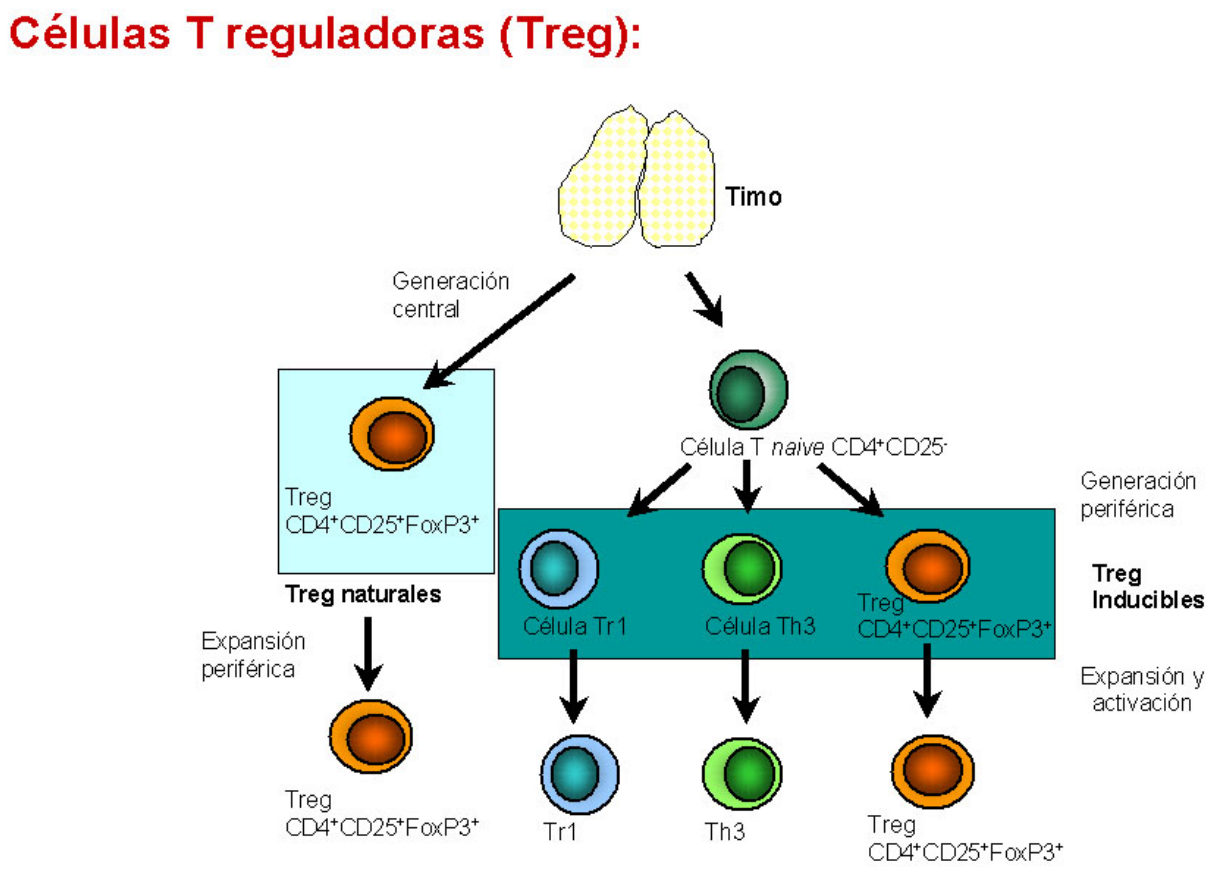
2. TOLERANCIA

El sistema inmune de los mamíferos posee la capacidad de reaccionar contra cualquier estructura química de cualquier organismo a través de los BCR y TCR, al mismo tiempo que evita una equivocada o excesiva respuesta inmune deletérea contra el propio huésped (53). La enorme diversidad de receptores necesaria para poder reconocer tal cantidad de estructuras es generada por modificaciones genómicas somáticas en los órganos linfoides centrales, i.e. en la médula ósea en el caso de los linfocitos B y en el timo en el caso de los linfocitos T. Dado que este proceso en el que se generan los BCR y TCR es deliberadamente azaroso, una fracción de estos receptores es capaz de reconocer componentes del propio organismo (53). Por consiguiente, con el fin de evitar que estos linfocitos autorreactivos desencadenen procesos autoinmunes, los organismos mamíferos han evolucionado distintos mecanismos de tolerancia. Si el receptor generado reacciona contra el propio organismo, la célula que expresa dicho receptor puede, o bien modificarlo de manera que ya no reaccione contra lo propio, o bien puede ser inducida a morir, de la manera en que lo planteó Burnet en su teoría de selección clonal (54). Estos procesos ocurren en los mismos órganos linfoides centrales y se los denomina tolerancia central. No obstante, un alto porcentaje de linfocitos T (y también linfocitos B) escapan a este proceso y son capaces de reconocer tejidos periféricos con una afinidad potencialmente peligrosa. De esta forma, linfocitos T autorreactivos están normalmente presentes en todos los individuos, aunque sólo entre un 3% y un 8% de la población desarrolla enfermedades autoinmunes, indicando que mecanismos de tolerancia periférica juegan un papel fundamental en el completo control de los linfocitos T potencialmente patogénicos (55-57). Los mecanismos de tolerancia periférica incluyen cambios bioquímicos intrínsecos y en la expresión de genes que reducen la habilidad de los linfocitos autorreactivos para responder a la activación de su receptor, lo que se conoce como anergia clonal. Aún cuando estas células autoreactivas puedan evadir los procesos descritos anteriormente, denominados colectivamente como ignorancia inmunológica, existen controles periféricos extrínsecos que pueden limitar el peligro de las células autoreactivas. Estos controles extrínsecos limitan el suplemento de factores de crecimiento

esenciales, coestímulos y mediadores proinflamatorios (53). Por último, cierto tipo de células T limitan la activación y expansión de linfocitos T autorreactivos. A este tipo de células T se las denomina células T reguladoras (Treg) y están especializadas en supresión inmune (58). Las células Treg poseen un papel crítico en la inducción de tolerancia periférica tanto contra antígenos propios como no propios.

2.1. Células Treg

En base a su origen, las células Treg se pueden dividir en dos grupos, las Treg naturales que se originan en el timo y las Treg adaptativas o inducibles que se originan en la periferia. Las Treg naturales son $CD4^+$, expresan constitutivamente el factor de transcripción Foxp3 y la cadena α del receptor de la IL-2 (CD25). Además, son específicas de antígenos propios y actúan predominantemente a través de mecanismos dependientes de contacto (59, 60). El grupo de las células Treg inducibles está compuesto por T reguladoras productoras de IL-10 (Tr1), productoras de TGFβ (Th3) y las inducibles $CD4^+Foxp3^+$ (59, 60).



2.1.1. Células Treg $CD4^+Foxp3^+$ naturales e inducibles

Las Treg naturales expresan el factor de transcripción Foxp3, un regulador maestro del desarrollo y la función de estas células. El gen foxp3 fue identificado como un gen defectivo en la cepa de ratones Scurfy. Esta mutación recesiva está ligada al cromosoma X y resulta letal en machos homozigotos, exhibiendo una hiperactivación de linfocitos T $CD4^+$ y un exceso de producción de citoquinas proinflamatorias (61). Mutaciones en el gen humano foxp3 causan la enfermedad genética IPEX (*immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome*), que es el equivalente humano de Scurfy (62). La transducción de linfocitos T *naive* con Foxp3 incrementa los niveles de expresión de CD25 y otros marcadores de superficie asociados con este tipo de Treg, como CTLA4 y GITR, al mismo tiempo que reprime la producción de IL-2, IFNγ e IL-4 (58). Las evidencias sugieren que las Treg naturales poseen un TCR con mayor afinidad por los ligandos tímicos de antígenos propios/MHC que otras células T, y que esta alta afinidad es la que recluta las células T al linaje Treg en el curso de la selección tímica (58). Por otro lado, los linfocitos T *naive* también pueden adquirir la expresión de Foxp3 en la periferia y la consecuente función supresora. Por ejemplo, la estimulación antigénica in vitro de células T *naive* en presencia de TGFβ aumenta la población de Treg $Foxp3^+$ (63-65). Además, el ácido retinoico producido por una población de células dendríticas intestinales, y en presencia de TGFβ, facilita la diferenciación de linfocitos T *naive* a Treg $Foxp3^+$ (66-69). Sin embargo, aún queda por determinar si la diferenciación de Treg $Foxp3^+$ en la periferia a partir de células T *naive* es funcionalmente estable in vivo y hasta que punto contribuye al *pool* periférico de Treg $Foxp3^+$. En cuanto a los mecanismos de supresión, las Treg naturales inhiben la proliferación de células T *naive* y su diferenciación en células T efectoras in vivo, suprimen las funciones efectoras de linfocitos T $CD4^+$ y $CD8^+$, de células NK, NKT, linfocitos B, macrófagos, y células dendríticas. Una vez activadas por un determinado antígeno, estas Treg pueden suprimir a las células T respondedoras independientemente de si comparten especificidad antigénica ente sí (58).

2.1.2. Células Tr1

La primera población de linfocitos T $CD4^+$ descrita como Tr1 fue aislada de humanos con inmunodeficiencia combinada severa (SCID) transplantados con células hematopoyéticas con antígenos leucocitarios incompatibles. A pesar de la falta de compatibilidad antigénica, se

encontró que los pacientes no desarrollaron la enfermedad del injerto contra el huésped, lo cual estaba asociado a altos niveles de IL-10 en sangre. La fuente principal de esta IL-10 resultó ser una subpoblación de linfocitos T derivados de los donantes que más tarde se denominó Tr1 (70, 71).

2.1.2.a. Fenotipo

La principal característica de las Tr1 reside en que una vez activadas vía el TCR, producen grandes cantidades de IL-10, muy bajas cantidades de IL-2 y no producen IL-4, ambas citoquinas potentes factores de crecimiento para las células T. A diferencia de las Tr1 murinas, las Tr1 humanas también producen IFNγ, pero a niveles que son un orden de magnitud inferior a los producidos por las Th1 (72). Las Tr1 también pueden producir TGFβ e IL-5, aunque dependiendo de las condiciones experimentales, el perfil de secreción de estas citoquinas puede variar. Como en el caso de las Treg naturales, las células Tr1 tienen baja capacidad proliferativa in vitro, aunque pueden ser expandidas en presencia de IL-2 e IL-15. El fenotipo anérgico de las Tr1 es debido a un efecto autocrino de la IL-10, ya que la respuesta proliferativa puede ser restablecida mediante el bloqueo específico con anticuerpos contra la IL-10 (71, 72). A pesar de su escasa proliferación in vitro, al ser activadas mediante el TCR los clones humanos de células Tr1 expresan niveles normales de marcadores de activación, como CD25, CD40L, CD69, HLA-DR y CTLA-4 (72).

2.1.2.b. Marcador

En los últimos años se han realizado distintos estudios con el fin de identificar un marcador específico de las Tr1, y hasta el momento, la única característica común entre estas células es la expresión de altos niveles de IL-10. Si bien la expresión de GATA-3 (ROG) por parte de las Tr1 puede ser utilizado para distinguirlas de las Treg $Foxp3^+$ productoras de IL-10 (73), ROG también es expresado por células Th activadas, limitando su utilidad como marcador de las Tr1. Las Tr1 humanas no activadas expresan receptores de quimioquinas asociados tanto a células Th1 (CXCR3 y CCR5) como a células Th2 (CCR3, CCR4, CCR8) (74).

2.1.2.c. Mecanismos de supresión

Las células Tr1 regulan las respuestas inmunes in vitro e in vivo tanto de linfocitos T *naive* como células efectoras Th1, Th2 y de memoria (70, 71, 75-78). Para ejercer sus funciones supresoras, las Tr1 precisan ser activadas por su TCR, lo cual provoca la secreción local de las citoquinas inmunomoduladoras IL-10 y TGFβ. De esta forma, una vez activadas de manera antígeno-específica, pueden suprimir las respuestas contra otros antígenos. Las citoquinas IL-10 y TGFβ actúan tanto en las células presentadoras de antígeno (APC) como en los linfocitos T. Así, IL-10 disminuye la expresión de moléculas coestimuladoras y la producción de citoquinas proinflamatorias en las APC, mientras que al mismo tiempo inhibe la producción de IL-2 y TNFα por los linfocitos T $CD4^+$. De manera similar, TGFβ disminuye las funciones presentadoras de antígenos de las APC e inhibe la proliferación y producción de citoquinas por las células T. Las funciones supresoras de las Tr1 pueden ser revertidas por anticuerpos bloqueantes contra IL-10 y TGFβ (79). Las Tr1 generadas in vitro a partir de la estimulación conjunta de CD3 y CD46, suprimen no sólo mediante IL-10, sino también por un mecanismo dependiente de granzima B/perforina (80-82).

2.1.2.d. Papel en desórdenes inmunológicos

Teniendo en cuenta que las Tr1 se originan en la periferia y que para suprimir respuestas inmunes necesitan ser activadas por el TCR, se ha propuesto que estas Treg están involucradas en el control de respuestas contra antígenos no propios. Sin embargo, existen evidencias que indican que las Tr1 también modulan respuestas contra antígenos propios y de esta forma, juegan un papel importante en procesos autoinmunes. Así, se han aislado de individuos sanos clones de células Tr1 reactivos contra MHC propio que inhiben de manera dependiente de IL-10 y TGFβ la proliferación de linfocitos T $CD4^+$ *naive* específicos de antígenos (83). También se encontró que un 80% de portadores sanos de *Pemphigus vulgaris* (PV) posee células Tr1 específicas para Desmogleina 3, el autoantígeno de PV, mientras que sólo el 17% de enfermos de PV presentan Tr1 específicas (77). De manera similar, mientras que en pacientes con diabetes tipo 1 los linfocitos T específicos del autoantígeno presentan una polarización extrema hacia un fenotipo proinflamatorio Th1, en individuos normales los linfocitos autorreactivos exhiben un claro perfil de Tr1 (84). Estudios en pacientes con artritis reumatoide demostraron que los niveles de Tr1 en sangre periférica y tejido sinovial son significativamente menores que en los controles. Además, la frecuencia de las Tr1 entre los linfocitos CD4 infiltrantes está inversamente correlacionada con la frecuencia de células Th1 y la severidad de la enfermedad (85). En pacientes con anemia

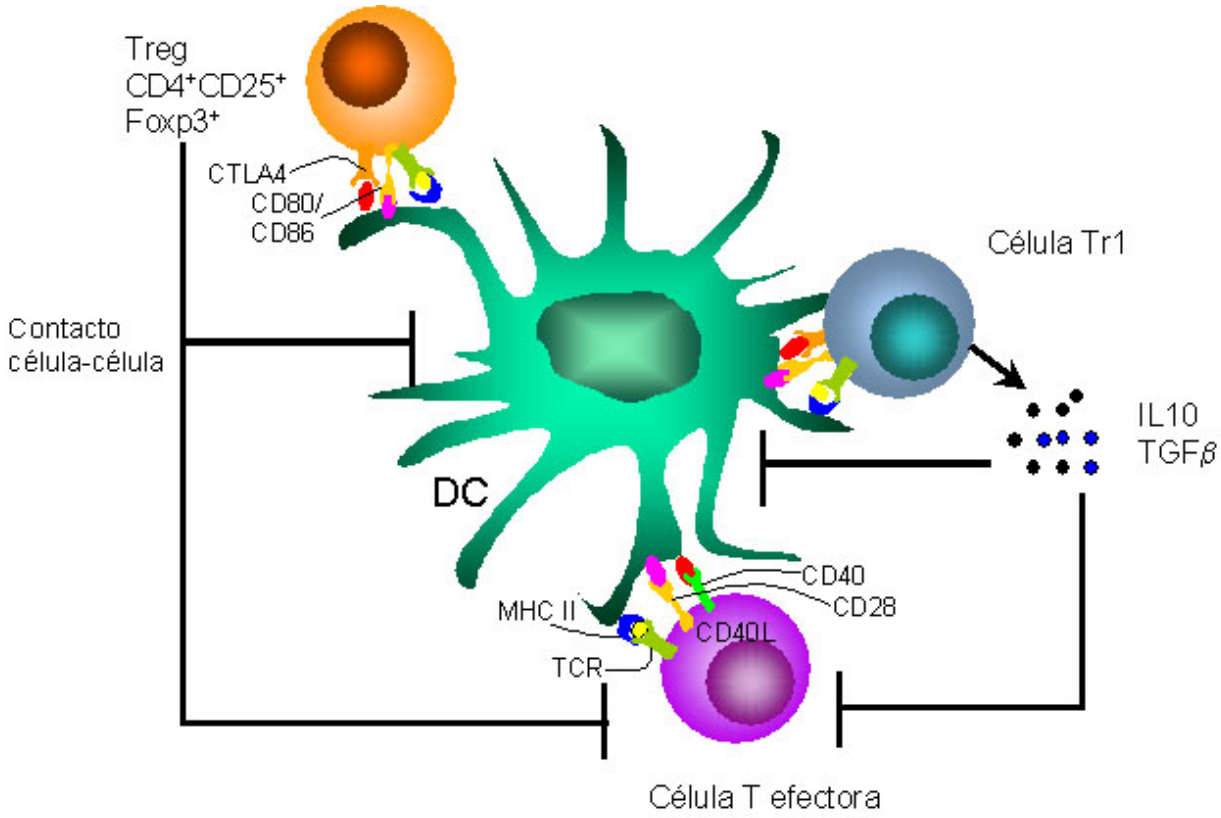
hemolítica autoinmune también se identificaron en sangre periférica células Tr1 específicas del principal autoantígeno en los glóbulos rojos (86). Por último, también se ha demostrado que, mientras que en humanos no alérgicos la mayoría de los linfocitos CD4 específicos de los alergenos presentes en sangre periférica son Tr1, en pacientes alérgicos la gran mayoría son células Th2 productoras de IL-4 (87).

2.1.2.e. Tratamiento de enfermedades inmunes

Debido a la naturaleza supresora de las Tr1 y la posibilidad de ser inducidas tanto in vitro (o ex vivo) como in vivo, estas células han sido utilizadas para el tratamiento de distintas patologías causadas por una exagerada o mal dirigida respuesta inmune, como es el caso de alergias (88), transplantes (89) y enfermedades autoinmunes (79). En un modelo de colitis inducido por transferencia de linfocitos T $CD4^{+}CD45RB^{high}$ en recipientes SCID, la transferencia conjunta de células Tr1 es capaz de prevenir el desarrollo de la inflamación intestinal presumiblemente mediante la secreción de IL-10 y TGFβ (71, 90). También se observó que las Tr1 resultan protectoras en modelos de esclerosis múltiple (75, 91) y diabetes (92).

2.1.2.f. Diferenciación

Las células Tr1 se diferencian tanto in vivo como in vitro a partir de precursores *naive* (79). In vitro, se ha observado que la estimulación repetitiva a través del TCR en presencia de altas concentraciones de IL-10 genera células Tr1 (71). Por otro lado, la activación en presencia de las drogas inmunosupresoras, vitamina D3 y dexametasona, induce la diferenciación de Tr1 humanas y murinas mediante la producción autocrina de IL-10 (75). En conjunto, estos datos sugieren que la IL-10 no sólo es fundamental en las propiedades supresoras de las Tr1 sino que también juega un papel central en la diferenciación de estas células. Sin embargo, si bien la IL-10 parece ser necesaria, en humanos parece no ser suficiente para una completa diferenciación de células Tr1 (76). Las células Tr1 humanas también pueden ser diferenciadas mediante la estimulación conjunta de CD3 con el regulador del complemento CD46 en presencia de IL-2 (82), por anticuerpos contra CD45RO/RB (93) y por la estimulación a través de CD2, el ligando de CD58 (94). En los últimos años se han reportado gran cantidad de evidencias que muestran que las células Treg (Tr1 y $Foxp3^{+}$) pueden ser diferenciadas por células dendríticas, siendo este uno de los principales mecanismos de tolerancia periférica (79).



2.3. Células Dendríticas

Las DC son células leucocitarias derivadas de médula ósea, distribuidas ampliamente por todo el organismo y que están especializadas en la captura, procesamiento y presentación de antígenos a células T (95). Las DC expresan un amplio repertorio de PRR y, en respuesta a señales de estos receptores, sufren una profunda transformación fenotípica y funcional, denominada activación o maduración. En esta transformación las DC migran al ganglio linfático y se convierten en células presentadoras de antígeno inmunogénicas, promoviendo la expansión y diferenciación de células T *naive* en células efectoras (96). De esta forma, las DC poseen un papel clave traduciendo información de la inmunidad innata a la inmunidad adaptativa. Las DC activadas por contacto con los patógenos normalmente presentan altos niveles de MHC cargados con péptidos derivados de los patógenos, los cuales pueden ser reconocidos por los TCR de los linfocitos T *naive* específicos del patógeno. Esto proporciona la primera señal de activación ("señal 1") a los linfocitos T. Además, las DC activadas por patógenos también expresan una variedad de moléculas coestimuladoras, que son reconocidas por receptores de las células T y transmiten señales importantes para la supervivencia y proliferación de las células T ("señal 2"). Las más ampliamente conocidas son las moléculas coestimuladoras CD80 y CD86, cuyo ligando en la célula T es CD28; y CD40 que es reconocido por CD40L. Por último, las DC activadas

producen mediadores que actúan en las células T promoviendo su diferenciación en células efectoras ("señal 3") (97). Así, la IL-12 es un mediador liberado por DC activadas que puede instruir el desarrollo de una respuesta inmune de tipo 1 mediada por células Th1. La integración de estas tres clases de señales por el linfocito T determina su destino. Mientras que las tres señales parecen ser necesarias para una completa generación de células T efectoras, la señal 1 en ausencia de las señales 2 y 3 puede inducir tolerancia matando a las células T, haciéndolas anérgicas, o induciendo la generación de Treg. De esta forma, las DC además de conectar la inmunidad innata y adaptativa en las respuestas inmunes, inducen tolerancia específica de antígenos en los linfocitos T.

2.3.1. Células dendríticas tolerogénicas

Se han propuesto distintos modelos para explicar como las DC pueden inducir o mantener tolerancia periférica en linfocitos T. Inicialmente, Steinmann *et al.* (98, 99) propusieron que en condiciones normales, la captura de antígenos por DC inmaduras, las cuales expresan bajos niveles de MHC y moléculas coestimuladoras, es capaz de inducir tolerancia hacia aquellos péptidos presentados a linfocitos T específicos. Por el contrario, si el mismo antígeno es capturado por las DC en condiciones de activación, el linfocito T recibirá las tres señales y se inducirá una potente respuesta T. En este modelo de tolerancia periférica, DC inmaduras constantemente transportan antígenos propios desde los tejidos periféricos a los ganglios linfáticos y bazo.

De manera coherente con este modelo, existen varias evidencias que sugieren que existe un flujo de antígenos de los tejidos hacia los ganglios linfáticos a través de DC movilizadas. Se ha identificado un grupo de DC ($OX41^-$) que transporta de manera constitutiva cuerpos apoptóticos derivados del epitelio intestinal a las áreas de linfocitos T de los ganglios linfáticos mesentéricos (100). Estas DC $OX41^-$ son pobres APC a pesar de presentar altos niveles de CD80 y CD86 y podrían tener un papel en la inducción y mantenimiento de la tolerancia. También se ha observado que en condiciones basales, DC transportan antígenos desde las vías respiratorias hacia los ganglios linfáticos torácicos (101). La función de las DC en estado basal in vivo ha sido examinada utilizando como sistema de transporte de antígenos un anticuerpo monoclonal contra un receptor endocítico específico de DC (DEC-205). De esta manera, los antígenos unidos covalentemente a anticuerpos contra DEC-205 son inyectados en ratones y presentados por DC no activadas a los linfocitos T en los ganglios linfáticos. El resultado es que linfocitos T $CD4^+$ y $CD8^+$ se vuelven tolerantes hacia el respectivo antígeno (102, 103). Estudios

subsecuentes mostraron que esta tolerancia está asociada a un incremento en la población de células Treg $CD4^+CD25^+$ (104). Es más, tratamientos con los correspondientes conjugados anti-DEC-antígeno resulta en una significativa mejoría en modelos murinos de enfermedades de hipersensibilidad por contacto, diabetes y esclerosis múltiple (105, 106). Estas observaciones sugieren que en estado basal, la principal función de las DC podría ser mantener la tolerancia periférica. En este sentido, se ha observado que existe una correlación directa entre la cantidad de antígeno expresado en la periferia y el grado de tolerancia de linfocitos T $CD8^+$ específicos de ese antígeno (107) y que antígenos expresados como transgenes en células β del páncreas son presentados por APC derivadas de médula ósea en los ganglios linfáticos drenantes, induciendo deleción de linfocitos T $CD8^+$ (108). De manera similar, mediante un sistema de doble transgénico se ha demostrado in vivo que DC que presentan epítopos de linfocito T citotóxicos (CTL) en estado basal inducen tolerancia periférica en células T $CD8^+$, mientras que si son presentados por DC activadas por anticuerpos anti-CD40 inducen inmunidad protectora (109). Estudios posteriores mostraron que la tolerancia en este sistema es intrínseca de las células T $CD8^+$ y depende de la expresión de PD-1 en las DC y de señales provenientes de CTLA-4 en los linfocitos T (110).

En cuanto a la inducción de Treg por DC, las primeras evidencias provinieron de experimentos con células humanas en los que se observó que DC inmaduras son capaces de inducir la diferenciación de células Tr1 tanto in vitro como in vivo (111, 112). Más tarde también se demostró que DC inmaduras pueden promover la diferenciación y expansión de células Treg $Foxp3^+$ (113, 114). De hecho, las DC parecen ser las APC más eficientes en la inducción de Treg $Foxp3^+$(115).

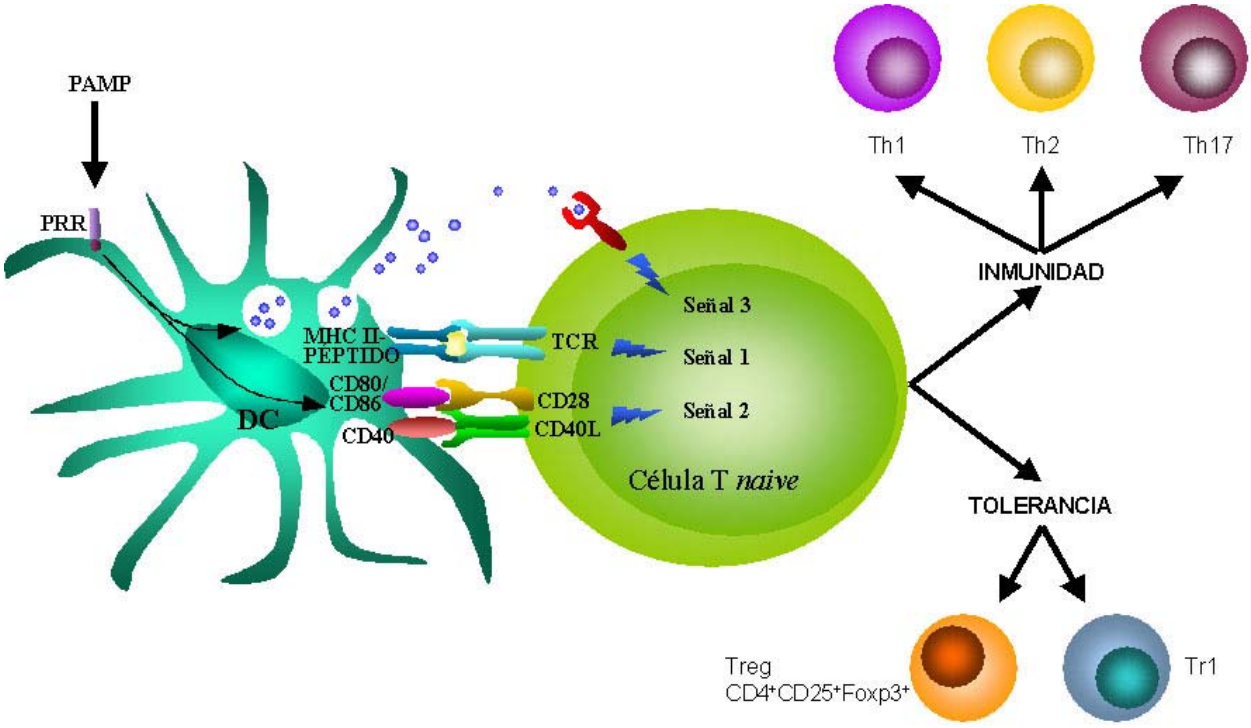
En conjunto, estas observaciones avalan la hipótesis según la cual el estado de activación de las DC resulta crucial en el destino de los linfocitos T, es decir que las DC inmaduras inducen tolerancia mientras que DC maduras son inmunogénicas. Sin embrago, la idea de que el estado de activación de las DC es binario, i.e. maduras o inmaduras, siendo las DC inmaduras inductoras de tolerancia por defecto y simplemente predecesoras no activadas de las DC maduras e inmunogénicas, ha sido cuestionada, al menos en parte, por varios resultados. En primer lugar, existen evidencias experimentales que sugieren que ciertas DC especializadas tendrían propiedades tolerogénicas intrínsecas, mientras que otros datos indican que la función de las DC estaría dictada por el microambiente particular en el que se encuentre durante su desarrollo o activación. En los tejidos linfoides asociados al tracto intestinal, por ejemplo, se ha identificado una población de DC $CD103^+$ en los ganglios linfáticos mesentéricos que promueve el desarrollo de Treg $Foxp3^+$ por un mecanismo dependiente de TGFβ y ácido retinoico (67, 69). En el bazo,

se ha encontrado que DC $CD8^{+}CD205^{+}$ inducen Treg $Foxp3^{+}$ funcionales a partir de células T $Foxp3^{-}$ mediante la producción de TGFβ, mientras que las DC $CD8^{-}DCIR2^{+}$ estimulan y expanden las Treg $Foxp3^{+}$ naturales (116). También se ha identificado una población minoritaria de DC $CD19^{+}$ en el bazo, que al ser estimuladas con CpG parece adquirir funciones supresoras en células T dependientes de la enzima IDO (117). Además, se ha identificado, también en el bazo, una población de DC $CD11c^{low}CD45RB^{high}$ que posee un fenotipo inmaduro incluso después de ser activadas in vitro con LPS o CpG y que induce la diferenciación de células Tr1 a través de la producción de IL-10 (118).

Las DC tolerogénicas también pueden ser inducidas por agentes biológicos o farmacológicos. Así, el tratamiento de DC con las citoquinas antiinflamatorias IL-10 (119) o TGFβ + IL-10 (120), las convierte en células tolerogénicas capaces de inducir la diferenciación de Treg in vitro e in vivo. En particular, la diferenciación de DC murinas derivadas de médula ósea en presencia de IL-10 induce la diferenciación de las DC tolerogénicas $CD11c^{low}CD45RB^{high}$ (118). De la misma forma, células estromales derivadas del bazo promueven el desarrollo de $CD11c^{low}CD45RB^{high}$ (121). El tratamiento de DC con la forma activa de la vitamina D3, 1,25(OH)2D3, inhibe la maduración de las DC, disminuyendo la producción de IL-12 y aumentado la de IL-10 con la consecuente disminución de la activación de células T (122). Además, DC tratadas con agonistas del receptor prostanoide D1 incrementan la población de Treg Foxp3 in vivo (123). Por último, las drogas inmunosupresoras como corticoesteroides, ciclosporina A y rapamicina, previenen la activación de las DC por mecanismos que incluyen la inhibición de la translocación nuclear de miembros específicos de la familia de factores de transcripción NF-κB, necesarios para una completa activación de las DC, induciendo tolerancia específica de antígenos en células T (124). La capacidad de las DC para inducir tolerancia dependiendo del ambiente también es aprovechada tanto por algunos patógenos como por algunos tumores, los cuales evaden al sistema inmune convirtiendo a las DC en tolerogénicas e inductoras de Treg (113, 125).

Las DC ocupan una posición crítica en el sistema inmune como conexión entre la inmunidad innata y la inmunidad adaptativa, siendo capaces de desencadenar respuestas específicas de antígeno tanto de tipo Th1 como Th2, y de inducir tolerancia dominante contra un determinado antígeno mediante la diferenciación de Treg. Esta flexibilidad que poseen las DC, sumada al casi ilimitado repertorio de reconocimiento que poseen los linfocitos T, las convierte en una herramienta con un potencial enorme para inducir respuesta inmune o tolerancia hacia prácticamente cualquier antígeno. Así, las DC pueden ser utilizadas para el tratamiento de

enfermedades en las que el objetivo sea estimular la respuesta inmune, como es el caso del cáncer (126), o pueden ser aplicadas para silenciarla, como ocurre en transplantes (127), alergias (128), y enfermedades inflamatorias crónicas y autoinmunes (129).



3. AUTOINMUNIDAD. MECANISMOS GENERALES

Las respuestas autoinmunes son similares a las respuestas inmunes fisiológicas hacia los patógenos en el sentido que son específicamente activadas por antígenos, pero con la diferencia que en este caso los antígenos son propios. El tipo de enfermedad autoinmune que se desarrolla cuando linfocitos autorreactivos son activados depende en gran medida de su blanco particular. Si, por ejemplo, el autoantígeno está presente en el páncreas, como es el caso de la diabetes mellitus dependiente de insulina, linfocitos T específicos de la insulina producida por las células β del páncreas dirigen el ataque del sistema inmune hacia este órgano y la enfermad se manifiesta con una progresiva destrucción de las células productoras de insulina (130, 131).

En principio, la destrucción que el sistema inmune realiza de los tejidos propios puede ocurrir ya sea por un reconocimiento específico de antígenos propios, seguido por una respuesta inmune efectora o como una consecuencia secundaria de una respuesta inflamatoria no específica. En respuestas inmunes normales dirigidas a la destrucción de patógenos, la consecuencia típica es la eliminación del invasor, después de la cual la respuesta inmune cesa. Sin embargo, en el escenario de la autoinmunidad, los antígenos propios no pueden ser eliminados fácilmente, debido a que se encuentra en exceso o son ubicuos. Por lo tanto, un

mecanismo clave para limitar el alcance de la respuesta inmune no puede aplicarse en las enfermedades autoinmunes, las cuales tienden a evolucionar a un estado crónico inflamatorio. Esta inflamación crónica, a su vez, provoca la liberación de más autoantígenos como consecuencia del daño tisular, quebrando una de las barreras de autoinmunidad conocida como *secuestration*, mediante la cual gran parte de los antígenos propios son normalmente mantenidos fuera del alcance del sistema inmune. De esta manera, se produce la activación de nuevos clones de linfocitos T específicos de los autoantígenos liberados, fenómeno que se denomina propagación de epítopo. Además, este estado de inflamación crónica, junto con la destrucción del tejido y la consecuente liberación de inductores endógenos de la inflamación, atrae y activa a más células efectoras no específicas como neutrófilos y macrófagos. El resultado es un proceso destructivo que se va amplificando (132).

Los linfocitos T juegan un papel fundamental en prácticamente todas, si no todas, las respuestas inmunes adaptativas, incluyendo las que son llevadas a cabo por los linfocitos B. Las células T pueden ser clasificadas en diferentes poblaciones según sus marcadores de superficie y función. Mientras que los linfocitos T citotóxicos, que incluyen tanto los linajes CD4 y CD8, son capaces de matar células diana, las células $CD4^+$ helper (Th) son aquellas capaces de modular las respuestas de las células B y T $CD8^+$. La función de las células Th $CD4^+$ está predominantemente determinada por las citoquinas que producen, y estas células T son clasificadas en distintos conjuntos dependiendo de su perfil de citoquinas, i.e. Th0, Th1, Th2 y Th17 (133, 134). Alteraciones en el balance entre estas poblaciones tienen la potencialidad de causar enfermedades autoinmunes. Dado que los linfocitos T poseen un papel central en las respuestas inmunes adaptativas, no resulta sorprendente que también jueguen un rol fundamental en la mayoría, sino en todas, de las enfermedades autoinmunes.

Las células Th1 y Th2 no representan distintos linajes, sino formas polarizadas de respuestas inmunes mediadas por células Th $CD4^+$. En ratones, la respuesta del tipo Th1 está caracterizada por la producción de IL-2, IFNγ y TNF, mientras la producción de IL-4, IL-5, IL-9, IL-10 e IL-13 está ausente. En contraste, la respuesta Th2 está caracterizada por la producción de IL-4, IL-5, IL-9, IL-10 e IL-13, mientras que la producción de IFNγ y TNF está ausente. Además de diferir en esta característica, existe gran cantidad de evidencias que indican que la polarización de estas dos poblaciones es antogonística, es decir, las citoquinas Th1 reprimen la respuesta Th2, y las citoquinas Th2 reprimen la respuesta Th1 (135, 136).

En general, una respuesta polarizada hacia Th1 resulta altamente protectora contra infecciones causadas por la mayoría de los microbios, especialmente los parásitos intracelulares. Esto se debe a la habilidad de las citoquinas Th1 para promover la producción de anticuerpos

capaces de opsonizar y fijar el complemento y al mismo tiempo activar células de la inmunidad innata como macrófagos y neutrófilos. Esta característica de la respuesta Th1, la de iniciar y mantener una respuesta inflamatoria, si bien resulta efectiva en la erradicación de agentes infecciosos, cuando es errónea o exagerada puede causar condiciones patológicas crónicas. De hecho, se cree que células Th1 juegan un papel crítico en un gran porcentaje de enfermedades autoinmunes, tales como la esclerosis múltiple y la artritis reumatoide (136).

3.1. Artritis reumatoide

La artritis reumatoide (AR) es una enfermedad crónica autoinmune que afecta principalmente a las articulaciones pequeñas de manos y pies y tejidos circundantes. Esta enfermedad está caracterizada por una crónica y progresiva inflamación simétrica de la membrana sinovial (sinovitis) y el consecuente deterioro del cartílago, hueso y, en última instancia, destrucción de la articulación (137). Si bien la principal manifestación clínica se produce en las articulaciones, la AR puede afectar distintos tejidos y órganos, por lo que suele estar agrupada dentro de las enfermedades autoinmunes sistémicas (138). La AR presenta una prevalencia cercana al 1% en EEUU y Europa Occidental y el pico de incidencia se encuentra entre los 45-55 años, siendo la incidencia 3 veces superior en mujeres (139).

Durante el progreso de la AR la membrana sinovial se engrosa e hipertrofia de manera que adquiere un aspecto edematoso, recibiendo el tejido en este estado el nombre de *pannus*. A medida que la enfermedad progresa, el *pannus* va invadiendo y destruyendo las estructuras articulares locales. En condiciones fisiológicas, la membrana sinovial es una estructura compuesta por una capa íntima que contiene sinoviocitos y una capa subíntima que contiene tejido conectivo, vasos sanguíneos y linfáticos y terminaciones nerviosas. En la AR la membrana sinovial presenta una marcada proliferación de los sinoviocitos, con infiltrados de linfocitos T $CD4^+$, linfocitos B y macrófagos, los cuales a veces se organizan en estructuras similares a folículos linfoides con centros germinales (137). El aumento en el número de sinoviocitos produce la hiperplasia de la capa íntima, mientras que la expresión local de enzimas líticas, tales como proteasas, digieren la matriz extracelular y destruyen la estructura articular (140).

La etiología de la AR es desconocida, aunque los datos existentes apuntan a un origen multifactorial, en el que intervienen factores genéticos, estocásticos y medioambientales. Si bien la naturaleza autoinmune de esta enfermedad reside principalmente en la presencia de anticuerpos contra la región Fc de IgG, conocidos como factor reumatoide, también se han

propuesto que proteínas expresadas normalmente en las articulaciones, como proteoglicano y colágeno de tipo II, funcionan como antígenos involucrados en la generación o perpetuación de una respuesta de linfocitos T patogénicos (137). Como en la mayoría de las enfermedades autoinmunes, gran parte del conocimiento sobre los mecanismos patogénicos de la AR, así como el diseño de nuevas terapias contra la enfermedad, han derivado de estudios en modelos animales.

3.1.1. Artritis inducida por colágeno (CIA)

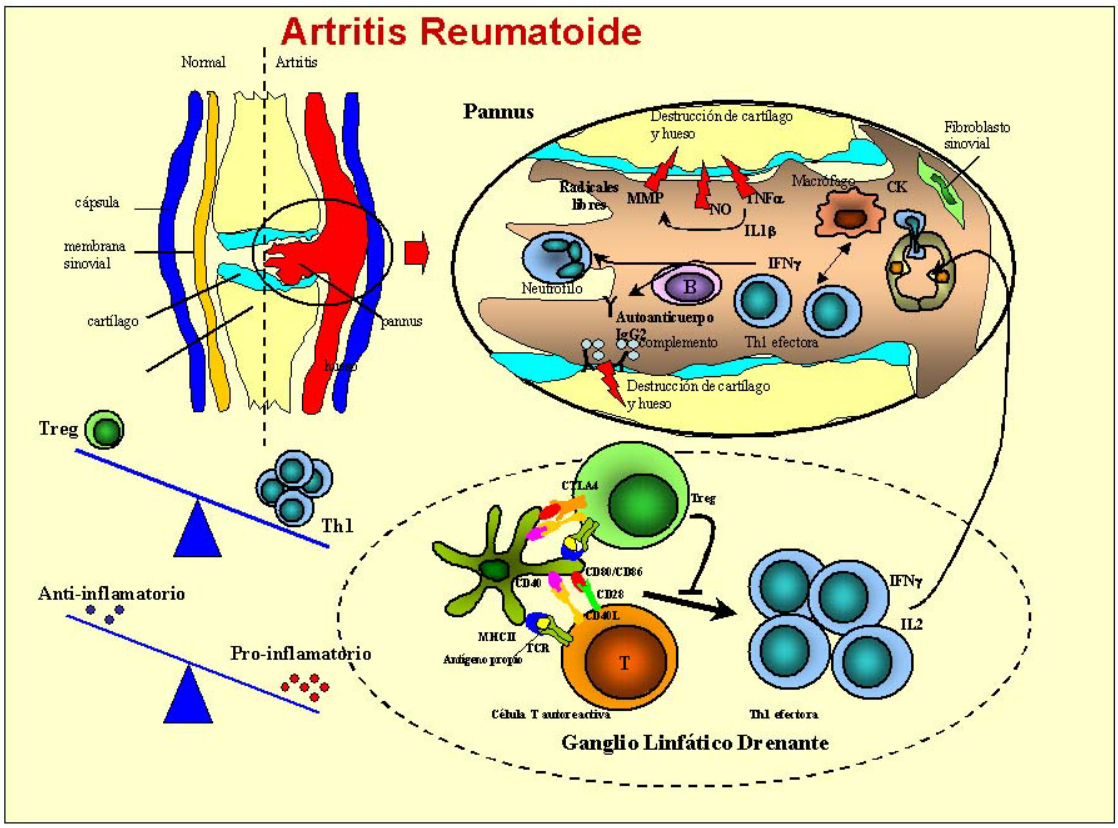
Se han establecido distintos modelos de AR, siendo el de artritis inducida por colágeno (CIA) el más ampliamente estudiado. La gran aceptación de este modelo reside en que además de estar claramente definido y ser reproducible, comparte varias características patofisiológicas con la AR (141). En primer lugar, la susceptibilidad a la CIA está claramente asociada a la expresión de genes específicos de MHC II, lo que se correlaciona con la asociación que se observa en humanos entre los alelos HLA-DR y la susceptibilidad a desarrollar AR. Además, la respuesta al autoantígeno tanto de linfocitos B como T son requeridas para establecer la patogenicidad. Por último, existe un significativo papel de los anticuerpos autorreactivos en la promoción de procesos inflamatorios vía la activación de la cascada de complemento (141). De hecho, el modelo de CIA ha sido utilizado extensamente con el fin de identificar mecanismos potencialmente patogénicos de autoinmunidad, incluyendo el papel que cumplen distintas poblaciones celulares en la aparición y progresión de la enfermedad. En los últimos años este modelo se ha convertido en una herramienta fundamental para el diseño y desarrollo de terapias, tales como el bloqueo del TNF, el cual es un mediador crítico en la patogénesis de la AR (142).

La inducción de artritis se realiza en cepas genéticamente susceptibles de ratón mediante la inmunización con colágeno de tipo II (CII) emulsionado en adyuvante completo de Freund (CFA). El resultado es una respuesta patogénica que incluye hiperplasia sinovial, infiltrado de células mononucleares y degradación del cartílago. Tanto la naturaleza antigénica del CII, como el método de administración y la presencia de CFA crean un ambiente favorable para la inducción de una respuesta Th1, por lo que los linfocitos T juegan un papel prominente en el moldeado de la respuesta autoinmune en CIA (141). Por un lado regulan al menos parte de la red de citoquinas que se producen en las articulaciones inflamadas, mientras que al mismo tiempo modulan la respuesta de los linfocitos B patogénicos. Es más, varios trabajos han demostrado que la subclase de anticuerpos anti-CII producidos está altamente relacionada con el grado de enfermedad. De hecho, el principal mecanismo en la inmunopatogénesis de este modelo parece

estar mediado por los autoanticuerpos contra CII (141, 143). Los anticuerpos anti-CII en CIA son principalmente del tipo IgG2a e IgG2b, los cuales alcanzan niveles máximos durante el pico de la enfermedad y parecen tener el potencial de iniciar una respuesta inflamatoria en las articulaciones, ya que la transferencia adoptiva de sueros de ratones artríticos a ratones *naive* es capaz de inducir una inflamación severa. Sin embargo, la transferencia pasiva de anticuerpos no causa una respuesta autoinmune completa, dado que una vez que son deplecionados, la respuesta inflamatoria decrece, indicando que además de la deposición de anticuerpos anti-CII en la articulación, otros factores son necesarios para establecer una artritis autoinmune (141, 143).

Teniendo en cuenta que los anticuerpos IgG2a e IgG2b son capaces de activar la cascada de complemento, la unión y acumulación de estos anticuerpos en la región articular podría explicar parte del inicio de la respuesta inflamatoria local. De hecho, tanto la depleción del complemento como deficiencias genéticas en alguno de sus componentes importantes convierten ratones susceptibles en resistentes a CIA (144-147).

La activación de la cascada de complemento, en particular C5a, recluta neutrófilos y macrófagos, los cuales son activados mediante los receptores FcγR y secretan quimioquinas y mediadores proinflamatorios, como IL-1β, TNFα, IL-6, MIP-1α, NO y prostaglandinas (PGE2). A su vez, estos factores activan las células residentes como los sinoviocitos, tanto del tipo fibroblastos como macrófagos, y además reclutan y activan más tipos celulares, incluyendo células NK, DC, linfocitos T y B. La producción de mediadores proinflamatorios como IL-1β, TNF-α e IL-6 por parte de los sinoviocitos y células infiltrantes induce la síntesis y secreción de enzimas que degradan la matriz extracelular. De esta forma el CII y otras proteínas de la matriz degradadas aumentan la cantidad de autoantígeno libre en la articulación, activando linfocitos T específicos de CII y otras proteínas de la matriz. El resultado final es la destrucción de la articulación orquestada por células y moléculas efectoras del sistema inmune (141, 143).



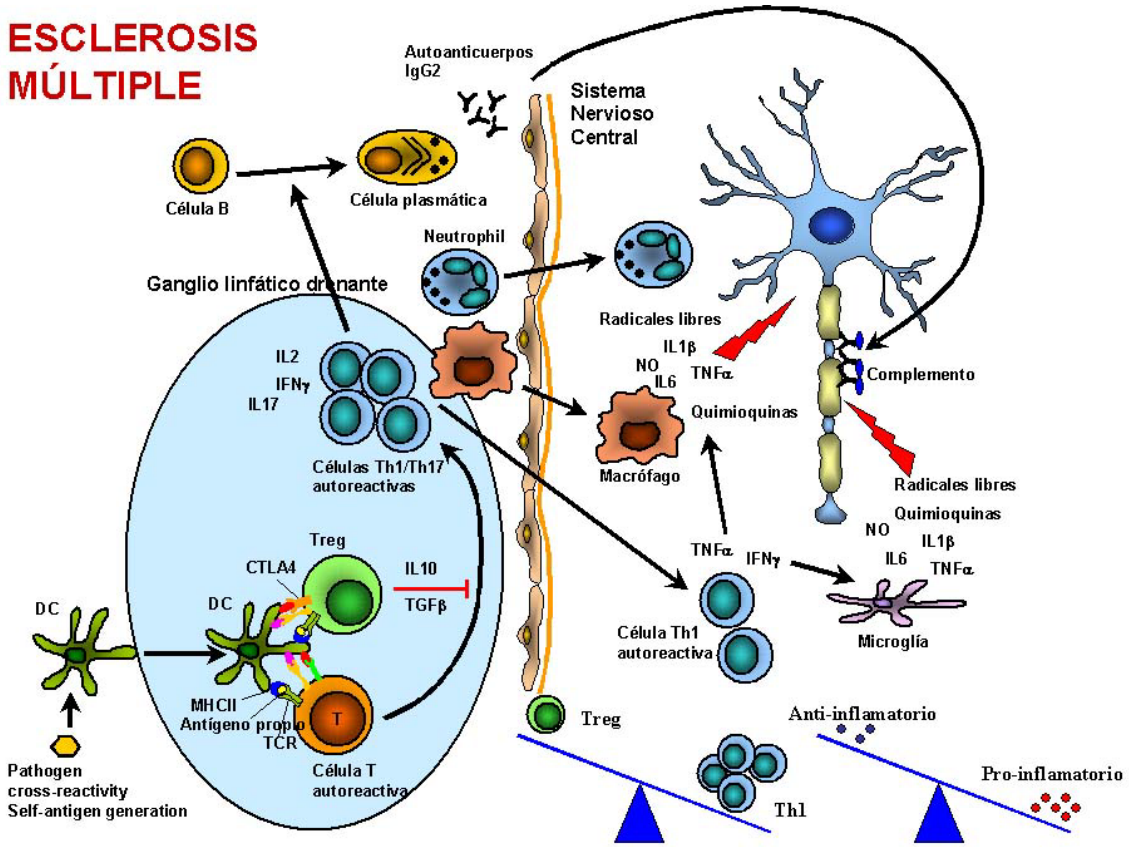
3.2. Esclerosis múltiple

La esclerosis múltiple (EM) es una enfermedad inflamatoria que afecta el sistema nervioso central (CNS), i.e. el cerebro y la médula espinal, y que habitualmente comienza a desarrollarse entre los 20 y 40 años (148, 149). La EM lleva a una discapacidad mediante deficiencias en la sensibilidad y en las funciones motoras, autonómicas y neurocognitivas. Además del componente inflamatorio agudo, también existe un proceso degenerativo que contribuye substancialmente a la progresión del deterioro. Este proceso degenerativo incluye pérdida axonal en las lesiones y daño difuso en la materia blanca distante de las áreas que se ven involucradas por histopatología o resonancia magnética. Si bien la enfermedad no lleva a un acortamiento de la vida, tiene importantes consecuencias socioeconómicas (148, 149). Existen dos formas principales de la EM. La EM remitente-recurrente (RR-EM) es la más frecuente (85%-90%) y su incidencia es dos veces superior en las mujeres. Entre un 40-50% de los pacientes con RR-EM dejan de tener ataques agudos y desarrollan posteriormente una enfermedad progresiva neurodegenerativa secundaria a la inflamación crónica del CNS. Esta forma se denomina EM secundaria progresiva (SP-EM). Por último, entre el 10% y 15% de los pacientes desarrollan desde el comienzo un deterioro clínico progresivo en ausencia de ataques

agudos, conocido como EM primaria progresiva (PP-EM) (150). Por el momento no está claro que factores son responsables de los distintos cursos de la enfermedad (150).

La EM es considerada una enfermedad mediada por linfocitos Th1 (150). Su base inmunológica se soporta tanto en la asociación que existe con genes del sistema inmune, en particular los MHC, como en la respuesta que exhibe esta enfermedad a terapias inmunosupresoras (151). Modelos animales como el de encefalomielitis autoinmune experimental (EAE) han proporcionado evidencias que muestran que las células T reactivas contra proteínas de la mielina causan enfermedades autoinmunes demielinizantes (150). El modelo de EAE ha servido como una herramienta fundamental para probar nuevas terapias contra la autoinmunidad mediada por respuestas del tipo Th1 (152).

Estudios en pacientes con EM y en EAE han llevado al siguiente escenario patogénico. En ciertas condiciones, como infecciones virales o inmunizaciones con péptidos o proteínas de la mielina con CFA, linfocitos T $CD4^+$ específicos de la mielina son activados y migran al CNS a través de la barrera hematoencefálica (BBB). Una vez en el CNS los linfocitos $CD4^+$ autorreactivos secretan citoquinas y quimioquinas que inician la cascada proinflamatoria local, lo cual provoca la afluencia de fagocitos mononucleares periféricos al CNS y la activación de monocitos/macrófagos y células de la microglia residentes. Los linfocitos T activados producen IFNγ y TNF-α que a su vez inducen la producción de NO por macrófagos, células de la microglía y astrositos. La presencia de estas células sumada a las citoquinas proinflamatorias IL-12 y TNF ha llevado al concepto que la EM y su modelo EAE son enfermedades autoinmunes mediadas por células Th1. Por otro lado, la activación de linfocitos Th1 autorreactivos lleva a la activación de linfocitos B y la consecuente producción de anticuerpos contra la mielina de manera local. Los anticuerpos contra la mielina forman parte de los mecanismos efectores de EAE mediante la opsonización de la mielina y la activación del complemento que lleva a la deposición del complejo de ataque a membrana y a la citolisis mediada por complemento (150, 153). Este ataque concertado por células T y B, el complemento y los mediadores inflamatorios producen áreas de desmielinización, lo cual afecta la conducción eléctrica a lo largo del axón y produce los defectos patofisiológicos.



3.3. Terapias

Actualmente, existen varios tratamientos disponibles para la EM y la AR. La mayoría de estas terapias o bien son drogas inmunosupresoras inespecíficas, como el metotrexato en el caso de la AR, o bien se basan en la interrupción de pasos específicos de la cascada inmunopatogénica, incluyendo: factores involucrados en el reclutamiento de linfocitos a los tejidos diana; enzimas que son críticas para la penetración de las células inmunes en los vasos sanguíneos y la matriz extracelular; citoquinas que median la patología dentro de los tejidos; varios tipos celulares que median el daño en el sitio de la enfermedad, así como los receptores clonales de estas células (TCR y BCR); y mediadores tóxicos como los componentes del complemento y el NO (154).

Si bien muchos de estos tratamientos resultan prometedores en las terapias contra la AR y la EM, ninguno esta exento de efectos secundarios. De hecho, la EM y la AR son enfermedades complejas en las que intervienen gran variedad de factores, y en particular, los diferentes genotipos brindan distintas susceptibilidades y gran heterogeneidad entre los pacientes. Por esto, las actuales terapias resultan, en el mejor de los casos, sólo parcialmente eficientes en una significativa proporción de los pacientes (155). Por lo tanto, todavía resulta de vital importancia la búsqueda de nuevas terapias para el tratamiento de estas enfermedades. Considerando que el

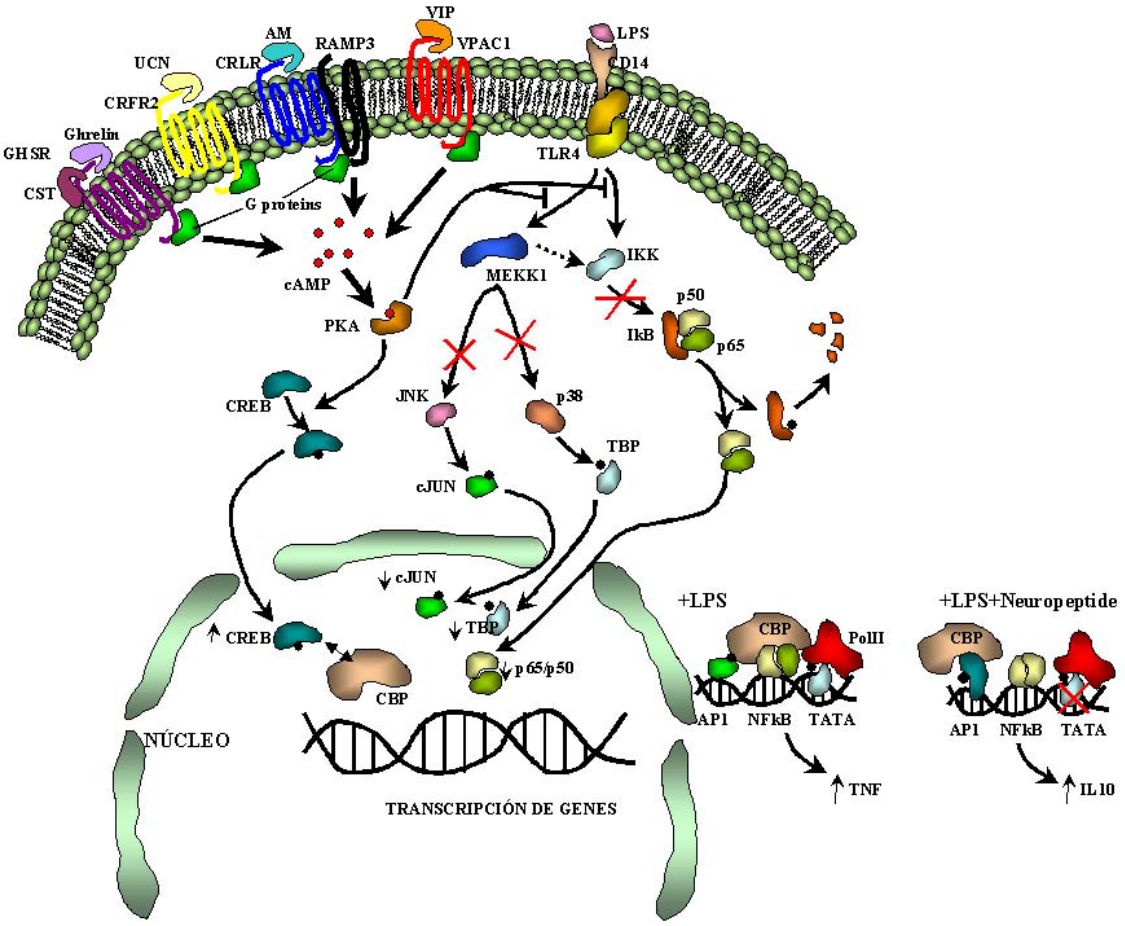
sistema inmune posee sus propios mecanismos para restablecer la homeostasis, los factores endógenos que produce y están involucrados en el establecimiento de la tolerancia y la resolución de la inflamación representan un atractivo enfoque terapéutico para enfermedades inflamatorias y autoinmunes. En particular, en los últimos años han surgido dentro de la lista de estos factores ciertos neuropéptidos, originalmente descritos como mediadores neuroendocrinos, pero que también son producidos por células inmunes.

4. Neuropéptidos

Durante años los sistemas inmune y neuroendocrino han sido considerados como dos redes independientes que mantienen el balance entre el huésped y el ambiente exterior. El sistema neuroendocrino responde a estímulos externos como temperatura, dolor y estrés, mientras que el sistema inmune responde a bacterias, virus, parásitos y daños tisulares. Sin embargo, en los últimos 20 años han surgido gran cantidad de evidencias que muestran que ambos sistemas responden al peligro de manera coordinada. De esta manera, el sistema inmune señala acerca de la presencia del peligro de una infección al cerebro, el cual orquesta la respuesta febril y sus efectos subsecuentes en el comportamiento (156). De manera análoga, el sistema inmune es regulado por el CNS en respuesta a estrés ambiental, ya sea directamente por el sistema autonómico nervioso o por el eje Hipotálamo-Pituitario-Adrenal (HPA) (157). Esta comunicación bidireccional reside en que los sistemas inmune y neuroendocrino comparten ligandos, como neuropéptidos, hormonas y citoquinas, y sus respectivos receptores. Los glucocorticoides y la norepinefrina constituyen dos ejemplos de factores endógenos inmunosupresores producidos por el eje HPA y el sistema nervioso simpático, respectivamente. Además, como respuesta a estímulos inflamatorios, varios neuropéptidos son liberados en las terminaciones periféricas de nervios eferentes y sensoriales que se encuentran próximos a las células inmunes (157).

Algunos neuropéptidos con propiedades inmunomoduladoras han emergido en los últimos años como potenciales candidatos para tratar las respuestas inmunes indeseadas que ocurren en los desórdenes inflamatorios y autoinmunes. El péptido intestinal vasoactivo (VIP), adrenomedulina (AM), grelina (GHR), urocortina (UCN) y cortistatina (CST) son neuropéptidos que no presentan homología entre sí y tanto las fuentes celulares principales como las muchas de las funciones neuroendocrinas son distintas (158-162). Sin embargo, estos neuropéptidos comparten ciertas características que los convierten en atractivos candidatos para restablecer la

homeostasis inmune. Son producidos por células inmunes, especialmente en condiciones inflamatorias o de estimulación antigénica (163-167). Los receptores están expresados en distintas células inmunes, en particular linfocitos T, DC, macrófagos/monocitos y neutrófilos (164, 165, 168-170). Por último, los receptores de estos neuropéptidos están acoplados a proteína G y activan la vía AMPc/PKA (164, 165, 168-170), la cual es considerada una vía de señalización inmunosupresora (171, 172).



4.1. Péptido intestinal vasoactivo (VIP)

VIP es un péptido de 28 aminoácidos que está estructuralmente relacionado con otras hormonas gastrointestinales como secretina, glucagón, péptido inhibitorio gástrico, péptido histidina metionina (PHM, en humanos; PHI en otros mamíferos), PACAP27, PACAP38 y CRF (173).

VIP es sintetizado a partir de un precursor proteico de 170 aminoácidos, prepro-VIP, que también contiene PHM/PHI. Prepro-VIP es metabolizado por una peptidasa en el retículo

endoplásmico, generando pro-VIP. Pro-VIP es a su vez cortado hasta generar VIP. VIP se encuentra localizado por todo el organismo. En particular, VIP está presente en las fibras nerviosas que inervan los órganos linfoides primarios y secundarios, i.e. timo, bazo, ganglios linfáticos y tejidos linfoides asociados a mucosas (164).

4.1.1. Producción de VIP por células inmunes

La primera evidencia de la presencia de VIP en células inmunes fue su identificación por inmuohistoquímica en mastocitos de rata (174). Posteriormente se ha identificado VIP en neutrófilos, linfocitos T $CD4^+$ y $CD8^+$, y linfocitos B de bazo (164). Además de expresar el mRNA de VIP y acumular la proteína en el citoplasma, los linfocitos secretan VIP en respuesta a a varios estímulos inflamatorios y mitogénicos (175). Se ha demostrado que linfocitos Th2, pero no Th1, sintetizan y secretan VIP una vez estimulados por el TCR (176).

4.1.2. Expresión receptores de VIP en células inmunes

VIP ejerce sus funciones biológicas a través de 3 receptores, VPAC1, VPAC2 y PAC1, que pertenecen a la familia de receptores de clase II (o familia B) acoplados a proteína G (GPCR) (177). Estos receptores activan las siguientes cascadas de transducción de señales: *(1)* estimulación de la adenilato ciclasa, producción de cAMP y activación de PKA; *(2)* activación de fosfolipasa C y *(3)* activación fosfolipasa D (178).

El receptor VPAC1 se expresa de manera constitutiva en linfocitos T y B de bazo y ganglios linfáticos, timocitos, monocitos y macrófagos peritoneales. Además, se ha demostrado su expresión en linfocitos T de lámina propia de intestino delgado y grueso. Por otro lado, el receptor VPAC2 se expresa de forma inducible en linfocitos T y monocitos/macrófagos estimulados con anti-CD3 o LPS, respectivamente. Con respecto al receptor PAC1, sólo se encontró expresión en macrófagos de forma constitutiva (164).

4.1.3. Efectos biológicos

Consecuentementemente con la amplia distribución de VIP y sus receptores en el organismo, este neuropéptido desempeña múltiples funciones de forma autocrina, paracrina, y endocrina. Si bien fue originalmente descrito por sus propiedades en el tracto gastrointestinal,

donde induce la relajación de la musculatura lisa, posteriormente se ha demostrado su acción en sistemas como el neuroendocrino, cardiovascular y respiratorio (tabla I).

4.1.3.a. Efectos de VIP endógeno en el sistema inmune

El papel de VIP endógeno en el sistema inmune deriva de estudios genéticos y funcionales en humanos, en ratones KO para VIP y ratones que o bien son KO para alguno de sus receptores o bien lo sobreexpresan. Así, ratones KO para VPAC2 muestran un aumento en una reacción Th1 como la respuesta de hipersensibilidad retardada (DTH), y una disminución en el título de anticuerpos IgE, probablemente debido a un aumento en la proporción entre las citoquinas Th1/Th2 producidas por los linfocitos T (179). De manera coherente, ratones que sobreexpresan VPAC2 presentan un aumento en respuestas de tipo Th2 (180, 181). Ratones deficientes en VIP o el receptor PAC1 muestran una mayor respuesta inflamatoria sistémica y son más susceptibles a morir por shock endotóxico (182, 183). En humanos, la expresión alterada de los receptores de VIP está relacionada con una aberrante respuesta de tipo Th1 en pacientes con EM y AR (184-186).

4.1.3.b. Efectos de VIP en células de la inmunidad innata

Existe una gran cantidad de resultados que indican que VIP es un potente desactivador de los macrófagos. Se ha demostrado que VIP inhibe, tanto in vitro como in vivo, la producción de NO y de las citoquinas proinflamatorias TNFα, IL6 y IL-12, al mismo tiempo que estimula la producción de los mediadores antiinflamatorios IL-10 e IL-1Ra, el receptor soluble de la IL-1β (164). VIP también inhibe la expresión de las quimioquinas KC, MIP2, MCP1, MIP1α, MIP1β y RANTES, las cuales actúan como quimioatractantes específicos de neutrófilos, monocitos/macrófagos y linfocitos T (164). De manera interesante, VIP tiene un efecto dual en la secreción de IL-6, dependiendo de la concentración de LPS utilizada como estímulo. Así, mientras que inhibe la producción de IL-6 a concentraciones superiores a 10 μg/ml, en macrófagos no estimulados o estimulados con concentraciones muy bajas de LPS, VIP estimula la secreción de IL-6 (187, 188). El uso de agonistas y antagonistas de los distintos receptores permitió establecer que aunque en menor medida PAC1 y VPAC2 están involucrados, VPAC1 es el principal receptor responsable de los efectos de VIP sobre los macrófagos (189). Esta capacidad de VIP para inhibir la producción de gran variedad de mediadores proinflamatorios

reside en el hecho que este neuropéptido regula distintas vías de transducción de señales y factores de transcripción que controlan la expresión de un amplio número de genes, muchos de los cuales están directamente implicados en la regulación de la inmunidad innata y adaptativa (189).

La disección molecular de los mecanismos implicados en las propiedades inmunomoduladoras de VIP en los macrófagos llevó a la identificación de dos vías, una independiente y otra dependiente de cAMP, siendo esta última la más estudiada (189). Los efectos de VIP sobre el NO y las citoquinas son ejercidos a nivel transcripcional mediante la regulación de varios factores de transcripción. En particular, VIP inhibe la translocación y transactivación de NF-κB (189).

Además de afectar la respuesta de los macrófagos, VIP también afecta a las DC, y consecuentemente, a la respuesta de linfocitos T. Mientras que en DC inmaduras, VIP induce quimiotaxis con una potencia similar a la quimioquina RANTES, en DC maduras inhibe la migración espontánea y la inducida por MIP-3β (190). En células de Langerhans estimuladas con LPS, VIP inhibe la producción de IL-12 e IL1β y aumenta la de IL-10 (191). Por otro lado, VIP inhibe en la producción de CXCL10, un quimioatractante de células Th1, y promueve la de CCL2, un quimioatrayente de células Th2, resultando en el reclutamiento de células Th2 efectoras in vitro e in vivo (192). Además, VIP afecta de manera diferencial la expresión de moléculas coestimuladoras según se trate de DC maduras o inmaduras. En DC inmaduras, VIP aumenta la expresión de CD86, con la concomitante proliferación de células T y su diferenciación en Th2 efectoras. Por otro lado, VIP disminuye la expresión de CD80 y CD86 en DC estimuladas con LPS, lo que reduce la capacidad de estas DC para estimular la proliferación de linfocitos T y la secreción de citoquinas Th1 y Th2 (193).

4.1.3.c. Efectos de VIP en células T

VIP puede afectar la diferenciación de linfocitos T $CD4^+$ y la proporción Th1/Th2 de dos maneras distintas. Por un lado, y como ya se mencionó, afectando las APC como las DC y macrófagos modifiacando el contexto de moléculas coestimuladoras y las citoquinas producidas por estas células. Por otro lado, VIP podría afectar directamente a linfocitos T durante su diferenciación. La presencia de VIP en cultivos de células T transgénicas con APC irradiadas produce un aumento de IL-4 y una disminución de IFNγ. Además, VIP también puede actuar sobre células Th1/Th2 efectoras. Tanto experimentos in vivo como in vitro indican que VIP

promueve la supervivencia y proliferación de células Th2, pero no Th1 efectoras. Es más, VIP es capaz de inducir células Th2 de memoria (164).

4.1.4. VIP como agente terapéutico

Teniendo en cuenta la capacidad para inhibir respuestas inflamatorias y su habilidad para desviar las respuestas T hacia un perfil Th2, VIP se convirtió en un factor terapéutico en distintos modelos de enfermedades inflamatorias y autoinmunes caracterizadas por una respuesta Th1.

4.1.4.a. Efecto de VIP en AR

El tratamiento de ratones con artritis inducida por CIA con VIP disminuye la frecuencia, retarda el desarrollo y reduce la severidad de la enfermedad (194). El efecto terapéutico de VIP está asociado con una notable reducción de los dos componentes deletéreos de la enfermedad, i.e. la respuesta inflamatoria y la autoinmune. De esta forma, VIP reduce la respuesta inflamatoria en las articulaciones mediante la reducción de la producción de distintos mediadores proinflamatorios y el aumento de la producción de IL-10 e IL-1Ra. Además, VIP reduce la respuesta de células T específicas del autoantígeno, desviando la respuesta hacia un perfil Th2, y por consiguiente, reduce el título de anticuerpos anti-colágeno del tipo IgG2a. De manera similar, también se ha encontrado que en células sinoviales de pacientes con artritis VIP inhibe la producción de TNFα e IL-8 (195).

4.1.4.b. Efecto de VIP en Enfermedad de Crohn

La enfermedad de Crohn es una enfermedad que se encuentra distribuida por todo el mundo, crónica, inflamatoria y que afecta al intestino delgado y a la mucosa del colon. Esta enfermedad, de etiología desconocida, está caracterizada por una exagerada respuesta inmune en los tejidos linfoides asociados al intestino, lo que produce una inflamación severa y prolongada de la mucosa intestinal. La inflamación local está asociada a una descontrolada producción de citoquinas proinflamatorias y a una activación y expansión oligoclonal de linfocitos T $CD4^+$, específicamente Th1. En un modelo murino de enfermedad de Crohn inducido por la administración intrarectal de TNBS, el tratamiento con VIP reduce la severidad clínica e histopatológica de la colitis, eliminando la pérdida de peso, la diarrea y la inflamación intestinal

(196). Como en el caso de CIA, la administración de VIP se encuentra asociada a una disminución de la respuesta inflamatoria y autoinmune de tipo Th1.

4.1.4.c. Efecto de VIP en shock séptico

Consecuentemente con su efecto inhibidor sobre la secreción de citoquinas proinflamatorias por macrófagos, la administración de VIP protege contra la mortalidad inducida por la inyección de altas dosis de LPS (197). Los ratones tratados con VIP no presentan las alteraciones histopatológicas carecterísticas del shock endotóxico, como coagulación intravascular diseminada, infiltración leucocitaria en distintos órganos, isquemia mesentérica y necrosis en los riñones. VIP actúa disminuyendo los mediadores inflamatorios TNFα, IFNγ, IL-6, IL-12 y NO y aumentando la producción de IL-10. Además, el efecto inhibitorio de VIP sobre la producción de quimioquinas es probable que también juegue un papel importante en su acción preventiva en la endotoxemia, ya que la migración de células inflamatorias a los órganos es uno de los eventos iniciales durante el desarrollo del shock séptico.

4.2. Grelina (GHR)

En 1999 Kojima *et al* identificaron y clonaron el péptido grelina (GHR), ligando endógeno de, hasta el momento, un GPCR huérfano (198). El gen de GHR codifica para un péptido de 117 aminoácidos, prepro-grelina, que posee un 82% de homología entre rata y humano. De manera similar a VIP, prepro-grelina es procesada en una forma madura de 28 aminoácidos que es secretada. GHR es la única hormona que es acilada en su tercer residuo de serina. Esta octanoilación es esencial para que la GHR se una a su receptor GHS-R y ejerza sus actividades biológicas. El estómago es la principal fuente de GHR periférica, aunque estudios recientes muestran que GHR se encuentra ampliamente distribuido por el organismo. El mRNA es expresado en el hipotálamo, yeyuno, duodeno, colon, pulmones, hígado, tejido adiposo y placenta (199). Además, también está expresado en tejidos linfoides como timo, bazo y ganglios linfáticos (199).

4.2.1. Producción de GHR por células inmunes

En cuanto a células del sistema inmune, el mRNA de GHR está expresado en líneas leucémicas B y T y en líneas mieloides (200). Tanto linfocitos T y B como DC, monocitos y neutrófilos secretan GHR (acilada y desacilada) cuando son activados (200, 201). En particular, se ha reportado que linfocitos T humanos expresan prepro-GHR en el aparato de Golgi, donde probablemente es procesada y clivada a su forma madura. Los linfocitos T expresan GHR de manera polarizada en asociación con las balsas lipídicas $GM1^+$ (201).

4.2.2. Expresión receptores de GHR en células inmunes

El gen del receptor de GHR, ghs-r, se expresa en dos formas provenientes de un *splicing* alternativo, ghs-r1a y ghs-r1b. GHS-R1a es un GPCR de 366 amino ácidos y siete dominios transmembrana (202), mientras que la variante GHS-R1b parece ser una receptor no funcional (203).

Si bien originalmente se creía que el receptor GHS-R1a estaba expresado exclusivamente en la pituitaria y el hipotálamo, más tarde se encontró que este receptor está ampliamente expresado en distintos órganos y varias formas de cáncer (199, 204). Al igual que su ligando GHR, el mRNA de GHS-R1a se encuentra expresado en órganos linfoides, linfocitos T, B, DC y monocitos (205). En linfocitos T humanos no activados, GHS-R se encuentra en la superficie celular de manera difusa, pero al ser activados vía el TCR, los niveles de mRNA y de proteína aumentan varias veces, al mismo tiempo que el GHS-R se redistribuye a las balsas lipídicas mostrando un patrón polarizado similar al de los receptores de quimioquinas (201).

4.2.3. Efectos biológicos

La primera función biológica que se le asignó a GHR fue su capacidad para inducir la liberación de la hormona de crecimiento (GH) de la glándula pituitaria. Más tarde, se encontró que GHR incrementa la adiposidad y es un potente inductor de la ingestión de alimentos (206, 207). El principal órgano productor de GHR es el estómago y durante estados de ayuno, GHR es liberado a la circulación sistémica, donde cruza la BBB para alcanzar el hipotálamo e inducir hambre (208, 209) (tabla I).

4.2.3.a. Efectos de GHR endógeno en el sistema inmune

Si bien existen ratones KO tanto para GHR (210) como para GHS-R (211), hasta el momento los datos sobre el funcionamiento del sistema inmune de estos ratones son escasos. Recientemente se demostró que los KO para GHR y GHS-R presentan una acelerada involución tímica asociada con la edad que se manifiesta con una reducción en la cantidad de células epiteliales tímicas y un aumento en los fibroblastos adipogénicos del timo (212, 213). Ratones de 24 meses de edad deficientes en GHR o GHS-R poseen un timo más pequeño, menor cantidad de timocitos y muestran una reducción en la diversidad del TCR en linfocitos T $CD4^+$ de bazo (212).

4.2.3.b. Efectos de GHR en células de la inmunidad innata

Se ha demostrado que GHR ejerce potentes efectos inhibitorios sobre la activación de monocitos y células endoteliales humanas. GHR inhibe la translocación de NF-κB y la secreción de IL-8 por células endoteliales estimuladas con TNF o H_2O_2 (214) y reduce la expresión y secreción de TNF, IL-6 e IL-1β y quimioquinas por monocitos y DC estimuladas con LPS o leptina (167, 201). Además, se ha reportado que GHR disminuye la producción de IL-6 y NO por macrófagos peritoneales de rata estimulados con LPS (215).

4.2.3.c. Efectos de GHR en células T

En linfocitos T de bazo estimulados vía el TCR, GHR inhibe la proliferación y la expresión de mRNA de citoquinas Th1 (IL-2 e IFNγ) y Th2 (IL-4 e IL-10) (216). En linfocitos T humanos estimulados por el TCR o leptina, y al igual que en el caso de monocitos, GHR inhibe la expresión y secreción de las citoquinas TNF, IL-6 e IL-1β. Además, GHR induce la movilización de calcio intracelular en linfocitos T (201).

4.2.4. GHR como agente terapéutico

GHR ha sido utilizada como agente terapéutico en distintos modelos de enfermedades no sólo por su capacidad para inhibir la activación de linfocitos T, DC, monocitos y células endoteliales, sino también por su habilidad para revertir estados energéticos desfavorables (217).

4.2.4.a. Efecto de GHR en AR

El tratamiento de ratas con CIA con un agonista del receptor de GHS-R disminuye la severidad de la enfermedad y los niveles séricos de IL-6 y NO (215). Por otro lado, se ha observado que los osteoblastos expresan GHS-R y que GHR aumenta su proliferación y diferenciación. Es más, la administración de GHR aumenta la formación de hueso (218).

4.2.4.b. Efecto de GHR en Enfermedad de Crohn

La administración de GHR a ratones con colitis inducida por TNBS aumenta la supervivencia y disminuye los signos clínicos e histopatológicos asociados con la enfermedad, tales como pérdida de peso, diarrea e inflamación. El efecto terapéutico de GHR está asociado a una disminución en la producción de un amplio espectro de mediadores inflamatorios y a una reducción en la respuesta autoinmune de tipo Th1 junto con un incremento en las poblaciones de Tr1/Th3 (219).

4.2.4.c. Efecto de GHR en shock séptico y sepsis

En modelos de endotoxemia, se ha demostrado que la administración de GHR aumenta la supervivencia y revierte la hipotensión y la pérdida de peso inducida por LPS (220, 221). Durante la endotoxemia, GHR exógena también reduce la concentración en suero de TNF, MCP-1, IL-1β, IL-6, IL-1 y los niveles de mRNA de TNF, IL-1β e IL-6 en bazo e hígado (201, 214). Por otro lado, se ha observado que durante la sepsis inducida por CLP el receptor GHS-R aumenta su expresión en el sistema vascular, mientras que la administración de GHR mejora la perfusión de los tejidos y reduce los niveles de endotelina-1 (222, 223)

4.3. Urocortina

Urocortina (UCN) es un péptido de 40 aminoácidos perteneciente a la familia CRF (corticotrophin-releasing factor), con un 90% de homología entre rata y humano e inicialmente caracterizado por sus efectos en el apetito y el sistema cardiovascular. UCN está ampliamente distribuido por los sistemas digestivo, nervioso central, cardiovascular, reproductivo, endocrino e inmune (159) (tabla II).

4.4. Adrenomedulina

Adrenomedulina (AM) es un péptido de 52 aminoácidos con un puente disulfuro entre los residuos 16 y 21, que es sintetizado como parte de una proteína precursora, prepro-adrenomedulina de 185 aminoácidos. AM está expresado en por todo el organismo, aunque las células endoteliales son su principal fuente (158) (tabla II).

4.3. Cortistatina

Cortistatina (CST) es un neuropéptido cíclico de 17 o 29 aminoácidos que presenta gran homología con somatostatina, con quien comparte muchas de sus propiedades farmacológicas y funcionales. Identificada originalmente en el CNS, CST también está presente en otros tejidos, incluyendo los pertenecientes al sistema inmune (168)(tabla II).

	VIP	GHR	AM	UCN	CST
Familia	PACAP secretin glucagon GHRH	motilin	CGRP	CRF	SOM
Expresión en el sistema inmune	mastocitos, PMN, linfocitos CD4 Th2 y CD8	monocitos, DC, linfocitos T y B	macrófagos, monocitos, granulocitos, linfocitos T (158, 166)	macrófagos, monocitos mastocitos, linfocitos T y B (159, 224).	macrófagos, monocitos, DC, linfocitos T (163, 225)
Receptores	VPAC1, VPAC2, PAC1	GHRS-R	CRLR/RAMP1, CRLR/RAMP2, CRLR/RAMP3	CRFR1 y CRFR2	Sstl-5 y GHS-R
Expresión de R en células inmunes	macrófagos, monocitos, PMN, DC, linfocitos T y B	monocitos, PMD, DC, linfocitos T y B	macrófagos, DC, linfocitos T y B	macrófagos, monocitos, DC, PMN, linfocitos T (226)	macrófagos, monocitos, DC, linfocitos T y B (168)
Efectos en el sistema inmune	↓ mediadores inflamatorios ↑ mediadores anti inflamatorios ↓ coestimuladoras macrófagos y DC ↓ proliferación células T ↓ expresión TLR ↓ respuesta Th1 ↑ respuesta Th2	↓ activacion células endoteliales ↓ secreción de TNF, IL-6, IL 1β por monocitos ↓ proliferación célulasT ↓ citoquinas de respuesta Th1 y Th2: IL-2, IFNγ, TNF, IL-4, IL1β ↑ timopoiesis	↓ TNF en células de Kupffer estimuladas con LPS (227) ↓ expresión de CD1 1β en PMN (228) ↓ apoptosis en células endoteliales (229) ↑ moléculas de adhesión en HUVEC (230)	↓ TNF en células de Kupffer estimuladas con LPS (231) ↑ apoptosis en macrófagos (232) ↑ desgranulación de mastocitos y permeabilidad vascular (233)	
Efecto terapéutico	Shock séptico, AR, EM, IBD, EAE, diabetes tipo I, uveoretinitis, pancreatitis, keratitis, síndrome de Sjogren	Shock séptico, AR, IBD, caquexia (217) .		EAE (234)	

	VIP	GHR	AM	UCN	CST
EFECTOS PRINCIPALES	vasodilatación ↑ volumen minuto del corazón relajación del músculo liso broncodilatación ↑ crecimiento analgesia hyperglicemia hipertermia efectos neurotróficos metabolismo óseo movilidad gástrica secreción de GI	vasodilatación ↑ volumen minuto del corazón ↑ apetito y adiposidad ↑ hormona de crecimiento ↑ secreción de GI ↑ movilidad gástrica	vasodilatación ↑ volumen minuto del corazón relajación del músculo liso broncodilatación	vasodilatación ↑ volumen minuto del corazón relajación del músculo liso broncodilatación ↓ ingesta de comidas ↑ secreción de ACTH	↓ actividad locomotora ↑ sueño de ondas lentas ↓ hormona de crecimiento ↓ proliferación celular

OBJETIVOS

Como se expuso anteriormente, por un lado existe una alta prevalencia de enfermedades inflamatorias y autoinmunes en las que se observa un claro desbalance entre mediadores proinflamatorios y antiinflamatorios y entre células Th1/Th17 autoreactivas y células Treg. Mientras que por otro lado, los neuropéptidos cortistatina, adrenomedulina, urocortina, grelina y VIP, aunque originalmente descritos como mediadores neuroendócrinos, *(1)* son producidos por células del sistema inmune, *(2)* sus receptores están expresados en células del sistema inmune, *(3)* estos receptores señalizan por la vía inmunosupresora AMPc/PKA. Por lo tanto, el objetivo general de esta tesis es evaluar la capacidad de cortistatina, adrenomedulina, urocortina, grelina y VIP para modular la respuesta inmune y restablecer la homeostasis en distintas enfermedades de origen inflamatorio y/o autoinmune.

Así, los objetivos concretos son:

1. Evaluar la habilidad de cortistatina, adrenomedulina, urocortina, grelina y VIP para regular la producción de mediadores críticos de la respuesta inflamatoria y la posible acción terapéutica en modelos de sepsis y shock séptico.
2. Determinar la capacidad de VIP para inducir tolerancia en condiciones de autoinmunidad mediante la generación de células dendríticas tolerogénicas.
3. Estudiar la capacidad de cortistatina para regular la respuesta inflamatoria y del tipo Th1 en artritis inducida por colágeno.

ANEXO ARTÍCULOS

Cortistatin, a new antiinflammatory peptide with therapeutic effect on lethal endotoxemia

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Cortistatin is a recently discovered cyclic neuropeptide related to somatostatin that has emerged as a potential endogenous antiinflammatory factor based on its production by and binding to immune cells. Because human septic shock involves excessive inflammatory cytokine production, we investigated the effect of cortistatin on the production of inflammatory mediators and its therapeutic action in various murine models of endotoxemia. Cortistatin down-regulated the production of inflammatory mediators by endotoxin-activated macrophages. The administration of cortistatin protected against lethality after cecal ligation and puncture, or injection of bacterial endotoxin or *Escherichia coli*, and prevented the septic shock-associated histopathology, such as infiltration of inflammatory cells and intravascular disseminated coagulation in various target organs. The therapeutic effect of cortistatin was mediated by decreasing the local and systemic levels of a wide spectrum of inflammatory mediators, including cytokines, chemokines, and acute phase proteins. The combined use of cortistatin and other antiinflammatory peptides was very efficient treating murine septic shock. This work provides the first evidence of cortistatin as a new immunomodulatory factor with the capacity to deactivate the inflammatory response. Cortistatin represents a potential multistep therapeutic agent for human septic shock, to be used in combination with other immunomodulatory agents or as a complement to other therapies.

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Abbreviations used: APP, acute phase protein; CLP, cecal ligation and puncture; MPO, myeloperoxidase; NO, nitric oxide; RANTES, regulated on activation, normal T cell expressed and secreted; SAA, serum amyloid A; VIP, vasoactive intestinal peptide.

Septic shock is a systemic response to severe bacterial infections, generally caused by Gram-negative bacterial endotoxins. Indeed, the administration of the endotoxin LPS to experimental animals leads to pathophysiologic changes similar to human septic shock syndrome, and lethal endotoxemia has been extensively used as an experimental model of Gram-negative septic shock (1). The septic shock syndrome is characterized by a hyperactive and out-of-balance network of endogenous proinflammatory cytokines, including TNFα, IL-12, IL-6, and IFNγ (2). The overproduction of inflammatory cytokines generates systemic activation, which affects vascular permeability and cardiac function and induces metabolic changes that can lead to tissue necrosis and eventually to multiple-organ failure and death. The current therapeutic strategies for human septic shock are designed to neutralize one or more of the inflammatory mediators, and none of them are entirely effective (3, 4). This illustrates the need for novel therapeutic approaches to down-regulate the exacerbated inflammatory response typical of endotoxemia.

Cortistatin is a recently discovered cyclic neuropeptide named after its predominantly cortical expression and ability to depress cortical activity (5). Cortistatin shows a high homology with somatostatin; however, based on nucleotide sequence and chromosomal location, they are products of separate genes. Cortistatin binds to all five cloned somatostatin receptors and shares many pharmacological and functional properties with somatostatin, including the depression of neuronal activity and inhibition of cell proliferation (6). However, cortistatin also has many distinct properties, including induction of slow-wave sleep and reduction of locomotor activity (6). Cortistatin, but not somatostatin, has been detected in various human immune cells, including lymphocytes, monocytes, macrophages, and dendritic cells (7–9). Therefore, some of the somatostatin immunomodulatory actions could be shared by cortistatin. Because cortistatin levels correlate with the degree of inflammatory cell differentiation and activation (7, 8), this peptide could function as a major endogenous regulatory factor in the immune system. In addition

to somatostatin receptors, cortistatin can bind to other hormone receptors that mediate antiinflammatory actions, such as the receptor for the growth hormone secretagogue ghrelin (10–12). The aim of this study is to investigate the potential antiinflammatory action of cortistatin and its therapeutic use in murine models of lethal endotoxemia.

RESULTS

Cortistatin inhibits production of inflammatory mediators by activated macrophages in vitro

Macrophages, major participants in innate immunity, contribute to the initiation of the inflammatory response by killing pathogens through phagocytosis, release of cytotoxic oxygen and nitrogen intermediates, and release of chemokine and cytokines that attract and activate other immune cells. Despite the beneficial role of inflammatory factors in host defense, their sustained production can lead to serious pathological conditions. Therefore, although necessary for the elimination of pathogens, macrophage activation leads to serious deleterious effects in the host if left unchecked. To investigate the potential antiinflammatory action of cortistatin, we evaluated first the effect of cortistatin on the production of several inflammatory mediators by peritoneal macrophages. Cortistatin inhibited the production of TNFα, IL-6, and nitric oxide (NO) by activated macrophages (Fig. 1 A). This effect was dose dependent with a maximal effect at 10^{-8} M (Fig. 1 B), a concentration that is within the physiological range (6). Interestingly, cortistatin showed higher inhibitory effect on TNFα, IL-6, and NO production than the structurally related peptide somatostatin, or the somatostatin receptor agonist octreotide (Fig. 1 B). In addition, although the somatostatin receptor antagonist cyclosomatostatin fully blocked the effect of somatostatin, it only partially reversed the inhibitory effect of cortistatin (Fig. 1 C), suggesting that cortistatin could exert its effects through both somatostatin receptor-dependent and -independent mechanisms.

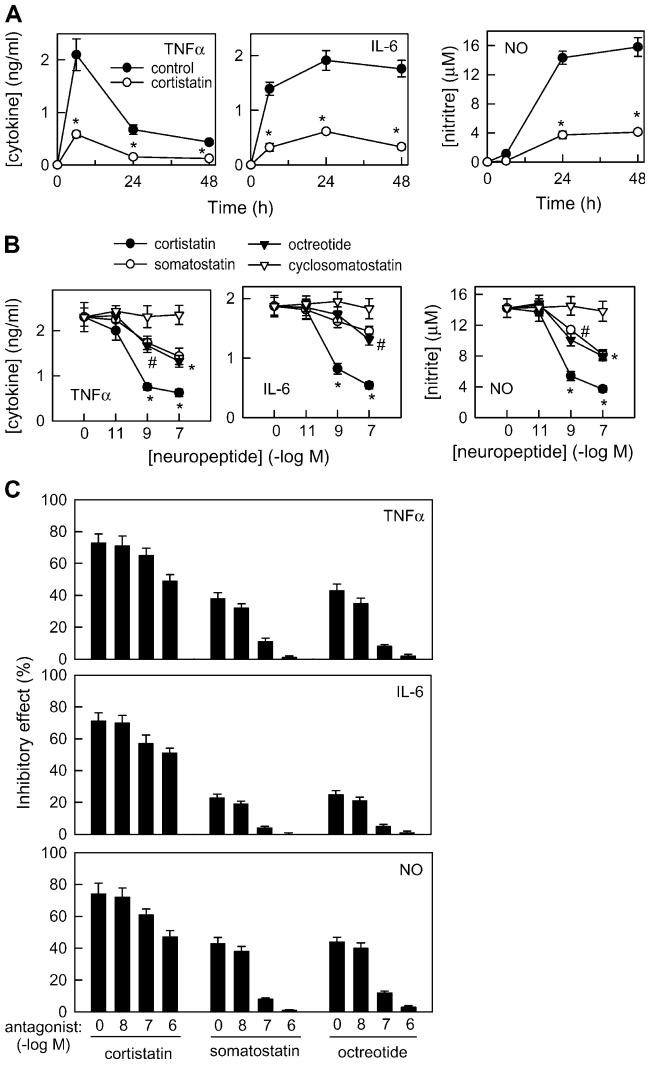

Figure 1. Cortistatin inhibits the production of inflammatory mediators by activated macrophages in vitro. (A) Peritoneal macrophages were stimulated with 1 μg/ml LPS in the absence (control) or presence of 10^{-8} M cortistatin. After different times, proinflammatory mediators were determined ($n = 6$). *, $P < 0.001$ versus untreated control mice. (B) Peritoneal macrophages were stimulated with 1 μg/ml LPS in the absence (control) or presence of different concentrations of cortistatin, somatostatin, octreotide, or cyclosomatostatin. After 24 h, proinflammatory mediators were determined ($n = 5$). *, $P < 0.001$, #, $P < 0.05$ versus untreated control mice. (C) Peritoneal macrophages were stimulated with 1 μg/ml LPS and treated with cortistatin, somatostatin, or octreotide (10^{-8} M) in the presence of different concentrations of the antagonist cyclosomatostatin ($n = 4$). Results show the percentage of inhibition of cytokine/NO production in comparison to LPS-treated controls.

Treatment with cortistatin protects against lethal endotoxemia and reduces the related histopathology

Because cortistatin inhibits the production of proinflammatory macrophage-derived factors crucial for the development of septic shock, we expected a protective effect in high-dose endotoxemia, a murine model for septic shock syndrome. Mice were injected with different doses of LPS, treated 30 min later with cortistatin (2 nmol/mouse; 250 μg/kg), and the survival was monitored. Treatment with cortistatin significantly protected against the lethal effect of LPS (Fig. 2 A). The protective effect was observed over a wide range of LPS doses, with cortistatin shifting the LD50 from 100 to 450 μg LPS (Fig. 2 B). Animals injected with cortistatin had a survival rate of 80%; even for the nonsurvivors, cortistatin doubled the time until death (Fig. 2 C). The effect of cortistatin was dose dependent, with doses as low as 0.5 nmol (50 μg/kg) being partially protective (Fig. 2 D). In contrast, somatostatin and octreotide, or the control peptide cyclosomatostatin were not protective (Fig. 2 E). Because the production of inflammatory cytokines occurs in a rapid sequence starting with TNFα, which reaches a maximum 2 h after LPS infusion (see Fig. 5), we next investigated the therapeutic effect of cortistatin in conditions of already established septic shock. Kinetic studies showed that cortistatin exerted a full protective action

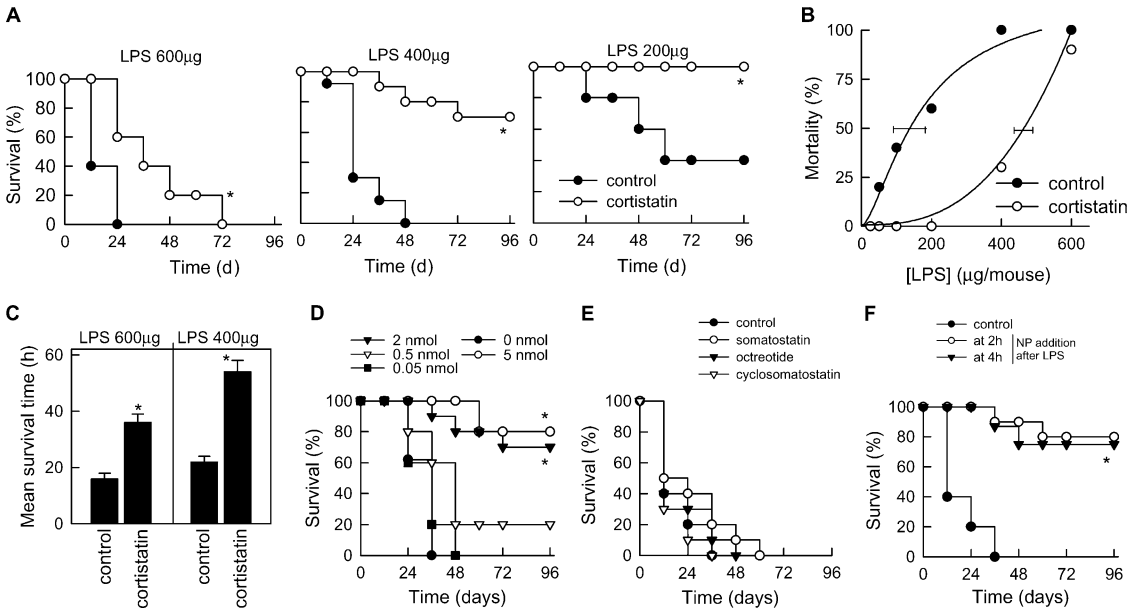

Figure 2. Treatment with cortistatin protects against lethal endotoxemia. (A–C) BALB/c mice were injected i.p. with different doses of LPS (control). Cortistatin (2 nmol/mouse) was injected i.p. 30 min after LPS administration. Survival was monitored during the next 96 h. Similar results were obtained in three identical independent experiments or when C57BL/6 mice were used. Mortality curves in B were used lo calculate LD50, and horizontal bars indicate the 95% confidence limits of LD50 determinations. (C) The average survival time was calculated for nonsurvivors in both the untreated and cortistatin-treated groups. (D) Mice were injected i.p. with 400 μg LPS and different doses of cortistatin (from 0 to 5 nmol/animal). (E) Mice were injected i.p. with 400 μg LPS and, 30 min later, treated with medium (control), cortistatin, somatostatin, octreotide or cyclosomatostatin (2 nmol/mouse). Survival was monitored during the next 96 h. (F) Cortistatin (2 nmol/mouse) was injected i.p. 2 or 4 h after LPS challenge. $n = 12$–20 mice/group. *, $P < 0.001$ versus untreated control mice.

when given up to 4 h after shock induction (Fig. 2 F), with decreased protection for later cortistatin administration (not depicted).

Endotoxemic animals suffered from disseminated intravascular coagulation with multiple organ failure as indicated by severe congestion, hemorrhage, hyperemia, fibrin deposits, edema, thrombosis, and massive accumulation of leukocytes in lungs and the intestinal tract, as well as severe congestion of the hepatic sinusoids, hepatocyte necrosis, segmental ischemia of the bowel with regions of hemorrhage or necrosis, and an infracted cecum (Fig. 3 A and not depicted). Neutrophil infiltration in the liver, lungs, and intestine of endotoxemic animals was confirmed through measurements of the myeloperoxidase (MPO) activity (Fig. 3 B). Endotoxic shock induction was accompanied by an early granulocyte (mainly neutrophils) infiltration in the peritoneum, followed by later recruitment of macrophages and lymphocytes (Fig. 3 C). In contrast, cortistatin-treated animals did not present any of the histopathologic alterations associated with septic shock, such as disseminated intravascular coagulation, leukocyte infiltration, and inflammation in various organs and mesenteric ischemia, tissue congestion, and hemorrhage (Fig. 3).

Cortistatin improves survival in experimental sepsis

We next investigated the potential therapeutic effect of cortistatin in the murine model of cecal ligation and puncture (CLP), a clinically relevant model for human sepsis because it causes lethal peritonitis produced by polymicrobial infection. Cortistatin treatment was started 4 h after the induction of sepsis, at a time when mice show clear signs of sepsis, including lethargy, piloerection, diarrhea, huddling, fever, and malaise. Cortistatin treatment significantly improved survival (Fig. 4 A), and attenuated the clinical manifestations of sepsis, such as lethargy, diarrhea, body weight loss, and hypothermia (not depicted). We also examined whether delayed cortistatin treatment protects mice against sepsis induced by direct i.p. injection of live *Escherichia coli*. The mortality induced by injection of 10^8 live *E. coli* cells was decreased by cortistatin (Fig. 4 B). In both models, we did not observe any late deaths (up to 3 wk), indicating that cortistatin treatment confers lasting protection.

Cortistatin treatment reduces local and systemic inflammatory responses in endotoxemic mice

We next evaluated the effect of cortistatin treatment on the production of inflammatory mediators that are mechanistically linked to endotoxemia. Cortistatin administration reduced the levels of endotoxin-induced inflammatory cytokines (TNFα, IFNγ, IL-6, IL-1β, and IL-12), chemokines (RANTES [regulated on activation, normal T cell expressed and secreted] and MIP-2), and NO, in serum (systemic) and in various target organs, including peritoneum, liver, lung, and intestine (Fig. 5). In addition, cortistatin administration

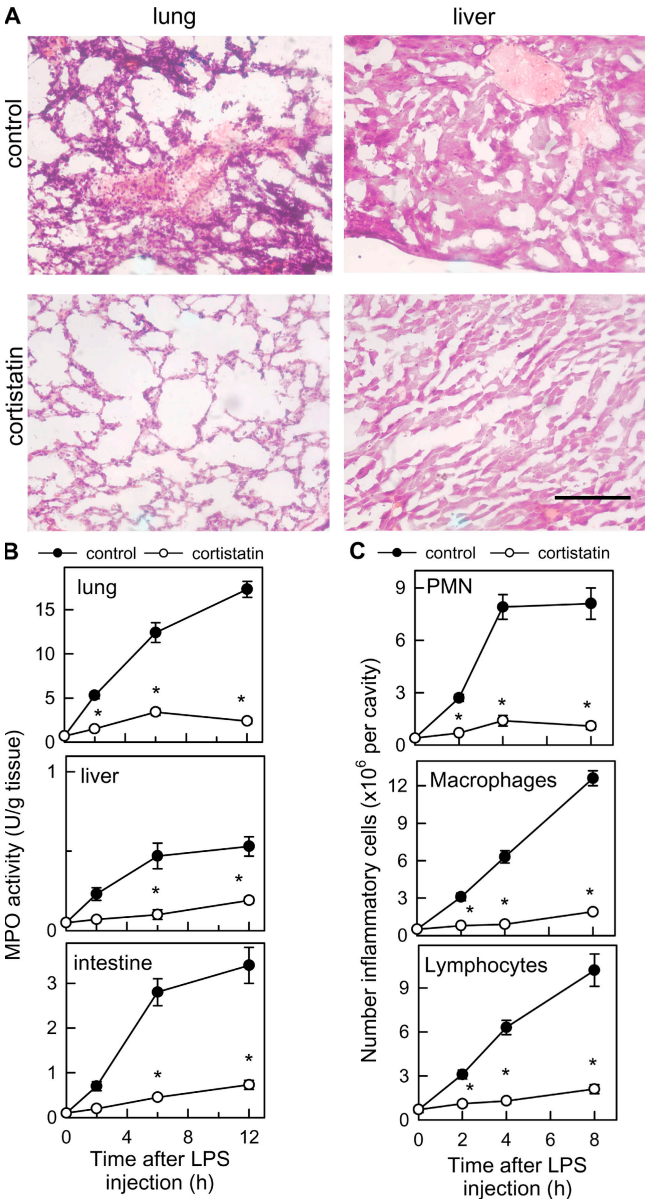

Figure 3. Cortistatin treatment reduces histopathologic signs of endotoxemia. Mice were injected i.p. with LPS (control). Cortistatin (2 nmol/mouse) was administered i.p. 30 min after LPS administration. (A) Cortistatin reduces inflammatory infiltration and disseminated coagulation in target organs. Histopathology analysis was determined in hematoxylin and eosin–stained sections of lung and liver obtained at 24 h of disease. Bar, 100 μm. (B) Cortistatin decreases MPO activity in lung, liver, and intestine. MPO content was determined at different times after LPS infusion. (C) Cortistatin reduces leukocyte recruitment in the peritoneal cavity. Peritoneal cell suspensions were obtained at different times after LPS injection, and numbers of macrophages, lymphocytes, and PMNs were determined by flow cytometry. $n = 8$–12 mice/group. *, $P < 0.001$ versus untreated control mice.

increased the systemic and local levels of the antiinflammatory cytokine IL-10 (Fig. 5), which has been shown to be protective in endotoxic shock (13).

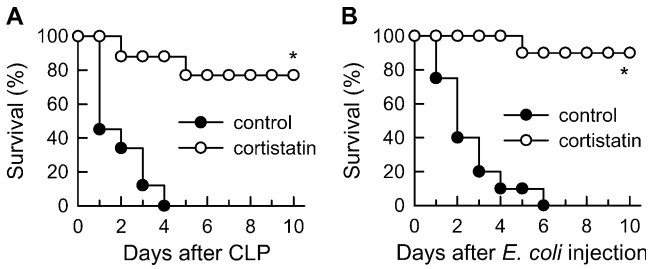

Figure 4. Cortistatin protects against other types of experimental sepsis. (A) Sepsis was induced in C57BL/6 mice after cecal ligation and puncture (CLP). 2 nmol cortistatin was administered i.p. twice at 6-h intervals beginning 4 h after CLP procedure. Survival was monitored over the next 8 d. $n = 10$ mice/group. *, $P < 0.001$ versus untreated control mice. (B) Endotoxemia was induced in BALB/c mice after i.p. injection of *E. coli* (10^8 cells), and medium (control) or 2 nmol cortistatin was administered i.p. 2 h later. $n = 8$ mice/group. *, $P < 0.001$ versus untreated control mice.

Coagulation abnormalities, especially disseminated intravascular coagulation and microthrombosis, are common features during sepsis (1–4, 14). Acute phase proteins (APPs) are involved in the initiation of aberrant coagulation during endotoxic shock (14). Cortistatin dramatically decreased the systemic levels of serum amyloid A (SAA) during septic shock (Fig. 5 B).

Cortistatin deactivates peritoneal macrophages during endotoxemia

The cortistatin-induced decrease in the levels of inflammatory factors in the target organs could result from the reduction in inflammatory cell infiltration. However, the fact that cortistatin down-regulates the in vitro production of various inflammatory factors by activated macrophages (Fig. 1) argues against this hypothesis. We determined the capacity of peritoneal macrophages isolated from untreated or cortistatin-treated endotoxemic mice to produce inflammatory mediators ex vivo. Although macrophages from mice with septic shock produced spontaneously high amounts of TNFα, IL-6, IL-12, RANTES, and MIP-2, macrophages isolated from cortistatin-treated animals produced significantly lower levels of proinflammatory cytokines and secreted high amounts of IL-10 (Fig. 6). Therefore, it is possible that the deactivation of resident and infiltrating macrophages is the major mechanism in the antiinflammatory action of cortistatin.

The combined therapy of cortistatin and other antiinflammatory neuropeptides protects against lethal endotoxemia

We next evaluated the effect of the combined treatment with cortistatin and another endogenous antiinflammatory peptide (15), the vasoactive intestinal peptide (VIP). Administration of cortistatin together with VIP significantly prevented the lethality (50% survival) induced by a high dose of LPS (600 μg/mouse), compared with mice treated with cortistatin or VIP alone that did not survive (Fig. 7, left). In addition, this

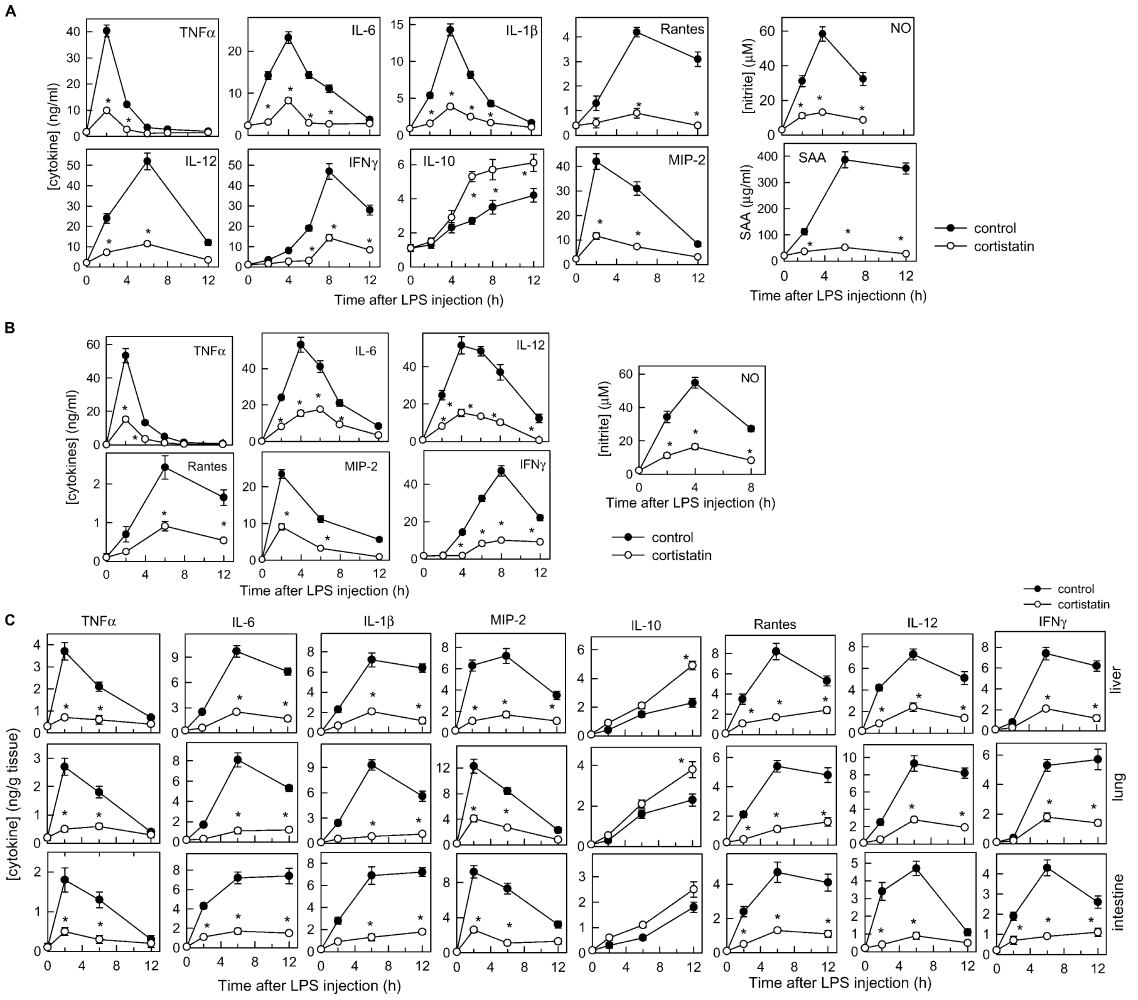

Figure 5. Treatment with cortistatin reduces local and systemic inflammatory responses in endotoxemic mice. Endotoxemia was induced by i.p. injection of LPS (400 μg/mouse). Mice were treated 30 min later with medium (controls) or with cortistatin (2 nmol/mouse). Serum (A), peritoneal fluid (B), and protein extracts from lung, liver, and small intestine (C) were collected at various time points after endotoxin injection, and the cytokine/chemokine, NO, and SAA contents were determined as described in Materials and methods. $n = 5$–8 mice/group. *, $P < 0.001$ versus untreated control mice.

combined treatment permits to reduce the effective therapeutic dose to 0.5 nmol cortistatin (Fig. 7, right). The synergistic effect of VIP and cortistatin in endotoxic shock could be the result of effects on different cells or to the involvement of different signal transduction pathways in the same target cell. We favor the latter because activated macrophages are the major players in the antiinflammatory activity of both peptides, and VIP acts through cAMP-dependent pathways (16), whereas the cortistatin effect should be cAMP independent (9).

DISCUSSION

Septic shock is a life-threatening complication of infections and the most common cause of death in intensive care units (750,000 cases per year in the United States with a mortality rate of 30%). Although our understanding of the pathogenesis of inflammation and sepsis has improved, until recently this has not translated into clinical benefit. The current strategies for human septic shock therapy are derived mainly from observations made in animal models. Promising experimental results prompted large-scale randomized clinical trials with a variety of agents, such as antiendotoxin monoclonal antibodies, glucocorticoids, or ibuprofen for nonspecific downregulation of inflammation, IL-1 receptor antagonists, or anti-TNFα antibodies (3, 4). Unfortunately, despite some promising results during the preliminary trials, all the major clinical studies of immunomodulators in sepsis were disappointing (17). There are several reasons for this lack of success. In animal models, the cascade of events starting with the initial stimulus, resulting in a cytokine cascade and leading to death follows a predictable time course. Thus, experimental protocols designed to block one cytokine cascade or another are relatively straightforward. However, in the human septic shock syndrome, the sequence of events is more complex,

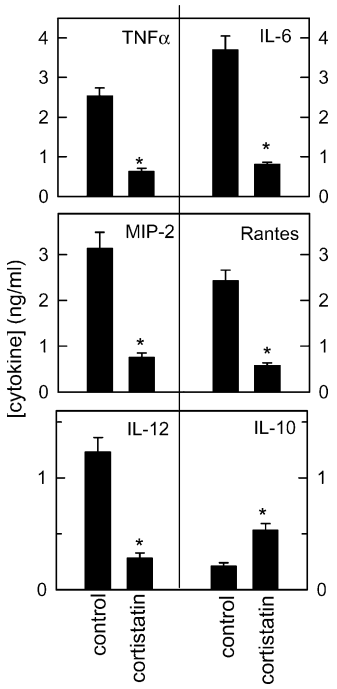

Figure 6. Cortistatin treatment deactivates peritoneal macrophages during endotoxemia. Mice were injected i.p. with LPS (control) and treated i.p. with cortistatin (2 nmol/mouse) 30 min later. Peritoneal macrophages were isolated 8 h after LPS injection and cultured with medium alone. After 48 h of culture, the concentration of inflammatory mediators in the culture supernatants was determined by ELISA ($n = 5$–6). *, $P < 0.001$ versus untreated control mice.

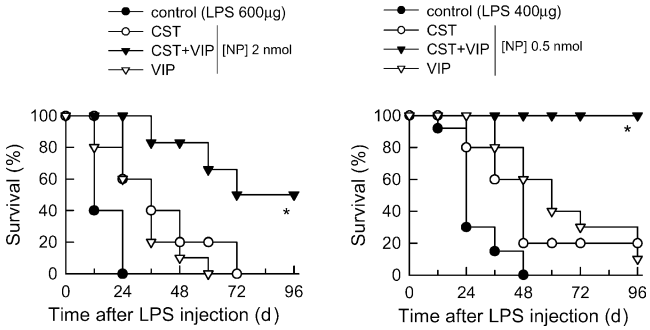

Figure 7. Combined therapy of cortistatin and other antiinflammatory neuropeptides protects against lethal endotoxemia. Septic shock was induced by i.p. injection of LPS (600 or 400 μg/mouse). Mice were treated 30 min later with medium (controls) or with various combinations of the neuropeptides (NP), cortistatin (CST), and vasoactive intestinal peptide (VIP) (2 or 0.5 nmol/mouse). Survival was monitored during 96 h. $n = 8$–10 mice/group. *, $P < 0.001$ versus mice treated with cortistatin or VIP alone.

and the course of the disease generally lasts days rather than hours, as in most animal models. Therefore, because of the complex pathophysiology of sepsis, consideration should be given to multidrug therapy, similar to the approach taken in AIDS and cancer therapy. The present work proposes a new therapy for endotoxemia based on the use of the newly discovered somatostatin-related peptide cortistatin. The administration of cortistatin protected against endotoxin-induced lethality. In addition, administration of cortistatin improved survival in two "true infection" models closer to sepsis in humans, such as cecal ligation and puncture and *E. coli* injection. Cortistatin prevented the septic shock-associated histopathology, including inflammatory cell infiltration and multiorgan intravascular disseminated coagulation. The therapeutic effect of cortistatin seems to be mediated through the decrease in the local and systemic levels of a wide spectrum of inflammatory mediators, including cytokines (TNFα, IL-6, IL-1β, IFNγ, and IL-12), chemokines (MIP-2 and RANTES) and acute phase proteins. The fact that the inhibitory effects of cortistatin on the release of inflammatory mediators in vitro by peritoneal macrophages resembled closely the cytokine profile in mice treated with cortistatin suggests that the deactivation of resident and infiltrating macrophages could be the major mechanism involved in the antiinflammatory action of cortistatin. However, because cortistatin receptors are ubiquitously expressed by monocytes/macrophages, dendritic cells, neutrophils, and endothelial cells (7–9), the participation of cells other than macrophages cannot be ruled out.

Among the proinflammatory cytokines involved in endotoxic shock, TNFα appears to play a central role. Indeed, increased serum TNFα levels appear during endotoxemia and TNFα injection induces shock, tissue damage, and death (18). During endotoxic shock, TNFα shows a spectrum of harmful effects. These infects include enhanced procoagulant activity of vascular endothelial cells, activation of neutrophils and macrophages, and increase in combination with IFNγ in the expression of adherent molecules resulting in increased neutrophil/monocyte adherence to endothelial cells and tissue infiltration. Lung and liver injury during endotoxemia is largely mediated through neutrophil and macrophage accumulation (19). The excessive production of free radicals by activated macrophages and neutrophils results in tissue damage. The decrease in NO serum levels in cortistatin-treated mice is beneficial because NO mediates hypotension and cytotoxicity, two pathologic hallmarks of septic shock (1–4). In addition, TNFα, IL-1β, and IL-6 affect hepatocytes by modifying their metabolic pathway toward gluconeogenesis and aminoacid uptake, as well as increased synthesis of coagulation and complement factors and of APPs. APPs are antiproteolitic enzymes that contribute to the procoagulant state and to the inhibition of fibrinolysis observed in sepsis. During septic shock, there is an increase in the so-called positive APPs (α1-antitrypsin, α2-macroglobulin, serum amyloid A, fibrinogen, and C-reactive protein), a decrease in negative APPs (protein C and antithrombin), and an increase in the thrombin-activated fibrinolytic inhibitor (14). Coagulation abnormalities, especially disseminated intravascular coagulation and microthrombosis, are common in patients with sepsis (1–4, 14). In our study, cortistatin dramatically decreased the systemic levels of the positive APP SAA during septic

shock. This correlates with the fact that animals treated with cortistatin did not exhibit disseminated coagulation in any of the organs studied. The fact that cortistatin reduced local and systemic levels of the chemokines MIP-2 (chemotactic for neutrophils) and RANTES (chemotactic for macrophages/monocytes) partially explains the absence of inflammatory infiltrates in the affected organs.

TNFα is at the pinnacle of a cascade of inflammatory mediators. During septic shock, TNFα precedes other proinflammatory cytokines (Fig. 5), and the administration of anti-TNFα antibodies reduces the levels of IL-6 and IFNγ in endotoxemic animals (20, 21). This suggests that IL-6 and IFNγ are downstream of TNFα in the cytokine cascade involved in septic shock, and that their production is dependent on TNFα. However, this conclusion is still under debate at least for IL-6. Thus, a specific inhibitor for TNFα was shown to reduce TNFα, but not IL-6 levels after lethal endotoxin challenge (22). In patients with septic shock, IL-6 appears to be a better predictor for survival because higher plasma levels of IL-6, but not TNFα, are evident in the nonsurvivor group (23). The importance of inflammatory cytokines other than TNFα in the pathogenesis of the endotoxic shock is also indicated by the fact that pretreatment with corresponding neutralizing antibodies protects against lethality (21, 24–26). Therefore, the regulation by cortistatin of a wide range of inflammatory mediators at both the local and systemic level, including mediators that appear later during the inflammatory response, might offer a therapeutic advantage over current therapies using neutralizing antibodies directed against a single mediator. This also explains the protective effect of delayed cortistatin administration in established endotoxemia.

Cortistatin shares many structural and functional properties with somatostatin. The lack of a significant phenotype in mice lacking a functional somatostatin gene (27) suggests a cortistatin compensatory effect. However, the proposed compensatory role of cortistatin is brought into question by the lack of an increase in cortistatin gene expression in the somatostatin KO mice (27). Cortistatin and somatostatin exhibit several distinct functions in the nervous system (5, 6). We find similar differences in our system. Somatostatin and octreotide, a somatostatin analogue already introduced in the clinical practice, deactivate some macrophage populations and are protective in certain inflammatory disorders, such as pancreatitis and liver injury (9, 28, 29). However, they are not protective in lethal septic shock (Fig. 2 E and references 9, 30). The superior potency of cortistatin in reducing inflammation as compared with somatostatin and octreotide might reside in the capacity of cortistatin to activate different receptors and transduction pathways. Although somatostatin and octreotide only bind to somatostatin receptors, cortistatin can bind to other receptors as well. This is supported by the fact that the somatostatin receptor antagonist cyclosomatostatin completely reversed the antiinflammatory effect of somatostatin and octreotide in vitro, whereas only partially reversing the effect of cortistatin. Ghrelin is a new orexigenic hormone recently identified as a potent antiinflammatory factor with therapeutic action in several inflammatory disorders (11, 12, 31), and cortistatin, but not somatostatin or octreotide, binds to the ghrelin receptor. In fact, a ghrelin receptor antagonist partially reversed the inhibitory effect of cortistatin on cytokine production by macrophages (unpublished data). Therefore, the possibility exists that cortistatin exerts its therapeutic effect in septic shock, at least partially, through ghrelin receptors or cortistatin-specific receptors not yet identified.

Of physiological relevance is the observation that the expression of cortistatin and its receptors increases in inflammatory cells in response to immune activation, especially after inflammatory stimuli (7, 8). Although the levels of cortistatin have not been yet measured in patients with sepsis, it is tempting to speculate that the body responds to an exacerbated inflammatory response by increasing the peripheral production of endogenous antiinflammatory factors, including cortistatin, in an attempt to restore homeostasis. Although cortistatin affects cortical and locomotor activities and sleep induction in the central nervous system, we did not observe any adverse effects of cortistatin in the concentration range used in our experimental system. Extending the use of cortistatin to human patients will depend on the dosage. The ability of delayed administration of cortistatin to ameliorate ongoing disease also fulfills an essential prerequisite for an antiendotoxemic agent, as treatment starts after the onset of sepsis. In summary, this work identifies cortistatin as a new immunomodulatory factor with the capacity to deactivate the inflammatory response. Cortistatin might represent a multistep therapeutic agent for human septic shock for use in combination with other immunomodulatory agents or complementary to other nonimmunomodulatory therapies.

MATERIALS AND METHODS

Induction of endotoxemia and study design. To induce endotoxemia, BALB/c and C57BL/6 mice (6–8 wk old; The Jackson Laboratory) were injected i.p. with different amounts (25–600 μg/mouse; 400 μg/mouse unless otherwise indicated) of LPS (from *Salmonella enteridis*; Sigma-Aldrich). Animals were treated i.p. with medium (controls) or with different concentrations (0.05–5.0 nmol/mouse; 6–550 μg/kg) of cortistatin 1–29 (American Peptides Company), somatostatin, octreotide, or cyclosomatostatin (Sigma-Aldrich) 30 min after challenge with LPS. To study the therapeutic effect of delayed administration of cortistatin on established endotoxemia, cortistatin (2 nmol/mouse) was injected i.p. 2 or 4 h after endotoxin administration. Animals were monitored daily for survival and other clinical signs including ruffled fur, lethargy, appearance of diarrhea, and body weight loss. Some animals were killed at different times after LPS injection, blood samples were collected by cardiac puncture, peritoneal exudates were obtained as previously described (15), and liver, lungs, and small intestine were collected. The blood samples were allowed to clot for 1 h at room temperature and serum was obtained after centrifugation for determination of cytokines, chemokines, and SAA. Tissue specimens were immediately frozen in liquid nitrogen for histological studies, protein extraction and cytokine determination, and MPO activity measurement. The peritoneal suspension was centrifuged for 5 min at 1,800 *g*, and cell-free supernatants (peritoneal fluid) were harvested and assayed for cytokine/chemokine production. Peritoneal cells were counted and adjusted in PBS/3 mM EDTA medium at 3×10^6 cells/ml. The number of viable cells in the different peritoneal subpopulations was determined by flow cytometry (FACScan; BD Biosciences). In brief, peritoneal lymphocytes, macrophages, and PMNs were gated according to their

different forward scatter and side scatter characteristics and counted. The large predominance of neutrophils (99%) in the PMN population was confirmed in cytospin preparations stained with May-Grunwald and Giemsa. For histopathologic evaluation, freshly collected liver, lung, and intestine were fixed in 10% buffered formalin phosphate, embedded in sucrose, frozen in dry ice using OCT compound, and cryosectioned. Cross sections were stained with hematoxylin/eosin using standard techniques.

Alternatively, sepsis was induced by CLP. In brief, C57BL/6 mice were anesthetized with ketamine (75 mg/kg, intramuscular injection) and xylazine (20 mg/kg, intramuscular injection) and a small abdominal midline incision was made. The cecum was exposed, mobilized, and ligated below the ileocecal valve and punctured through both surfaces twice with a 22-gauge needle and the stool was extruded (1 mm). The cecum was then placed back into its normal intra-abdominal position and the abdomen was closed with a running suture of 6–0 prolene. All animals received subcutaneous resuscitative normal saline (20 ml/kg body weight) 4 h after surgery. Medium (controls) or cortistatin (2 nmol) were administered i.p. twice at 6-h intervals beginning 4 h after CLP. Survival was monitored once daily for 10 d.

For other sepsis model, BALB/c mice were injected i.p. with bacterial suspension containing 10^8 live *E. coli* cells (DH5-α; Invitrogen). 2 nmol cortistatin was administered i.p. 2 h after *E. coli* injection.

All experiments were performed according to the Institutional Guidelines for the Care and Use of Laboratory Animals in Research and the approval of the local committee in the Consejo Superior de Investigaciones Cientificas.

Cytokine, SAA, and nitric oxide determination. For cytokine determination in tissues, protein extracts were isolated by homogenization of lung, liver, and small intestine pieces (50 mg tissue/ml) in 50 mM Tris-HCl, pH 7.4, with 0.5 mM DTT, and 10 μg/ml of a cocktail of proteinase inhibitors containing phenylmethylsulfonyl fluoride, pepstatin, and leupeptin (Sigma-Aldrich). Samples were centrifuged at 30,000 *g* for 20 min and stored at −80°C until cytokine determination. Cytokine and chemokine levels in the serum, tissue protein extracts and culture supernatants were determined by a specific sandwich ELISA by using capture/biotinylated detection Abs obtained from BD Biosciences and Peprotech according to the manufacturer's recommendations. SAA levels were determined in serum samples by a murine ELISA kit (Tridelta Development). The amount of NO formed was estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay. Before the Griess reaction, all nitrate was converted to nitrite using 5% vanadium trichloride. Equal volumes of culture supernatants (90 μl) and Griess reagents (90 μl of 1% sulfanilamide/0.1% *N*-[naphthyl]ethylenediamine dihydrochloride in 2.5% H_3PO_4) were mixed, and the absorbance was measured at 550 nm. The amount of nitrite was calculated from a $NaNO_2$ standard curve.

MPO assay. Neutrophil infiltration in the lung, liver, and small intestine was monitored by measuring MPO activity by using a method reported previously (32). In brief, tissue specimens were homogenized at 50 mg/ml in phosphate buffer (50 mM, pH 6.0) with 0.5% hexadecyltrimethylammonium bromide. Samples were frozen, thawed three times, and centrifuged at 30,000 *g* for 20 min. The supernatants were diluted 1:30 with assay buffer consisting in 50 mM phosphate buffer pH 6.0 with 0.167 mg/ml o-dianisidine (Sigma-Aldrich) and 0.0005% H_2O_2, and the colorimetric reaction was measured at 450 nm between 1 and 3 min in a spectrophotometer (Beckman Instruments). MPO activity per gram of wet tissue was calculated as: MPO activity (U/g wet tissue) = (A_{450}) (13.5)/tissue weight (g), where A_{450} is the change in the absorbance of 450nm light from 1 to 3 min after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1 U MPO activity is the amount of enzyme that will reduce 1 μmol peroxide/min.

Macrophage cultures. Resident macrophages were obtained by peritoneal lavage with RPMI 1640 medium. Peritoneal cells were washed in cold medium and incubated in complete medium (RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum) at a concentration of 10^6 cells/ml. After 2 h at 37°C, nonadherent cells were removed by extensive washing. At least 95% of the adherent cells were macrophages as judged by morphological and phagocytic criteria and by flow cytometry. Macrophage monolayers were incubated with complete medium in the absence (unstimulated) or presence of LPS (1 μg/ml, from *E. coli* serotype 055:B5; Sigma-Aldrich). In some experiments, cortistatin, somatostatin, or octreotide (Sigma-Aldrich) were added at different concentrations (from 10^{-7} to 10^{-12} M) at the initiation of the culture, in the absence or presence of the somatostatin antagonist cyclosomatostatin (Sigma-Aldrich). Cell-free supernatants were collected at different times and cytokine/chemokine levels were determined as described in the Cytokine, SAA, and nitric oxide determination section.

Data analysis. All values are expressed as mean ± SD of mice/experiment. The differences between groups were analyzed by Mann-Whitney U test and, if appropriate, by Kruskal-Wallis analysis of variance test. Survival curves were analyzed by the Kaplan-Meyer log-rank test.

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Urocortin and Adrenomedullin Prevent Lethal Endotoxemia by Down-Regulating the Inflammatory Response

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Urocortin 1 (UCN) and adrenomedullin (AM) are two neuropeptides that have emerged as potential endogenous anti-inflammatory factors based on their production by and binding to immune cells. Because human septic shock involves excessive inflammatory cytokine production, we investigated the effect of UCN and AM in the production of inflammatory mediators and their therapeutic actions in two models of septic shock. Both peptides down-regulated the production of inflammatory mediators by endotoxin-activated macrophages. The administration of UCN or AM protected against lethality after cecal ligation and puncture or after injection of bacterial endotoxin and prevented septic shock-associated histopathology, such as infiltration of inflammatory cells and intravascularly disseminated coagulation in various target organs. The therapeutic effect of UCN and AM was mediated by decreasing the local and systemic levels of a wide spectrum of inflammatory mediators, including cytokines, chemokines, and the acute phase protein serum amyloid A. Importantly, UCN or AM treatment was therapeutically effective in established endotoxemia. In conclusion, UCN and AM could represent two multistep therapeutic agents for human septic shock to be used in combination with other immunomodulatory agents or complementary as anti-inflammatory factors to other therapies. ***(Am J Pathol 2006, 168:1921–1930; DOI: 10.2353/ajpath.2006.051104)***

Septic shock is a systemic response to severe bacterial infections generally caused by gram-negative bacterial endotoxins. The administration of the endotoxin lipopolysaccharide (LPS) in experimental animals leads to pathophysiologic changes similar to human septic shock syndrome, and lethal endotoxemia has been extensively used as an experimental model of gram-negative septic shock.[1] The severe pathological consequences of the septic shock syndrome give rise to a hyperactive and out-of-balance network of endogenous pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin (IL)-12, IL-6, and interferon-γ (IFNγ).[2] The overproduction of inflammatory cytokines generates systemic activation, which affects vascular permeability and cardiac function, induces metabolic changes that can lead to tissue necrosis, and eventually progresses to multiple-organ failure and death. Despite significant progress in understanding its pathophysiology, septic shock continues as the most common cause of death and morbidity in intensive care units. Promising experimental results prompted large-scale randomized clinical trial with a variety of agents designed to neutralize one or more of the inflammatory mediators involved in its pathology.[3,4] But unfortunately, despite some promising results during preliminary trial, all of the major clinical studies of immunomodulators in sepsis have yielded disappointing results. Because of the complex pathophysiology of human sepsis, consideration should be given to multidrug therapy, similar to the approach taken in cancer and acquired immune deficiency syndrome therapy.

Urocortin 1 (UCN) and adrenomedullin (AM) are two recently discovered neuropeptides that are structurally related to corticotropin-releasing factor (CRF) and calcitonin-gene-related peptide (CGRP), respectively.[5,6] Although UCN and AM were initially known by their cardiovascular protective activities,[6,7] several indications suggest that they could act as endogenous immunomodulatory factors, with predominant anti-inflammatory effects. UCN and AM and their receptors are expressed by several immune cells, including macrophages/mono-

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cytes and T cells, in lymphoid organs and the gastrointestinal tract, and their expressions increase on inflammatory conditions.[5,6,8–15] UCN inhibits experimental autoimmune encephalomyelitis,[16] endotoxin-induced TNF-α production by Kupffer cells,[17] and inflammatory bowel disease.[18] In addition, UCN present in the mucosa of patients suffering from *Helicobacter pylori*-induced gastritis increases during the stage of eradication and amelioration of inflammation, whereas in patients resistant to treatment, its levels remain low.[19] On the other hand, AM inhibits TNF-α production by activated macrophages and ameliorates inflammation-induced colitis.[13,18,20,21] Based on their potential anti-inflammatory actions, we investigate here the potential therapeutic effect of UCN and AM in two murine models of septic shock. We report that treatment of endotoxemic mice with UCN or AM significantly reduces the mortality and pathology by down-regulating a broad spectrum of inflammatory factors.

Materials and Methods

Induction of Endotoxemia and Study Design

To induce endotoxemia, BALB/c and C57BL/6 mice (6 to 8 weeks old; The Jackson Laboratories, Bar Harbor, ME) were injected intraperitoneally (i.p.) with different amounts (25 to 600 μg/mouse; 400 μg/mouse unless otherwise indicated) of LPS (from *Salmonella enteridis*; Sigma, St. Louis, MO). Animals were treated i.p. with medium (controls) or with different concentrations (0.05 to 5.0 nmol/mouse; 6 to 550 μg/kg) of UCN or AM (American Peptides Company, Sunnyvale, CA) 30 minutes after challenge with LPS. Heat-inactivated UCN, CGRP, CRF, or the AM fragment AM_{20-50} (2 nmol), administered 30 minutes after LPS injection, were used as control peptides. In addition, combinations of AM, UCN, and cortistatin were used in some LPS-induced endotoxemia experiments. To study the therapeutic effect of delayed administration of AM and UCN on established endotoxemia, the peptides were injected i.p. (2 nmol/mouse) 2 or 4 hours after endotoxin administration. Animals were monitored every 12 hours for survival and other clinical signs including ruffled fur, lethargy, appearance of diarrhea, and body weight loss. Animals were sacrificed at different times after LPS injection, blood samples were collected by cardiac puncture, peritoneal exudates were obtained as described below, and liver, lungs, and small intestines were collected. The blood samples were allowed to clot for 1 hour at room temperature, and serum was obtained after centrifugation for determination of cytokines, chemokines, and serum amyloid A (SAA). Tissue specimens were immediately frozen in liquid nitrogen for histological studies, protein extraction, cytokine determination, and myeloperoxidase (MPO) activity measurement. The peritoneal suspension was centrifuged for 5 minutes at 1800 $\times$ *g*, and cell-free supernatants (peritoneal fluid) were harvested and assayed for cytokine/chemokine production. Peritoneal cells were counted and adjusted in phosphate-buffered saline/3 mmol/L ethylenediamine tetraacetic acid medium at 3×10^6 cells/ml. The number of viable cells in the different peritoneal subpopulations was determined by flow cytometry (FACScan; BD Biosciences, Mountain View, CA). Briefly, peritoneal lymphocytes, macrophages, and polymorphonuclear cells (PMNs) were gated according to their different forward scatter and side scatter characteristics and counted. The large predominance of neutrophils (99%) in the PMN population was confirmed in cytospin preparations stained with May-Grunwald and Giemsa. For histopathological evaluation, freshly collected liver, lungs, and intestines were fixed in 10% phosphate-buffered formalin, sectioned, and stained with hematoxylin and eosin using standard techniques.

Alternatively, sepsis was induced by cecal ligation and puncture (CLP). Briefly, C57BL/6 mice were anesthetized with ketamine (75 mg/kg, intramuscular injection) and xylazine (20 mg/kg, intramuscular injection), and a small abdominal midline incision was made. The cecum was exposed, mobilized, and ligated below the ileocecal valve and punctured twice with a 22-gauge needle, and the stool was extruded (1 mm). The cecum was then placed back into its normal intra-abdominal position, and the abdomen was closed with a running suture of 6-0 prolene. All animals received subcutaneous resuscitative normal saline (20 ml/kg body weight) 4 hours after surgery. Medium (controls), UCN (2 nmol), or AM (2 nmol) was administered i.p. twice at 6-hour intervals beginning 4 hours after CLP. Survival was monitored twice daily for 10 days.

Cytokine, SAA, and Nitric Oxide Determination

For cytokine determination in tissues, protein extracts were isolated by homogenization of lung, liver, and small intestine pieces (0.5 mg tissue/ml) in 50 mmol/L Tris-HCl, pH 7.4, with 0.5 mmol/L dithiothreitol and 10 μg/ml of a cocktail of proteinase inhibitors containing phenylmethylsulfonyl fluoride, pepstatin, and leupeptin (Sigma). Samples were centrifuged at 30,000 $\times$ *g* for 20 minutes and stored at -80°C until cytokine determination. Cytokine and chemokine levels in the serum, tissue protein extracts, and culture supernatants were determined by a specific sandwich enzyme-linked immunosorbent assay (ELISA) by using capture/biotinylated detection antibodies from BD Pharmingen (San Diego, CA) and Preprotech (Rocky Hill, NJ) according to the manufacturers' recommendations. SAA levels were determined in serum samples by a murine ELISA kit (Tridelta Development, Wicklow, Ireland). The amount of nitric oxide (NO) formed was estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay. Equal volumes of culture supernatants (90 μl) and Griess reagents (90 μl of 1% sulfanilamide/0.1% *N*-[naphthyl]ethyl-enediamine dihydrochloride in 2.5% H_3PO_4) were mixed, and the absorbance was measured at 550 nm. The amount of nitrite was calculated from a $NaNO_2$ standard curve.

MPO Assay

Neutrophil infiltration in the lungs, liver, and small intestines was monitored by measuring MPO activity. Briefly,

tissue specimens were homogenized at 50 mg/ml in phosphate buffer (50 mmol/L, pH 6.0) with 0.5% hexadecyltrimethylammonium bromide. Samples were frozen and thawed three times and centrifuged at 30,000 × *g* for 20 minutes. The supernatants were diluted 1:30 with assay buffer consisting in 50 mmol/L phosphate buffer, pH 6.0, with 0.167 mg/ml *o*-dianisidine (Sigma) and 0.0005% H_2O_2, and the colorimetric reaction was measured at 450 nm between 1 and 3 minutes in a spectrophotometer (Beckman Instruments, Irvine, CA). MPO activity per gram of wet tissue was calculated as follows: MPO activity (U/g wet tissue) = (A_{450}) (13.5)/tissue weight (g), where A_{450} is the change in the absorbance of 450 nm light from 1 to 3 minutes after the initiation of the reaction. The coefficient 13.5 was empirically determined such that 1 U of MPO activity is the amount of enzyme that will reduce 1 μmol peroxide/minute.

Macrophage Cultures

Resident macrophages were obtained by peritoneal lavage with RPMI 1640. Peritoneal cells were washed in cold medium and incubated in complete medium (RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin, 2 mmol/L L-glutamine, 50 μmol/L 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum) at a concentration of 10^6 cells/ml. After 2 hours at 37°C, nonadherent cells were removed by extensive washing. At least 95% of the adherent cells were macrophages as judged by morphological and phagocytic criteria and by flow cytometry. Macrophage monolayers were incubated with complete medium in the absence (unstimulated) or presence of LPS (1 μg/ml, from *Escherichia coli* serotype 055: B5; Sigma). UCN or AM were added at different concentrations (from 10^{-7} to 10^{-13} mol/L) at the initiation of the culture. Cell-free supernatants were collected at different times, and cytokine/chemokine levels were determined as described above.

Data Analysis

All values are expressed as mean ± SD of mice per experiment. The differences between groups were analyzed by Mann-Whitney *U*-test and, if appropriate, by Kruskal-Wallis analysis of variance test. Survival curves were analyzed by the Kaplan-Meyer log-rank test.

Results

UCN and AM Inhibit the Production of Inflammatory Mediators by Activated Macrophages in Vitro

Macrophages, major participants in innate immunity, contribute to the initiation of the inflammatory response by killing pathogens through phagocytosis, release of cytotoxic oxygen and nitrogen intermediates, and release of chemokines and cytokines that attract and activate other immune cells. Despite the beneficial role of inflammatory factors in host defense, their sustained production can lead to serious pathological conditions. Therefore, although necessary for the elimination of pathogens, macrophage activation leads to serious deleterious effects in the host if left unchecked. To investigate the potential anti-inflammatory effect of AM and UCN, we evaluated first the effects of both peptides on the production of several inflammatory mediators by peritoneal macrophages. UCN and AM inhibited the production of the pro-inflammatory factors TNF-α, IL-6, and NO by activated macrophages (Figure 1). This effect was dose dependent with a maximal effect at 10^{-8} mol/L (not shown), a concentration that is within the physiological range.[5–7,9,13]

Treatment with UCN and AM Protects against Lethal Endotoxemia and Reduces the Related Histopathology

Because UCN or AM inhibits the production of pro-inflammatory macrophage-derived factors crucial for the development of septic shock, we expected a protective effect in high-dose endotoxemia, a murine model for septic shock syndrome. Mice were injected with different doses of LPS and treated 30 minutes later with UCN or AM (2 nmol/mouse; 400 μg/kg), and the survival was monitored. Treatment with UCN or AM significantly protected against the lethal effect of LPS (Figure 2A). This effect was not exclusive of BALB/c mice, and similar protective effects were obtained when C57BL/6 mice were used (not shown). The protective effect was observed over a wide range of LPS doses, with UCN and AM shifting the LPS dose causing 50% mortality (LD50) from 100 to 350 and 450 μg of LPS, respectively (Figure 2B). Animals injected with UCN or AM had a survival rate of 70 and 50%, respectively; even for the nonsurvivors, UCN and AM almost doubled the time until death (Figure 2C). The effect of both peptides was dose dependent, with doses as low as 0.5 nmol (100 μg/kg) being partially protective (Figure 2D). In contrast, CRF (a UCN-related peptide), CGRP (an AM-related peptide), heat-inactivated UCN, or the AM fragment AM_{20-50} (both used as control peptides) was not protective (Figure 2E). Our results demonstrate a slightly, although no significantly, higher effect of UCN than AM in preventing endotoxemia. These differences could be due to different expression levels of the receptors for UCN and AM in the cells involved in this response, to different pathway-coupling efficiencies, or to different peptide degradation rates in circulation or tissues.

Because the production of inflammatory cytokines occurs in a rapid sequence starting with TNF-α, which reaches a maximum at 2 hours after LPS infusion (see below), we next investigated the therapeutic effect of UCN and AM on conditions of already established septic shock. Kinetic studies showed that both peptides exerted a full protective action when given up to 4 hours after shock induction (Figure 2F), with decreased protection for later UCN/AM administration (not shown). The ability of delayed administration of UCN and AM to ameliorate

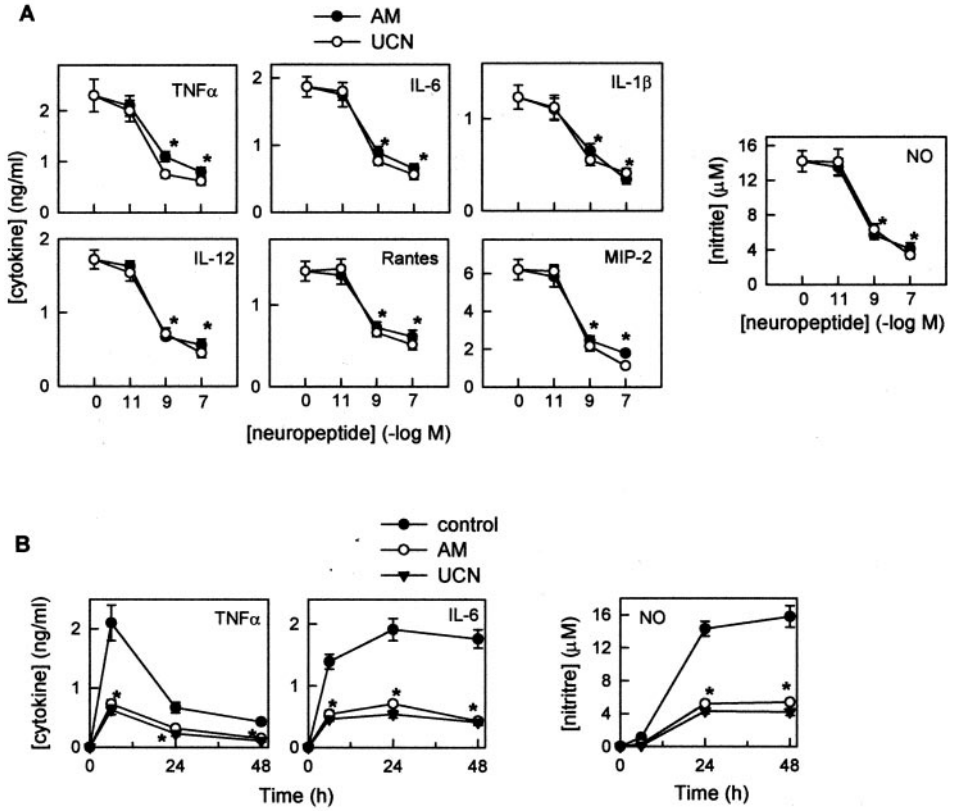

Figure 1. UCN and AM inhibit the production of inflammatory mediators by activated macrophages *in vitro*. **A:** Peritoneal macrophages were stimulated with LPS (1 μg/ml) in the absence or presence of different concentrations of AM or UCN. After different times (6 hours for TNF-α and 24 hours for IL-6, IL-12, IL-1β, RANTES, and MIP-2), pro-inflammatory mediators were assayed as described in Materials and Methods ($n = 6$). **B:** Peritoneal macrophages were stimulated with LPS (1 μg/ml) in the absence (control) or presence of AM or UCN (10^{-8} mol/L) for different times, and the inflammatory factors were determined in the supernatants ($n = 6$). $^*P < 0.001$ versus untreated control mice.

ongoing disease fulfills an essential prerequisite for any anti-endotoxemic agent, because treatment is started after the onset of sepsis in patients.

Endotoxemic animals suffered from disseminated intravascular coagulation with multiple organ failure as indicated by severe congestion, hemorrhage, hyperemia, fibrin deposits, edema, thrombosis, and massive accumulation of leukocytes in lungs (Figure 3A) and the intestinal tract (not shown), as well as severe congestion of the hepatic sinusoids and hepatocyte necrosis (Figure 3A) and segmental ischemia of the bowel with regions of hemorrhage or necrosis and an infracted cecum (not shown). Endotoxic shock induction was accompanied by an early granulocyte (mainly neutrophils) infiltration in the peritoneum, followed by later recruitment of macrophages and lymphocytes (Figure 3B). Neutrophil infiltration in the liver, lungs, and intestines of endotoxemic animals was confirmed through measurements of the MPO activity (Figure 3C). In contrast, animals treated with UCN or AM did not present any of the histopathological alterations associated with septic shock, such as disseminated intravascular coagulation, leukocyte infiltration, and inflammation in various organs and mesenteric ischemia, tissue congestion, and hemorrhage (Figure 3).

UCN and AM Improve Survival in Experimental Sepsis

We next investigated the potential therapeutic effect of UCN and AM in the murine model of CLP, a clinically relevant model for human sepsis because it causes lethal peritonitis produced by polymicrobial infection. Treatment with UCN or AM was started 4 hours after the induction of sepsis, at a time when mice show clear signs of sepsis, including lethargy, piloerection, diarrhea, huddling, fever, and malaise. UCN and AM treatments significantly improved survival (Figure 4) and attenuated the clinical manifestations of sepsis, such as lethargy, diarrhea, body weight loss, and hypothermia (not shown).

Treatment with UCN or AM Reduces Local and Systemic Inflammatory Responses in Endotoxemic Mice

We next evaluated the effect of UCN or AM on the production of inflammatory mediators that are mechanistically linked to endotoxemia. The administration of UCN or AM reduced the levels of endotoxin-induced inflammatory cytokines (TNF-α, IFNγ, IL-6, IL-1β, and IL-12), chemokines

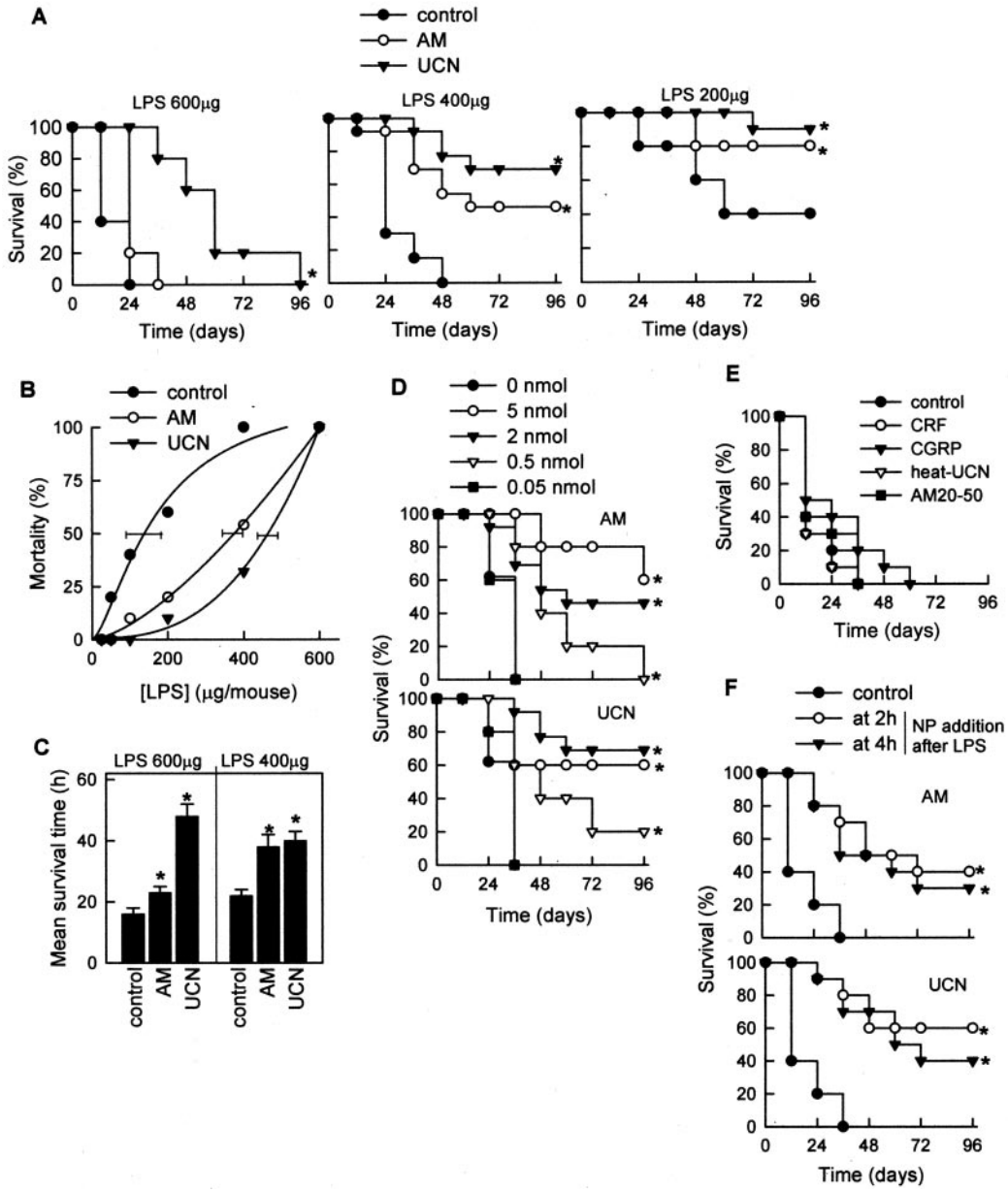

Figure 2. Treatment with UCN or AM protects against lethal endotoxemia. **A–C:** BALB/c mice were injected i.p. with different doses of LPS (control). AM or UCN (2 nmol/mouse) was injected i.p. 30 minutes after LPS administration. Survival was monitored over the next 96 hours. Similar results were obtained in three identical independent experiments or when C57BL/6 mice were used. Mortality curves in **B** were used to calculate LD50, and horizontal bars indicate the 95% confidence limits of LD50 determinations. In **C**, the average survival time was calculated for nonsurvivors in both the untreated and UCN/AM-treated groups. **D:** Mice were injected i.p. with 400 μg of LPS and different doses of UCN or AM (from 0 to 5 nmol/animal). **E:** Mice were injected i.p. with 400 μg of LPS and 30 minutes later, treated with medium (control), CRF, CGRP, or heat-inactivated UCN or the AM-fragment AM_{20-50} (2 nmol/mouse). Survival was monitored over the next 96 hours. **F:** UCN or AM (2 nmol/mouse) was injected i.p. 2 or 4 hours after LPS challenge. n = 12–20 mice/group. $^*P < 0.001$ versus untreated control mice.

(regulated upon activation normal T cells express sequence (RANTES) and macrophage inflammatory protein-2 (MIP-2)), and NO in serum (systemic) and in various target organs, including peritoneum, liver, lung, and intestine (Figure 5). In addition, UCN/AM administration increased the systemic and local levels of the anti-inflammatory cytokine IL-10 (Figure 5), which has been shown to be protective in endotoxic shock.[22]

Coagulation abnormalities, especially disseminated intravascular coagulation and microthrombosis, are common features during sepsis.[1–4,23] Acute phase proteins (APPs) are involved in the initiation of aberrant coagulation during endotoxic shock.[23] Therefore, we also investigated the effect of UCN and AM on the production of the APP SAA during endotoxic shock. Both peptides dramatically decreased the systemic levels of SAA during septic shock (Figure 5A).

UCN and AM Deactivate Peritoneal Macrophages during Endotoxemia

The UCN/AM-induced decrease in the levels of inflammatory factors in the target organs could result from the

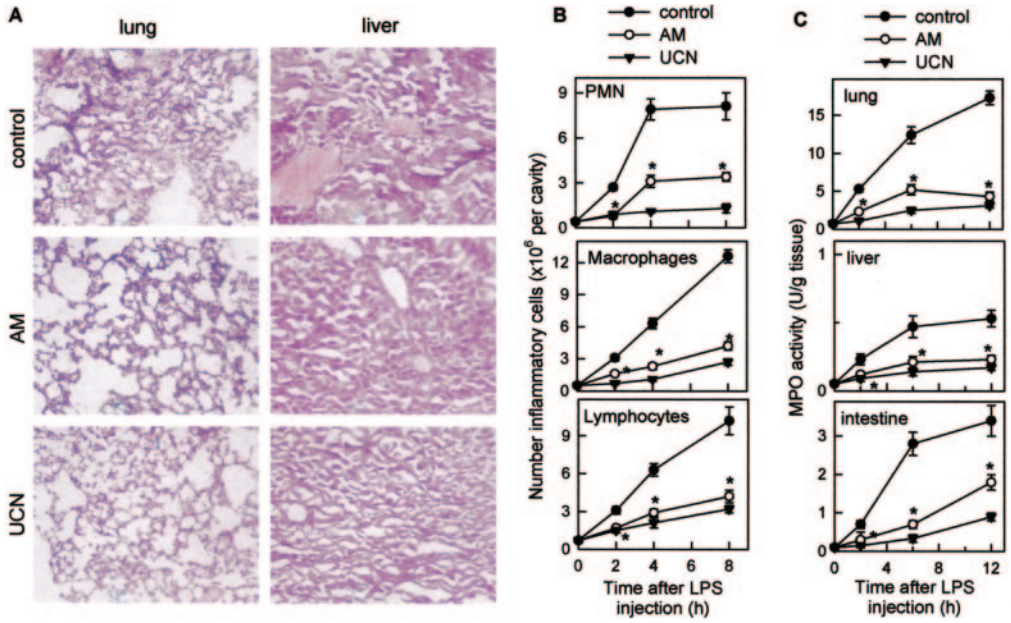

Figure 3. AM and UCN reduce endotoxemia-associated histopathology. Mice were injected i.p. with LPS (control). UCN or AM (2 nmol/mouse) was administered i.p. 30 minutes after LPS administration. **A:** UCN and AM reduced inflammatory infiltration and disseminated coagulation in target organs. Histopathology analysis was determined in hematoxylin and eosin-stained sections of lung and liver obtained at 24 hours of disease (×150). **B:** UCN and AM reduce leukocyte recruitment in the peritoneal cavity. Peritoneal cell suspensions were obtained at different times after LPS injection, and numbers of macrophages, lymphocytes, and PMNs were determined by flow cytometry. **C:** UCN and AM decrease MPO activity in lungs, liver, and intestine. MPO content was determined at different times after LPS infusion. $n = 8$–12 mice/group. $^*P < 0.001$ versus untreated control mice.

reduction in inflammatory cell infiltration. However, the fact that both peptides down-regulate the *in vitro* production of various inflammatory factors by activated macrophages (Figure 1) argues against this hypothesis. We determined the capacity of peritoneal macrophages isolated from untreated or UCN/AM-treated endotoxemic mice to produce inflammatory mediators *ex vivo*. Although macrophages from mice with septic shock produced spontaneously high amounts of TNF-α, IL-6, IL-12, RANTES, and MIP-2, macrophages isolated from UCN/AM-treated animals produced significantly lower levels of pro-inflammatory cytokines and secreted high amounts of IL-10 (Figure 6). Therefore, it is plausible that the deactivation of resident and infiltrating macrophages is the major mechanism in the anti-inflammatory action of UCN and AM.

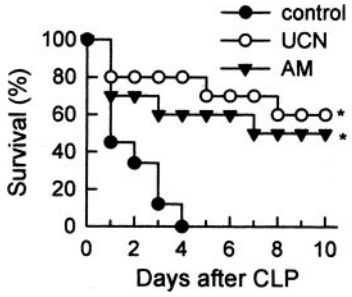

Figure 4. UCN and AM protect against CLP-induced experimental sepsis. Sepsis was induced in C57BL/6 mice after CLP. UCN or AM (2 nmol) was administered i.p. twice at 6-hour intervals beginning 4 hours after CLP procedure. Survival was monitored over the next 8 days. $n = 10$ mice/group. $^*P < 0.001$ versus untreated control mice.

The Combined Therapy of UCN, AM, and Cortistatin Protects against Lethal Endotoxemia

We next evaluated the effect of the combined treatment with UCN, AM, and another endogenous anti-inflammatory peptide,[24] cortistatin. Administration of UCN together with AM significantly prevented the lethality (20% survival) induced by a high dose of LPS (600 μg/mouse) (Figure 7, left), compared with mice treated with UCN or AM alone (Figure 2A). In addition, although endotoxic mice treated with cortistatin alone did not survive (not shown), the combined treatment with cortistatin and UCN or cortistatin and AM increased survival to 50% (Figure 7, left). This combined treatment permits reduction of the effective therapeutic dose to 0.5 nmol UCN/AM (Figure 7, right). This synergistic effect of UCN, AM, and cortistatin could be the result of effects on different cells or of the involvement of different signal transduction pathways in the same target cell. In the case of cortistatin, we favor the latter because activated macrophages are the major players in the anti-inflammatory activity of the three peptides, and both UCN and AM act through cAMP-dependent pathways,[5,6] whereas the cortistatin effect is partially cAMP independent.[25] However, because receptors for AM and UCN are ubiquitously present in monocytes/macrophages, dendritic cells, neutrophils, and endothelial cells (M. Delgado, unpublished data),[5,6,13] the participation of cells other than macrophages in such anti-inflammatory action cannot be excluded, and if so, AM and UCN could differentially affect these cells.

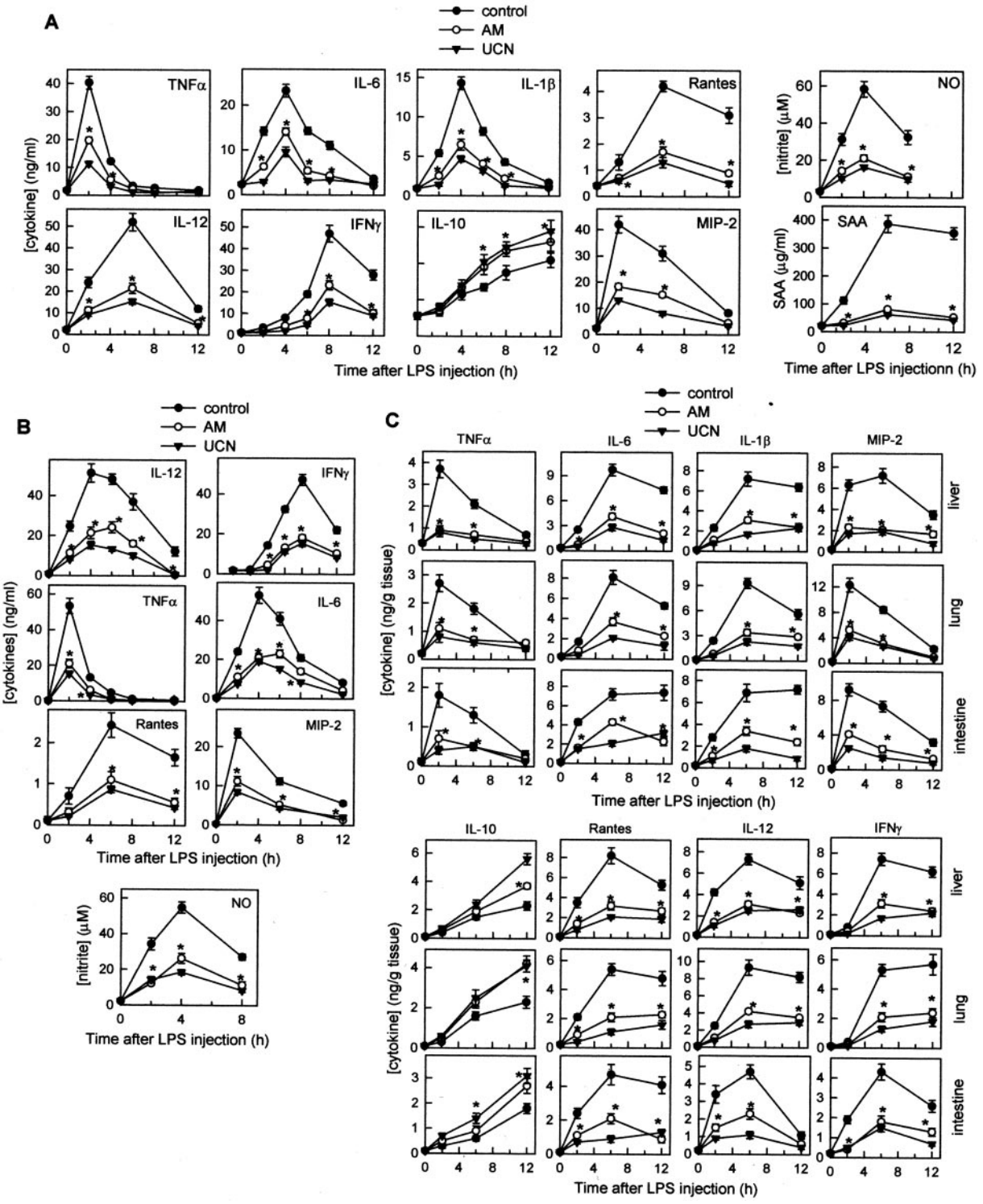

Figure 5. Treatment with UCN or AM reduces local and systemic inflammatory responses in endotoxemic mice. Endotoxemia was induced by i.p. injection of LPS (400 μg/mouse). Mice were treated 30 minutes later with medium (controls) or with UCN or AM (2 nmol/mouse). Serum (**A**), peritoneal fluid (**B**), and protein extracts from lungs, liver, and small intestines (**C**) were collected at various time points after endotoxin injection; and the cytokine/chemokine, NO, and SAA contents were determined as described in Materials and Methods. $n = 6–8$ mice/group. $^*P < 0.001$ versus untreated control mice.

Discussion

The present work proposes a new therapy for endotoxemia based on the use of the neuropeptides UCN and AM. The administration of UCN or AM protected against endotoxin-induced lethality. In addition, treatment with UCN or AM improved survival in a "true infection" model resembling sepsis in humans, such as cecal ligation and puncture. UCN and AM prevented septic shock-associated histopathology, including inflammatory cell infiltra-

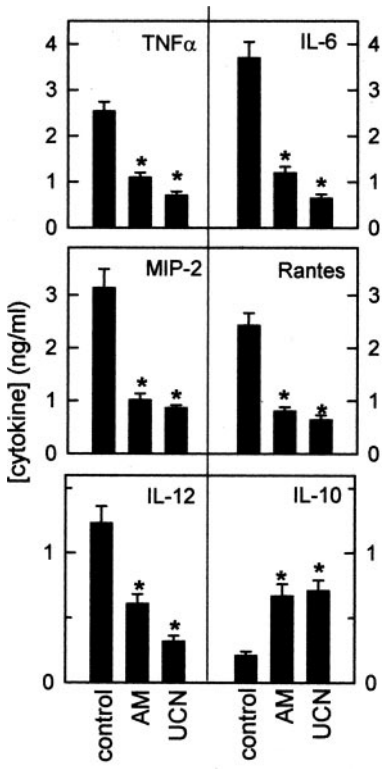

Figure 6. Treatment with UCN or AM deactivates peritoneal macrophages during endotoxemia. Mice were injected i.p. with LPS (control) and treated i.p. with UCN or AM (2 nmol/mouse) 30 minutes later. Peritoneal macrophages were isolated 8 hours after LPS injection. Recovered macrophages (12×10^6 cells for control, 3×10^6 cells for UCN/AM) were cultured with medium alone at 5×10^5 cells/ml for 48 hours, and the concentration of inflammatory mediators in the culture supernatants was determined by ELISA. $n = 5–6$ mice/group. $^*P < 0.001$ versus untreated control mice.

tion and multiorgan intravascular disseminated coagulation. The therapeutic effect of both peptides seems to be mediated through the decrease in the local and systemic levels of a wide spectrum of inflammatory mediators, including cytokines (TNF-α, IL-6, IL-1β, IFNγ, and IL-12), chemokines (MIP-2 and RANTES), and SAA. Our results suggest that deactivation of resident and infiltrating macrophages is the major mechanism in the anti-inflammatory action of UCN or AM in septic shock. In agreement

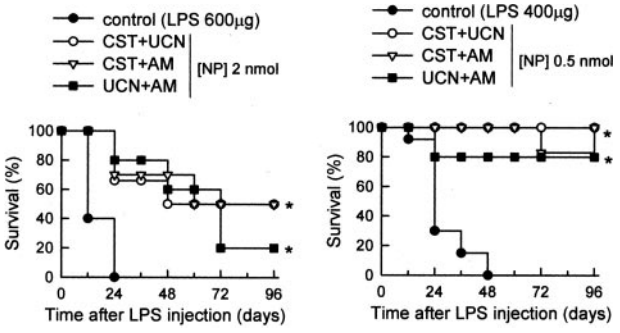

Figure 7. Combined therapy of UCN, AM, and cortistatin protects against lethal endotoxemia. Septic shock was induced by i.p. injection of LPS (600 or 400 μg/mouse). Mice were treated 30 minutes later with medium (controls) or with various combinations of the neuropeptides UCN, AM, and cortistatin (CST) (2 or 0.5 nmol/mouse). Survival was monitored over 96 hours. $n = 8–10$ mice/group. $^*P < 0.001$ versus mice treated with cortistatin, UCN, or AM alone.

with our results, UCN and AM were previously reported to decrease the production of TNF-α by macrophages and Kupffer cells.[13,17,20,21] Although AM has been found to increase IL-6 in a macrophage cell line,[20] in agreement with our results, AM reduces IL-6 production by synovial cells from rheumatoid arthritis patients and decreases serum IL-6 levels on human sepsis.[26,27]

Among the pro-inflammatory cytokines involved in endotoxic shock, TNF-α appears to play a central role. Increased serum TNF-α levels appear during endotoxemia, and TNF-α enhances procoagulant activity of vascular endothelial cells, activates neutrophils and macrophages, and increases adherent molecule expression, which in turn enhances neutrophil/monocyte infiltration and contributes to tissue injury.[28] However, the central importance of inflammatory cytokines other than TNF-α in its pathogenesis is still debatable.[29–33] IL-6 appears to be a better predictor for survival in patients with septic shock. In this sense, the fact that UCN or AM showed therapeutic effects administered 4 hours after induction of endotoxemia, once the TNF-α peak occurred suggests that both peptides could exert their effects independently of TNF-α inhibition. Thus, UCN and AM decrease the production of IL-6, NO, and various chemokines in endotoxemic mice. The UCN/AM-induced decrease of serum NO is beneficial because it should avoid the NO-mediated hypotension and cytotoxicity, two pathological hallmarks of septic shock.[1–4] The fact that UCN or AM reduced local and systemic levels of the chemokines MIP-2 (chemotactic for neutrophils) and RANTES (chemotactic for macrophages/monocytes) partially explains the absence of inflammatory infiltrates in the affected organs. An additional mechanism is the UCN/AM-induced increase of IL-10, a delayed production of anti-inflammatory cytokine therapeutic for sepsis syndromes.[34] Moreover, UCN and AM decreased the systemic levels of SAA, a hepatic APP that contributes to the disseminated intravascular coagulation and microthrombosis observed in sepsis.[1–4,23] Therefore, the regulation by UCN and AM of this wide range of inflammatory mediators at both local and systemic levels, including mediators that appear later during the inflammatory response, explains the protection against established endotoxemia by UCN and AM and might offer a therapeutic advantage over current therapies using neutralizing antibodies directed against a single mediator. Additional mechanisms other than the anti-inflammatory effect could contribute to the therapeutic action of both peptides in endotoxic shock. For example, based in their vasodilatory properties promoting hyperdinamic circulation, AM and UCN could reduce the circulatory collapse and organ damage characteristic of sepsis.[13,35–37] In addition, the infusion of UCN and AM increase the levels of β-adrenergic agonists,[38,39] widely recognized as potent anti-inflammatory factors.

UCN is structurally related to CRF. Depending on the site of secretion, CRF has shown both pro- and anti-inflammatory actions. Central secretion of CRF activates the hypothalamus-pituitary-adrenal axis that ultimately induces the release of corticosteroids and subsequent anti-inflammatory actions.[40–42] In contrast, peripherally secreted CRF is largely pro-inflammatory.[43–45] In contrast

to CRF, the anti-inflammatory effect of UCN in systemic inflammation and experimental autoimmune encephalomyelitis is corticosteroid independent.[17] The present study and other evidence suggest that UCN may counteract the pro-inflammatory effects of CRF at inflammation sites.[35] Interestingly, UCN preferentially binds to the CRF receptor type 2, and the distribution of UCN but not of CRF correlates with the distribution of the CRF receptor type 2 in the periphery.[5]

AM is structurally related to CGRP and binds to the calcitonin-related-like receptor (CRLR).[6] Because patients and animals with sepsis show increased serum CGRP levels,[46] CGRP has been suspected as an immunomodulatory factor. However, in contrast to AM, CGRP does not efficiently down-regulate the inflammatory response and fails preventing sepsis (Figure 2).[47,48] The different effectiveness showed by AM and CGRP could reside in the differential binding of both peptides to different binding complexes, composed by the CRLR and various receptor activity-modifying proteins (RAMP1, RAMP2, and RAMP2). Thus, CGRP specifically binds to the CRLR-RAPM1 complex, whereas AM preferentially binds to CRLR associated to RAMP2 and RAMP3.[6] We have recently observed that murine macrophages specifically express CRLR-RAMP2/3 complexes (E. Gonzalez-Rey, unpublished data). This partially explains the higher potency of AM versus CGRP on the inflammatory response.

Of physiological relevance is the observation that the expression of UCN and AM is increased in activated inflammatory cells[5,6,9–15] and in several inflammatory conditions, including rheumatoid arthritis, gastritis, ulcerative colitis, and endotoxemia.[13,19,45] Therefore, it is tempting to speculate that the body responds to an exacerbated inflammatory response by increasing the peripheral production of endogenous anti-inflammatory factors, including UCN and AM, in an attempt to restore homeostasis. Extending the use of UCN and AM to the human system, however, will depend on the peptide dosage and the expression of UCN/AM receptors in human immune cells, because species-related differences in expression have been found. In this sense, the widespread presence of UCN and AM receptors in all tissues of the body could limit their therapeutic application, because both peptides have differing actions affecting the cardiovascular system, stress and anxiety. However, we and others[5,6] have not observed any adverse effects of the peptides, in terms of hypotension and anxiety-like behavior, at the concentrations used, probably because a short period of treatment with the peptides is enough to get a significant disease remission.

In summary, this work identifies UCN and AM as potent immunomodulatory factors with the capacity to deactivate the inflammatory response. UCN and AM might represent multistep therapeutic agents for human septic shock for use in combination with other immunomodulatory agents or complementary to other nonimmunomodulatory therapies.

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Growth Factors, Cytokines, Cell Cycle Molecules

Neuropeptides Rescue Mice from Lethal Sepsis by Down-regulating Secretion of the Late-Acting Inflammatory Mediator High Mobility Group Box 1

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Originally described as a nuclear protein that bends DNA, the high mobility group box 1 protein (HMGB1) has recently emerged as a necessary and sufficient late mediator of severe sepsis. HMGB1 is therefore a molecular target that provides a wide window for clinical intervention in sepsis. Vasoactive intestinal peptide (VIP) and urocortin are two well known anti-inflammatory neuropeptides that protect against several immune disorders by regulating a wide panel of inflammatory mediators. In this study, we demonstrate the therapeutic effect of VIP and urocortin in various models of established sepsis: both agents reduced lethality induced by cecal ligation and puncture or by injection of live *Escherichia coli*. The therapeutic effect of VIP and urocortin was accompanied by a decrease in systemic levels of HMGB1. In addition, administration of recombinant HMGB1 completely reversed the protective effect of VIP and urocortin in experimental sepsis. *In vitro* and *ex vivo* studies show that both VIP and urocortin down-regulate translocation of HMGB1 from the nucleus to the cytoplasm and its subsequent secretion by activated macrophages, suggesting that macrophages are major targets in the inhibitory activity of these neuropeptides. To our knowledge, VIP and urocortin are the first endogenous inhibitors of HMGB1 secretion shown to improve sepsis survival in a clinically relevant time frame. ***(Am J Pathol 2008, 172:1297–1302; DOI: 10.2353/ajpath.2008.070969)***

High mobility group box 1 (HMGB1), a chromatin-binding protein, was recently described as a late inflammatory factor secreted by monocytes and macrophages.[1] Numerous evidences indicate that HMGB1 is a necessary and sufficient late mediator of severe sepsis.[2] Patients and animals with sepsis or endotoxemia present high levels of systemic HMGB1, and administration of HMGB1 to mice causes epithelial cell dysfunction and lethal multiple organ damage.[1,3,4] In addition, blocking of HMGB1 improves survival and prevents organ failure in septic mice.[4,5] Therefore, the late kinetic action of HMGB1 provides a wider time frame for the treatment of sepsis. Anti-inflammatory mediators are secreted by the host innate immune system during the ongoing process to restore homeostasis. However, the endogenous factors involved in the control of HMGB1 secretion are poorly known.

Vasoactive intestinal peptide (VIP) and urocortin (UCN) are two neuropeptides widely distributed that exert multiple functions in the body. VIP and UCN are produced by several immune cells, especially under inflammatory stimuli, and have potent anti-inflammatory effects.[6] The capacity of these neuropeptides to regulate a wide range of inflammatory mediators makes them attractive therapeutic candidates for the treatment of inflammatory and autoimmune diseases, such as endotoxemia, rheumatoid arthritis, and inflammatory bowel disease.[6] The aim of this work was to investigate the effect of VIP and UCN on the secretion of HMGB1 and their potential therapeutic effect in severe established sepsis.

Materials and Methods

Animal Models

Animal experimental protocols were reviewed and approved by the Ethical Committee of the Spanish Council of Scientific Research. To induce endotoxemia, BALB/c mice (6 to 8 weeks old; Jackson Laboratories, Campbell, CA) were injected i.p. with lipopolysaccharide (LPS) (100

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μg/mouse, Sigma-Aldrich, St. Louis, MO), or with a bacterial suspension containing 10^8 live *Escherichia coli* (DH5α). To induce sepsis, cecum of anesthetized BALB/c mice was ligated 5.0 mm from the cecal tip and punctured once with a 22 gauge needle, and the stool was then extruded (1 mm). Vehicle (controls), VIP (1 nmol, American Peptides, Sunnyvale, CA), or UCN (1 nmol, American Peptides) were administered i.p. starting at 12 or 24 hours after the cecal ligation and puncture (CLP), 2 hours after *E. coli* injection or 30 minutes after LPS infusion. The effective concentrations of neuropeptides used in the study were chosen based on previous experiments performed in our laboratory. In some experiments, recombinant HMGB1 (100 μg/mouse, HMGBiotech, Milan, Italy) was administered i.p. in VIP- and UCN-treated animals 18 hours after CLP. Animals were monitored daily for survival and clinical signs (ruffled fur, lethargy, diarrhea, and piloerection). Sera were obtained at different time points by cardiac puncture.

Cell Culture

BALB/c peritoneal macrophages or RAW264.7 cells were cultured at 10^6 cells/ml in RPMI medium 1640 (with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, and antibiotic-antimycotic mixture) for 2 hours, washed with Opti-MEM medium (Invitrogen, Carlsbad, CA) 2 hours later, and stimulated for 24 hours with LPS in the presence or absence of VIP or UCN in Opti-MEM. Supernatants were precipitated with trichloroacetic acid for HMGB1 determination.

Cytokine Determination

Cytokine contents in sera were determined by Multiplex (Bio-Rad, Hercules, CA) and BD CBA Flex Set (Becton Dickinson) assays following the manufacturer's recommendations.

HMGB1 Western Blot Analysis

Serum was filtered and concentrated through Centricon YM-100 and YM-10 (Millipore, Billerica, MA), respectively. Proteins in concentrated sera and cell culture supernatants were separated on 12% SDS-polyacrylamide gels and transferred to immunoblot membranes. Blots were blocked with 5% dry milk in PBS-Tween, incubated with a rabbit anti-HMGB1 antibody (BD PharMingen, 1:5000) and with a peroxidase-conjugated anti-rabbit antibody (DakoCytomation, Carpinteria, CA), and developed with ECL plus substrate (Amersham, Piscataway, NJ). HMGB1 expression was expressed as densitometric units relative to CLP or LPS control samples on the same blot.

Immunofluorescence

Macrophages adhered to coverslides were fixed in 4% paraformaldehyde for 20 minutes at room temperature and then incubated with glycine 30 mmol/L for 5 minutes. After washing three times with PBS, cells were permeabilized with 0.2% Triton X-100 for 15 minutes, and blocked with 2% bovine serum albumin for 1 hour. Cells were incubated with rabbit anti-HMGB1 antibody (dilution 1:2000 in PBS/2% bovine serum albumin) for 12 hours at 4°C. Slides were then washed and incubated with FITC-labeled goat anti-rabbit antibody (Invitrogen, Molecular Probes, 1:500). After extensive washing, samples were mounted in 4′,6-diamidino-2-phenylindole-containing Vectashield medium (Vector Laboratories, Burlingame, CA) and acquisition was performed with a microscope system (Cell R IX81; Olympus, Center Valley, PA), 63× and 100× objectives, illumination system (MT20; Olympus), and camera (Orca CCD; Hamamatsu).

Results

Late Administration of UCN and VIP Protect against Severe Sepsis

Because VIP and UCN inhibit the production of certain inflammatory mediators,[6] we first investigated the potential therapeutic action of both neuropeptides in the murine CLP model. CLP is a clinically relevant model for human sepsis that causes a polymicrobial peritonitis, bacteremia, and sepsis, and is considered a critical preclinical test for any new treatment of severe sepsis.[7–9] Delayed administration of VIP or UCN to mice with severe sepsis significantly reduced the mortality caused by cecal perforation (Figure 1A). We did not observe any late death (up to 3 weeks), indicating that VIP or UCN treatments confer lasting protection against experimental sepsis. Even when the initiation of the treatment was delayed to 24 hours after the induction of sepsis, VIP and

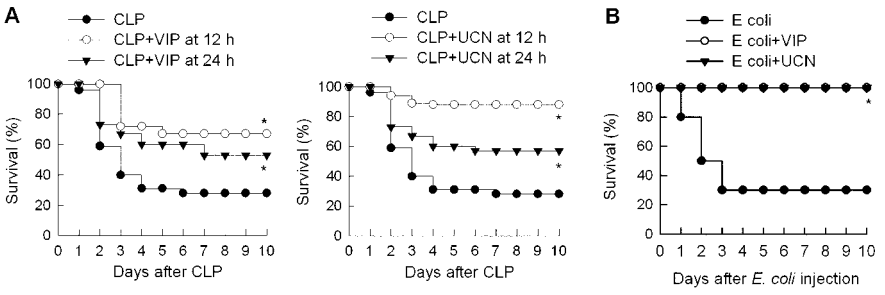

Figure 1. Delayed administration of UCN and VIP improve survival in established sepsis. **A:** Sepsis was induced by CLP, and mice were treated with vehicle or with UCN or VIP (1 nmol every 8 hours for 3 days) starting at 12 hours or 24 hours after sepsis induction. Survival was monitored for 10 days. $^*P < 0.05$ versus CLP alone (two-tailed Fisher's exact test). $n = 14$ to 32 mice/group. **B:** UCN and VIP protect against lethality induced by i.p. injection of 10^8 live *E. coli* (DH5α). UCN or VIP (1 nmol) were injected 2, 5, and 8 hours after bacterial injection. $^*P < 0.05$ vs. *E. coli* alone (two-tailed Fisher's exact test). $n = 10$ mice/group.

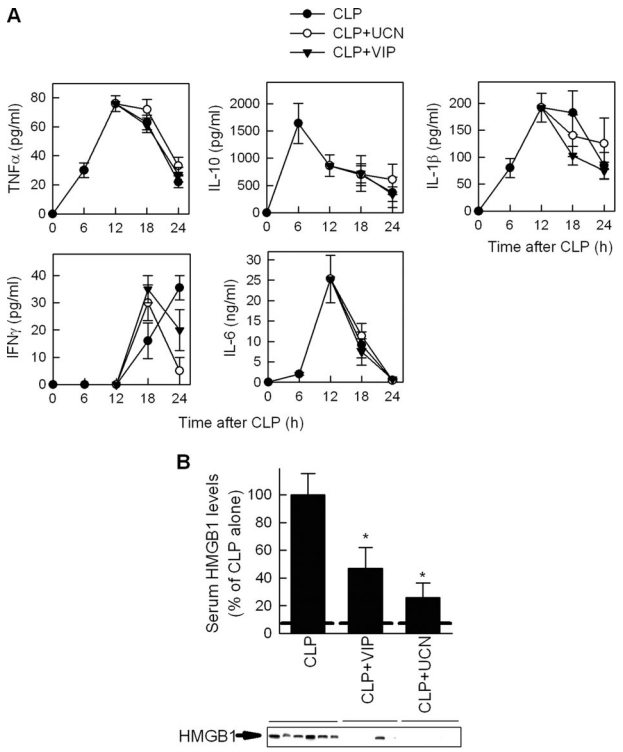

Figure 2. VIP and UCN inhibit HMGB1 release in sepsis. Mice were subjected to CLP and treated with vehicle or with VIP or UCN (1 nmol) at 12 and 18 hours after sepsis induction. **A:** Serum was collected at different times after CLP, and the levels of different cytokines determined as described in Materials and Methods. Data are mean ± SEM No significant differences were found between untreated or neuropeptide-treated mice. $n = 5$ to 10 mice per point. **B:** Serum was collected 24 hours after sepsis induction and the HMGB1 levels were determined by Western blot (HMGB1-specific band is indicated by an **arrow**) and expressed as band densities relative to control samples (CLP alone) on the same blot. Dashed line represents basal serum HMGB1 levels in normal mice. Data are mean ± SEM $^*P < 0.05$ versus CLP alone (Mann-Whitney test). $n = 7$ to 10 mice per group.

UCN increased the survival from 20%, to 53% and 57%, respectively (Figure 1A). The delayed administration of the neuropeptides also attenuated the clinical signs of sepsis, including lethargy, diarrhea, huddling, and piloerection. Furthermore, administration of UCN or VIP to animals with sepsis induced with *E. coli* increased survival from 30% to 100% (Figure 1B). Previous studies showed that VIP and UCN prevent lethal endotoxemia by down-regulating a wide spectrum of early inflammatory mediators, including tumor necrosis factor-alpha (TNFα), interleukin (IL)-6, IL-1β, nitric oxide, and several chemokines.[6] However, the therapeutic effect shown in the present study by VIP and UCN on established sepsis was observed when neuropeptides were administered after the peak of early inflammatory mediators.[10,11] In fact, delayed administration of VIP or UCN did not significantly affect the systemic levels of pro-inflammatory (TNFα, IL-1β, IL-10 and IL-6) and anti-inflammatory (IL-10) cytokines in animals with severe sepsis (Figure 2A). Thus, these data suggest that the therapeutic effect of UCN and VIP in sepsis is mediated by the control of a late mediator other than the early inflammatory cytokines.

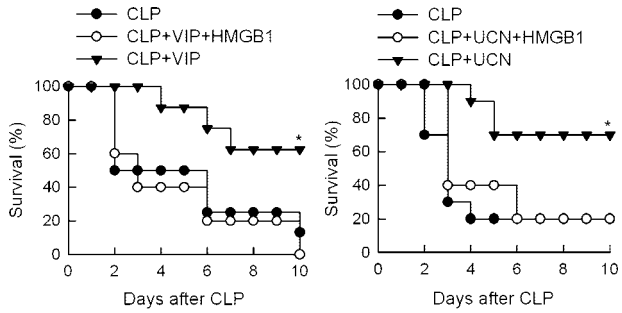

Figure 3. HMGB1 reversed the therapeutic effect of VIP and UCN in sepsis. Sepsis was induced by CLP and mice were treated with vehicle (CLP) or with UCN or VIP (1 nmol every 8 hours for 3 days) starting at 12 hours after sepsis induction. Recombinant HMGB1 (100 μg/mouse) was added to VIP- or UCN-treated animals at 18 hours after CLP. Survival was monitored for 10 days. $^*P < 0.05$ versus CLP alone and versus HMGB1-treated animals (two-tailed Fisher's exact test). $n = 5$ to 10 mice per group.

UCN and VIP Down-regulate HMGB1 Levels in Septic Mice

Although the pathophysiology of sepsis is unlikely attributable to a single molecule, several studies suggest that HMGB1 is a necessary and sufficient late mediator of the lethal multiple organ failure associated with severe sepsis.[1,4,5] Therefore, we next investigated whether VIP and UCN could attenuate circulating HMGB1 levels during sepsis. Consistent with previous observations,[4] sepsis induction resulted in increased systemic levels of HMGB1 (Figure 2B). Delayed treatment of septic mice with VIP or UCN significantly reduced the circulating levels of HMGB1 (Figure 2B). Interestingly, the relative efficiency in their protective effects on sepsis lethality (Figure 1A) correlated with the decrease on HMGB1 levels caused by both neuropeptides (Figure 2B). Moreover, exogenous administration of recombinant HMGB1 totally reversed the therapeutic effect of VIP and UCN in CLP-induced sepsis (Figure 3). Taken together these results indicate that VIP and UCN rescue mice from septic death by down-regulating the release of HMGB1.

UCN and VIP Inhibit the Secretion of HMGB1 by Activated Macrophages

The late appearance of HMGB1 in the serum of septic mice parallels the kinetic of HMGB1 release by activated macrophages.[1,4] In addition, macrophages have been previously described as the major targets in the anti-inflammatory effect of VIP and UCN.[6] Therefore, we next evaluated whether macrophages are direct cell targets of the inhibitory effect of VIP and UCN on the secretion of HMGB1. We treated macrophages with LPS in the presence or absence of a range of UCN or VIP concentrations for 24 hours and determined the presence of HMGB1 in the culture supernatants by Western blot. Whereas resting macrophages scarcely secreted HMGB1, stimulation with the bacterial endotoxin LPS induced the release of high levels of HMGB1 (Figure 4A and B). VIP and UCN dose-dependently inhibited LPS-induced HMGB1 release, showing a maximal effect at 10^{-7} M (Figure 4A and B), a concentration that is within the physiological range.[6]

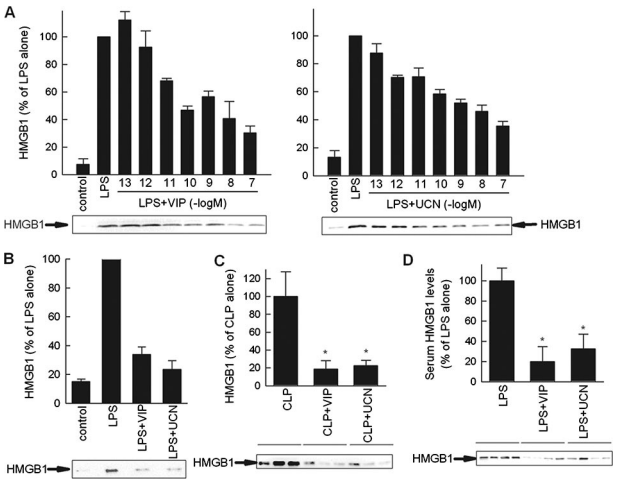

Figure 4. VIP and UCN reduce HMGB1 secretion by targeting macrophages. **A** and **B:** VIP and UCN inhibit the secretion of HMGB1 in endotoxin-activated RAW 264.7 (**A**) or peritoneal (**B**) macrophages were cultured with medium (control) or stimulated with LPS (1 μg/ml) and different concentrations of VIP or UCN (10^{-7} M for peritoneal macrophages) for 24 hours. HMGB1 content in culture supernatants was assayed by Western blotting and expressed as densitometric units relative to the LPS-treated condition on the same blot (HMGB1-specific band is indicated by an **arrow**). Data are mean ± SEM (n = 3 to 5). **C:** VIP and UCN deactivate peritoneal macrophages during sepsis. Mice were subjected to CLP and treated i.p. with VIP or UCN (1 nmol/mouse) 12 hours later. Peritoneal lavage was obtained 18 hours after sepsis induction. Peritoneal macrophages were isolated and cultured with medium at 10^6 cells/ml for 36 hours. The concentration of HMGB1 in the culture supernatants was determined by Western blotting and expressed as band densities relative to control samples (CLP alone) on the same blot. Data are mean ± SEM n = 8 per group $^*P < 0.001$ versus CLP alone (Mann-Whitney test). **D:** VIP and UCN down-regulate LPS-induced HMGB1 secretion *in vivo*. Endotoxemia was induced by i.p. injection of LPS (100 μg/mouse). Mice were treated 30 minutes later with medium (controls) or with VIP (1 nmol) or UCN (1 nmol). Serum was collected 24 hours after endotoxin administration and circulating HMGB1 levels were determined by Western blot (HMGB1-specific band is indicated by an **arrow**), and expressed as band densities relative to control samples (LPS alone) on the same blot. Data are mean ± SEM $^*P < 0.05$ versus LPS alone (Mann-Whitney test). n = 7 to 10 mice per group.

Although several administrations of VIP and UCN were needed to achieve a significant therapeutic effect *in vivo*, probably due to the short half-life of these peptides in circulation, a single administration of both neuropeptides was enough to signal macrophages and exert a long-term inhibition of HMGB1 *in vitro*. Thus, similar to their effect in other cytokines,[6] VIP and UCN still down-regulated HMGB1 secretion when they were added 4 hours after LPS stimulation, and when macrophages were exposed to VIP or UCN for 1 hour, and then extensively washed and stimulated with LPS (data not shown).

To determine whether our *in vitro* findings were relevant to the secretion of HMGB1 by macrophages during sepsis, we evaluated the impact of the treatment with VIP or UCN on the activation of peritoneal macrophages of septic mice *ex vivo*. Although macrophages isolated from septic animals spontaneously produced high amounts of HMGB1, macrophages isolated from animals treated with VIP or UCN secreted much lower levels of HMGB1 (Figure 4C). Because endotoxemia induced by LPS injection triggers a systemic inflammatory response characterized by the secretion of HMGB1 in the systemic circulation,[1] we then asked whether UCN and VIP could also decrease the release of HMGB1 induced by LPS *in vivo*. We administered UCN or VIP simultaneously with a LD_{40} injection of LPS and determined the serum HMGB1 levels 24 hours after the induction of endotoxemia. Consistent with previous observations,[1,12,13] LPS alone induced the release of HMGB1 into the serum, and both UCN and VIP reduced the levels of circulating HMGB1 (Figure 4D).

Taken together, these results suggest that the deactivation of resident and infiltrating macrophages could be the major mechanism involved in the therapeutic action of VIP and UCN on sepsis.

UCN and VIP Inhibit the Translocation of HMGB1 from the Nucleus to the Cytoplasm

HMGB1 lacks a secretory signal peptide and is secreted via a nonclassic secretory pathway.[14] Activation of monocytes and macrophages results in the accumulation of HMGB1 into cytoplasmic vesicles that display the features of secretory lysosomes.[14] Because UCN and VIP inhibit HMGB1 release by activated macrophages, we sought to determine whether both neuropeptides affect the relocalization of HMGB1 in LPS-stimulated peritoneal macrophages. Cells were cultured for 12 hours with LPS in the absence or presence of UCN or VIP, double stained with anti-HMGB1 antibodies and with the nuclear dye 4′,6-diamidino-2-phenylindole, and analyzed by fluorescence microscopy. Nonstimulated cells displayed a strong staining of HMGB1 mostly restricted to the nucleus, as indicated by the colocalization with 4′,6-diamidino-2-phenylindole. LPS stimulation resulted in an in-

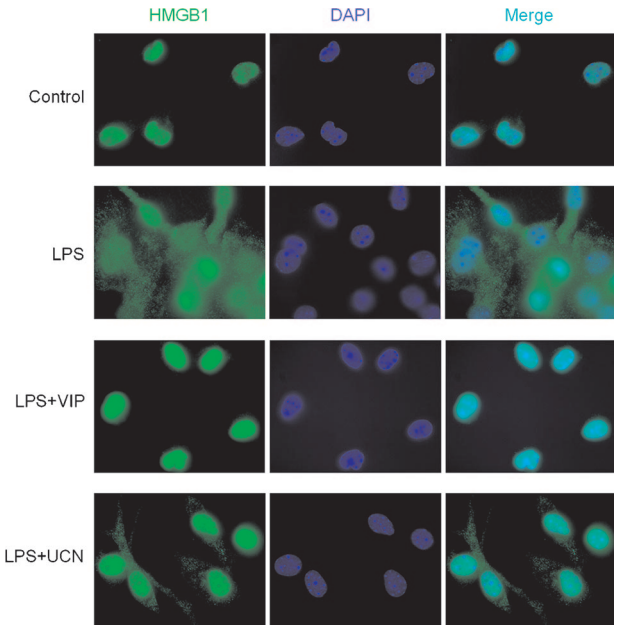

Figure 5. VIP and UCN impair cytoplasmic translocation of HMGB1 in activated macrophages. Peritoneal macrophages were cultured with medium (control), or stimulated with LPS (100 ng/ml) in the absence or presence of VIP (10^{-7} M) or UCN (10^{-7} M). After 12 hours, macrophages were fixed, permeabilized, stained with 4′,6-diamidino-2-phenylindole (blue channel) and anti-HMGB1 antibody (green channel), and the nuclear-cytoplasmic translocation of HMGB1 was determined by fluorescent microscopy (percentages of cells with HMGB1 translocated to the cytoplasm were: 11% unstimulated cells, 74% LPS-stimulated cells, 36% UCN-treated cells, and 13% VIP-treated cells). Data are representative of four independent experiments.

creased translocation of HMGB1 into the cytoplasm, where it displayed a punctuate staining (Figure 5). Both neuropeptides prevented HMGB1 redistribution to the cytoplasm and retained the protein in the nucleus (Figure 5).

Discussion

Sepsis is a major cause of morbidity and mortality in neonatal and medical intensive care units with an annual incidence of 750,000 patients per year in the United States.[15] Sepsis results from excessive stimulation of the host immune system by pathogen components to produce various pro-inflammatory cytokines, and their overproduction causes a systemic inflammation that can lead to lethal multiple organ damage. Despite continuing progress in the development of antibiotics and other supportive care therapies, there is a lack of effective therapy for sepsis.[16] Indeed, therapies directed to neutralize pro-inflammatory cytokines can prevent the development of septic shock in animal models, but clinical trials of these therapies have failed to improve the outcome of patients with sepsis.[17] The failure of these clinical trials may reside in the kinetics of cytokines such as TNFα and IL-1β, which are released early in the development of a systemic inflammatory response and normalize before the specific treatment is implemented. In recent years, various evidence indicates that HMGB1 is a necessary and sufficient late mediator of severe sepsis, and therefore, its targeting provides a wider window for clinical intervention.[1–5] In the present work, we show that VIP and UCN are physiological inhibitors of HMGB1 release. Both neuropeptides protect from the lethal effect of *E. coli* and CLP-induced sepsis and this protection is paralleled by a decrease in the systemic levels of HMGB1.

The inhibitory effects of VIP and UCN on HMGB1 release by peritoneal macrophages *in vitro* resembled closely the HMGB1 profile in endotoxemic and septic mice treated with the neuropeptides. This suggests that the deactivation of resident and infiltrating macrophages could be the major mechanism involved in the therapeutic action of VIP and UCN on established sepsis. However, we cannot exclude the involvement of other cells such as dendritic cells, natural killer cells, pituicytes, enterocytes, and endothelial cells, which secrete HMGB1 on stimulation.[2] Our results indicate that VIP and UCN inhibit HMGB1 release by interfering with its translocation from the nucleus to the cytoplasm, an essential step for HMGB1 secretion.[14]

We previously showed that VIP and UCN prevent endotoxin-induced production of TNF *in vivo* and *in vitro*.[6] However, the therapeutic use of these neuropeptides to improve survival in established sepsis was unknown. Sepsis and septic shock are two different syndromes and are likely to be mediated by different agents, such as TNF and HMGB1, respectively.[10,11] For example, therapies based in blockade of TNFα were efficient in endotoxemia,[18,19] but failed in septic humans and mice.[10,11] Furthermore, most septic patients do not have significant increased levels of TNFα, but they present high amounts of serum HMGB1.[16,17] Here, we observe that VIP and UCN are still protective against septic lethality even if administered 24 hours after sepsis induction. This wide therapeutic window can be mainly explained by the inhibition of HMGB1 secretion. Indeed, the exogenous administration of HMGB1 to septic animals abrogated the therapeutic effect of both neuropeptides. Moreover, the therapeutic window of UCN and VIP in severe sepsis has been uniquely achieved by specific blocking of HMGB1, such as neutralizing antibodies against HMGB1, ethyl pyruvate and nicotine.[4,5,12,13] However, antibody-blocking strategies increase the formation of antibody-antigen complexes and clinical studies designed to block cytokines were disappointing. Therefore, inhibition of HMGB1 secretion by VIP and UCN might represent a therapeutic advantage compared to anti-HMGB1 antibodies.

Besides sepsis, HMGB1 is also involved in the progression of other inflammatory and autoimmune diseases such as arthritis, inflammatory bowel disease, and ischemia/reperfusion.[2] UCN and VIP have been shown to exert protective actions in these disorders.[6] We have found that UCN reduces HMGB1 levels in serum of collagen-induced arthritic mice (A.C. and M.D., unpublished results). This finding supports the concept that VIP and UCN are endogenous inhibitors of HMGB1 release and suggests that inhibition of HMGB1 could be a general mechanism of action of both neuropeptides.

Of physiological relevance is the observation that the secretion of VIP and UCN dramatically increase in certain pathological inflammatory conditions, such as sepsis, endotoxemia and rheumatoid arthritis.[6,20–26] Interestingly, both VIP and HMGB1 peaks coincide in time in sepsis. In addition, deficient mice for VIP or VIP-receptor are significantly more susceptible to endotoxic shock.[27,28] Therefore, it is tempting to speculate that the body responds to an exacerbated inflammatory response by increasing the peripheral production of endogenous anti-inflammatory factors, including these neuropeptides, in an attempt to restore homeostasis. Thus, VIP and UCN emerge as natural anti-inflammatory peptides that regulate critical late events related to the overwhelmed systemic inflammatory response to infection that causes sepsis. The fact that the control of the systemic HMGB1 levels has lately emerged as one of the most promising therapeutic strategies for sepsis, point outs to neuropeptides such as VIP and UCN as feasible therapeutic agents for the treatment of this disorder.

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Ghrelin Protects against Experimental Sepsis by Inhibiting High-Mobility Group Box 1 Release and by Killing Bacteria[1,2]

Alejo Chorny, Per Anderson, Elena Gonzalez-Rey, and Mario Delgado[3]

Sepsis, a life-threatening complication of infections and the most common cause of death in intensive care units, is characterized by a hyperactive and out-of-balance network of endogenous proinflammatory cytokines. None of the current therapies are entirely effective, illustrating the need for novel therapeutic approaches. Ghrelin (GHR) is an orexigenic peptide that has emerged as a potential endogenous anti-inflammatory factor. In this study, we show that the delayed administration of GHR protects against the mortality in various models of established endotoxemia and sepsis. The therapeutic effect of GHR is mainly mediated by decreasing the secretion of the high mobility box 1 (HMGB1), a DNA-binding factor that acts as a late inflammatory factor critical for sepsis progression. Macrophages seem to be the major cell targets in the inhibition of HMGB1 secretion, in which GHR blocked its cytoplasmic translocation. Interestingly, we also report that GHR shows a potent antibacterial activity in septic mice and in vitro. Remarkably, GHR also reduces the severity of experimental arthritis and the release of HMGB1 to serum. Therefore, by regulating crucial processes of sepsis, such as the production of early and late inflammatory mediators by macrophages and the microbial load, GHR represents a feasible therapeutic agent for this disease and other inflammatory disorders. ***The Journal of Immunology,*** **2008, 180: 8369–8377.**

Sepsis is the third leading cause of death in developed societies and the most common cause of death in many intensive care units. Despite extensive research on the pathophysiology of sepsis and the technical advances, sepsis incidence is constantly rising (1.5–8% per year), and the increase in survival statistics of septic patients are merely due to the improvements in supportive treatment (1). The pathogenesis of sepsis is characterized by overwhelmed inflammatory and immune responses that can lead to tissue damage, multiple organ failure, and death. Most therapeutic strategies have targeted proinflammatory mediators, but they did not improve survival of patients when studied in large multicenter clinical trials (2). Therapies designed to block one single cytokine, such as TNF-α and IL-1β, have shown limited efficacy probably due to the early and transient kinetic of these inflammatory cytokines. In the last years, different evidences indicate that the high mobility group box 1 (HMGB1)[4] is a necessary and sufficient late mediator of severe sepsis and, therefore, its targeting provides a wider window for clinical intervention (3).

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[2] A.C. and M.D. designed research, performed research, and wrote the paper. E.G.-R. and P.A. performed research.

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[4] Abbreviations used in this paper: HMGB1, high mobility group box 1; GHR, ghrelin; CLP, cecal ligation and puncture; CIA, collagen-induced arthritis; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; FAM-GHR, carboxyfluorescein-labeled GHR.

Originally described as a nuclear protein that bends DNA, HMGB1 was recently found to be secreted by activated monocytes and macrophages (4), acting as a late proinflammatory factor (5) and promoting epithelial cell dysfunction (6). High levels of systemic HMGB1 are present in humans and animals with sepsis or endotoxemia (4, 7). Administration of recombinant HMGB1 to mice causes gut barrier dysfunction and lethal multiple organ damage (4, 6). In addition, passive immunization with neutralizing Abs against HMGB1 improves survival and prevents organ failure in septic mice (8, 9). Although the secretion of HMGB1 might be critical for the successful elimination of pathogens, it needs to be limited, because its excessive release results in severe inflammation and collateral tissue damage. In general, inflammatory responses are self-controlled by anti-inflammatory mediators secreted by host innate immune system during the ongoing process, but the endogenous factors involved in the control of HMGB1 secretion are poorly known.

Classically considered as neuroendocrine mediators, certain neuropeptides and hormones are also produced by immune cells and have recently emerged as potent endogenous anti-inflammatory factors involved in the control of the immune homeostasis (10). Ghrelin (GHR) is a 28-aa acylated polypeptide mainly produced by stomach cells, initially identified as an endogenous circulating ligand for the growth hormone secretagogue receptor, has potent orexigenic actions and is involved in the control of growth hormone secretion, energy expenditure, and adiposity (11). GHR and its receptors were recently detected in immune cells (12), indicating that this peptide may play a role in the immune system. Indeed, GHR has been described as a potential anti-inflammatory peptide that inhibits the production of proinflammatory cytokines by activated human T cells, monocytes, endothelial cells, and by animals with septic shock (12–14). In addition, GHR delays the onset, decreases the frequency and reduces the disease severity and mortality in experimental colitis, down-regulating both inflammatory and Th1-driven autoimmune responses (15).

In this study, we demonstrate that GHR is an endogenous inhibitor of HMGB1 release. Consistently, delayed administration of GHR attenuates the circulating levels of HMGB1, rescues from lethal experimental sepsis and attenuates the severity in arthritis. Notably, administration of recombinant HMGB1 to GHR treated mice reversed the therapeutic effect of the neuropeptide. Interestingly, this anti-inflammatory effect is accompanied by a potent bactericidal effect of GHR. In vitro and ex vivo studies indicate that macrophages are the major cell targets of GHR in the inhibition of HMGB1 secretion.

Materials and Methods

Animal models

Animal experimental protocols were reviewed and approved by the Ethical Committee of the Spanish Council of Scientific Research. To induce endotoxemia, BALB/c mice (6–8 wk old; The Jackson Laboratory) were injected i.p. with different amounts (25–600 μg/mouse; 400 μg/mouse unless otherwise indicated) of LPS (Sigma-Aldrich), or with a bacterial suspension containing 10^8 live *E. coli* (DH5α). To induce sepsis, cecum of anesthetized BALB/c mice was ligated 5.0 mm from the cecal tip and punctured once with a 22-gauge needle, and the stool was then extruded (1 mm). Vehicle (controls) or GHR (American Peptides) were administered i.p. starting at 12 or 24 h (at 12, 18, 24, 36, 48, and 72 h) after cecal ligation and puncture (CLP), 2 h (a single administration) after *E. coli* injection or at different times (a single administration) after LPS infusion. The effective concentrations of GHR used in the study were chosen based in previous experiments performed in our laboratory. In some experiments, recombinant HMGB1 (100 μg/mouse; HMGBiotech) was administered i.p. in GHR-treated animals 18 h after CLP. Animals were monitored daily for survival and clinical signs. Sera were obtained at different time points by cardiac puncture.

To induce collagen-induced arthritis (CIA) DBA/1J mice (7–10-wk-old; The Jackson Laboratory) were injected s.c. with 200 μg of type II collagen (Sigma-Aldrich) emulsified in CFA containing 200 μg of *M. tuberculosis* H37 RA (Difco). At day 21 after primary immunization, mice were boosted s.c. with 100 μg of type II collagen in CFA. GHR treatment consisted in the administration i.p. of GHR (1 nmol) on five consecutive days starting at day 24 when all mice showed established arthritis (clinical score >2). Control group was injected i.p. with PBS (untreated). Mice were analyzed by two blinded examiners every other day and monitored for signs of arthritis onset measuring the clinical score as described (16). Serum samples were collected at day 35 postimmunization for HMGB1 determination.

Cell culture

BALB/c peritoneal macrophages or RAW264.7 cells were cultured at 10^6 cells/ml in RPMI medium 1640 (with 10% heat-inactivated FBS, 2 mM glutamine, and antibiotic-antimycotic mixture) for 2 h, washed with Opti-MEM (Invitrogen) 2 h later, and stimulated for 24 h with LPS in the presence or absence of GHR in Opti-MEM. Supernatants were assayed for cytokine contents and precipitated with trichloroacetic acid for HMGB1 determination.

Cytokine and NO determination

Cytokine contents in sera and culture supernatants were determined by Multiplex assay (Bio-Rad) or by a specific sandwich ELISA by using capture/biotinylated detection Abs (BD Pharmingen) following the manufacturer's recommendations. NO amounts were estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay. Serum amyloid A levels in sera were determined by ELISA (Tridelta).

HMGB1 Western blot analysis

Serum was filtered and concentrated through Centricon YM-100 and YM-10 (Millipore), respectively. Proteins in concentrated sera and cell culture supernatants were separated on 12% SDS-polyacrylamide gels and transferred to Immuno-blot membranes. Blots were blocked with 5% dry milk in PBS-Tween 20, incubated with a rabbit anti-HMGB1 Ab (1/5000; BD Pharmingen) and with a peroxidase-conjugated anti-rabbit Ab (Dako-Cytomation), and developed with ECL plus substrate (Amersham). HMGB1 expression was expressed as densitometric units relative to CLP, LPS, or CIA control samples on the same blot.

Antimicrobial assays

To determine the antimicrobial activity of GHR in vivo, peritoneal lavage fluid recovered from mice 24 h post-CLP was analyzed for bacterial CFU/ml by dilution plating onto trypticase soy agar with 5% sheep blood (BD Biosciences) and colony counting following overnight incubation.

To determine the bactericidal activity of GHR in vitro, 10^4 *E. coli* (DH5α, mid-exponential growth phase) were incubated with different concentrations of GHR (added at time 0 and 3h) for 6 h at 37°C with shaking. After stopping the reaction with PBS at 4°C (dilution 1/100), the samples were plated, incubated overnight at 37°C, and the number of CFU/ml calculated.

To analyze membrane potential and permeability, 5×10^5 *E. coli* were incubated with 5 μM of GHR (added twice in an interval of 30 min) for 1 h at 37°C with shaking and then stained either with propidium iodide (PI, 10 μg/ml; Sigma-Aldrich) or bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($(DiBAC_4(3))$, 1 μg/ml; Molecular Probes) for 10 min at room temperature. After incubation, cells were washed and analyzed on a FACSCalibur flow cytometer (BD Biosciences). To determine the binding of GHR to the bacteria, 5×10^5 *E. coli* cells were incubated with GHR (4 μM or 9 μM) plus carboxyfluorescein-labeled GHR ((FAM-GHR), 1 μM; Phoenix Pharmaceuticals) for 30 min at 37°C with shaking. After incubation, cells were washed, immobilized on poly-L-lysine-covered glass slides for 1 h at 4°C, mounted in Vectashield medium (Vector Laboratories) and visualized by confocal microscopy (LEICA TCS SP5). Alternatively, FAM-GHR treated cells were analyzed by flow cytometry.

Immunofluorescence

Macrophages adhered to coverslides were fixed in 4% paraformaldehyde for 20 min at room temperature and then incubated with glycine 30 mM for 5 min. After washing three times with PBS, cells were permeabilized with 0.2% Triton X-100 for 15 min, and blocked with 2% BSA for 1 h. Cells were incubated with rabbit anti-HMGB1 Ab (dilution 1/2000 in PBS/2% BSA/0.1% Tween 20) for 12 h at 4°C. Slides were then washed and incubated with FITC-labeled goat anti-rabbit Ab (1/500; Molecular Probes). After extensive washing, samples were mounted in DAPI-containing Vectashield medium and acquisition was performed with a microscope system (Cell R IX81; Olympus).

Results

Late administration of GHR rescues from sepsis lethality and inhibits HMGB1 release

Different evidences have suggested the role of GHR in inflammation (12, 13, 15). Wu and coworkers (14, 17) have recently shown that GHR protects rats from experimental sepsis by down-regulating the production of some inflammatory cytokines. We first confirmed the therapeutic action of GHR in systemic inflammation and septic shock syndrome by using a model of endotoxemia induced by high-dose of endotoxin and of bacterial peritonitis by injection of *E. coli*. Early administration of GHR protected against septic death caused by a wide range of bacterial endotoxin LPS doses and 10^8 live *E. coli* (Fig. 1, *A* and *B*). The GHR effect on LPS-induced endotoxemia was dose-dependent, showing maximal protection at doses between 1 and 5 nmol (survival for 5, 1, 0.5, and 0.1 nmol were 80, 80, 50, and 10%, respectively). The protective effect of GHR on endotoxemia seems to be mediated through the regulation of the secretion of a wide array of inflammatory mediators, including several cytokines and chemokines, NO, and an acute phase protein (Fig. 1*C*). Because the production of inflammatory cytokines occurs in a rapid sequence starting with TNF-α, which reaches a maximum 2 h after LPS infusion, we next asked whether delayed administration of GHR could still protect in conditions of already established endotoxemia. Kinetic studies show that GHR exerts a full protective effect when administered 4 h after LPS injection (Fig. 2*A*). Even when the treatment was delayed until 12 h following the endotoxemia induction, GHR retained significant protective action (Fig. 2*A*). Notably, most of the inflammatory mediators returned to baseline at the time of GHR administration (Fig. 1*C*). Thus, the inhibitory effect of GHR on the release of the panel of early inflammatory mediators could only

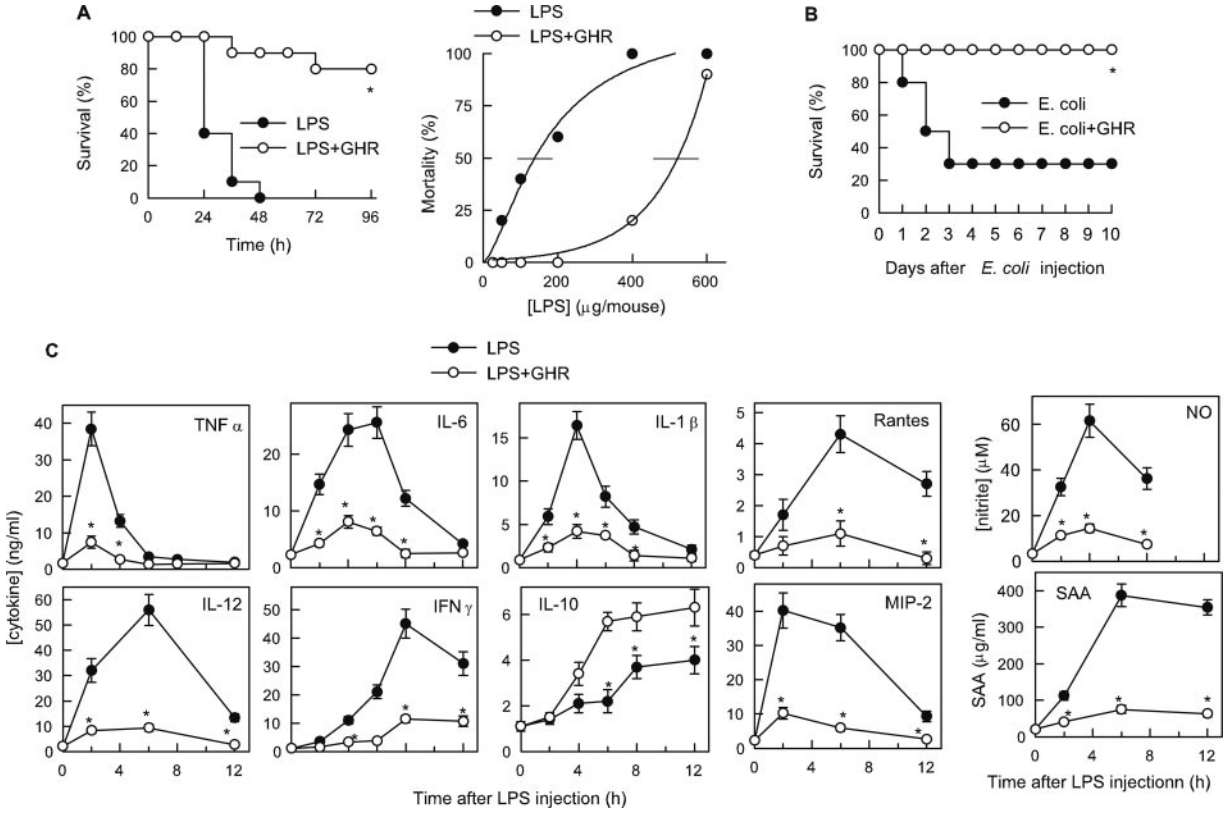

FIGURE 1. GHR protects against endotoxemia and *E. coli* induced lethality. *A*, Mice were injected i.p. with different doses of LPS (400 μg/mouse *in left panel*). GHR (1 nmol/mouse) was injected i.p. 30 min after LPS administration. Survival was monitored during the next 96 h. *Right panel*, Mortality curves were used to calculate LD_{50}, and horizontal bars indicate the 95% confidence limits of LD_{50} determinations. GHR treatment shifted the LD_{50} from 150 to 450 μg LPS. *B*, GHR protects against lethality induced by i.p. injection of 10^8 live *E. coli*. GHR (1 nmol/mouse) was injected 2 h after bacterial injection. $n = 10$–20 mice/group. *, $p < 0.001$ vs untreated control mice (two-tailed Fisher's exact test). *C*, GHR treatment reduces the systemic levels of early inflammatory mediators in endotoxemic mice. Endotoxemia was induced by i.p. injection of LPS (400 μg/mouse). Mice were treated 30 min later with medium (controls) or with GHR (1 nmol/mouse). Serum was collected at various time points after endotoxin injection. Serum cytokine and chemokine contents were determined by ELISA and NO levels were estimated from the accumulation of the stable NO metabolite nitrite by the Griess assay. Serum amyloid A (SAA) contents were determined by ELISA. $n = 5$–8 mice/group. Data are mean ± SD. *, $p < 0.001$ vs untreated control mice (Mann-Whitney *U* test).

explain the therapeutic effect of GHR in a short therapeutic window. However, the significant therapeutic effect shown by GHR in established endotoxemia could only be sustained by a potential effect on late mediators. Therefore, we next investigated whether GHR could attenuate circulating HMGB1 levels during endotoxemia. Consistent with previous observations (4), endotoxin administration resulted in increased systemic levels of HMGB1 (Fig. 2*B*). Treatment of endotoxemic mice with GHR reduced the secretion of HMGB1 into the circulation (Fig. 2*B*). Taken together, these data indicate that early administration of GHR prevents endotoxin-induced lethality by attenuating the release of both early and late inflammatory mediators.

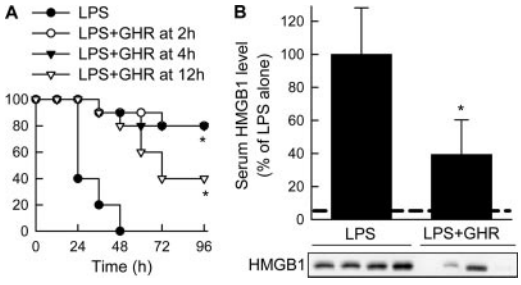

FIGURE 2. Delayed GHR treatment protects against established endotoxemia and reduces the systemic levels of HMGB1 levels. *A*, Endotoxemia was induced by i.p. injection of LPS (400 μg/mouse). GHR (1 nmol/mouse) was injected i.p. at 2, 4, or 12 h after LPS challenge. Survival was monitored during the next 96 h. $n = 10$ mice/group. *, $p < 0.001$ vs untreated control mice (two-tailed Fisher's exact test). *B*, Mice were injected with LPS (100 μg/mouse) and 30 min later with medium or with GHR (1 nmol/mouse). Serum was collected 24 h after endotoxin administration and circulating HMGB1 levels were determined by Western blot, and expressed as band densities relative to control samples (LPS alone) on the same blot. Dashed line represents basal serum HMGB1 levels in normal mice. Data are mean ± S.E.M. $n = 7$–10/group *, $p < 0.05$ vs LPS alone (Mann-Whitney *U* test).

There is general agreement among investigators that endotoxin administration may represent a model of systemic inflammation or endotoxic shock but not sepsis (2). On the contrary, the CLP model of peritonitis is considered for many researchers to be the most reliable animal model for human sepsis and a critical preclinical test for any new treatment of severe sepsis (18, 19). For example, TNF-α-blockade therapies were efficient in lethal endotoxemia and *E. coli* injection models (20–22), but failed in septic humans and CLP mice (23, 24). Sepsis induction by CLP results in increased circulating HMGB1 levels, with a peak at ~18 h after surgery (8). By contrast, other proinflammatory cytokines, such as TNF-α and IL-1β, reach their peak in the first hours of disease progression. Because GHR inhibits HMGB1 secretion in endotoxemia, we investigated whether GHR could improve sepsis survival. We induced sepsis by CLP and started GHR treatment 12 or 24 h after CLP procedure, i.e., after the early mediators' peak. GHR administration to mice

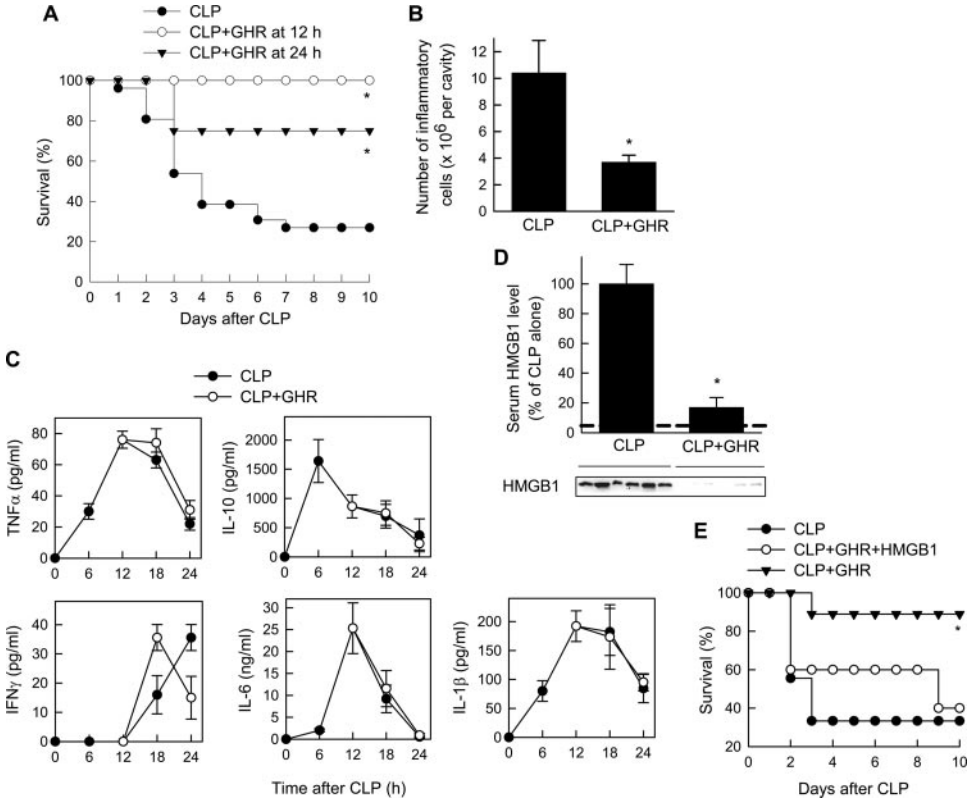

FIGURE 3. GHR improves survival and inhibits HMGB1 release in sepsis. *A*, Sepsis was induced by CLP. GHR treatment (1 nmol GHR/mouse at 12, 18, 24, 36, 48, and 72 h) was started 12 or 24 h later and survival was monitored for 10 d. $n = 20$/group. *, $p < 0.01$ vs CLP (two-tailed Fisher's exact test). *B*, GHR reduces leukocyte recruitment to the peritoneal cavity. Mice were subjected to CLP and treated i.p. with GHR (1 nmol/mouse) 12 h later. Peritoneal lavage was obtained 18 h after sepsis induction the number of inflammatory cells determined. Data are mean ± SEM. $n = 10$/group. *, $p < 0.001$ vs CLP alone (Mann-Whitney *U* test). *C*, Mice were subjected to CLP and treated with vehicle or with GHR (1 nmol) at 12 and 18 h after sepsis induction. Serum was collected at different times after CLP and the levels of different cytokines determined as described in *Materials and Methods*. Data are mean ± SEM. No significant differences were found between untreated or GHR-treated mice. $n = 5$–10 mice/point. *D*, Mice were subjected to CLP and GHR treatment was started 12 h later. Serum was collected 24 h after sepsis induction and the HMGB1 levels were determined by western blot and expressed as band densities relative to control samples (CLP alone) on the same blot. Dashed line represents basal serum HMGB1 levels in normal mice. Data are mean ± SEM. $n = 16$/group. *, $p < 0.001$ vs CLP alone (Mann-Whitney *U* test). *E*, HMGB1 reversed the therapeutic effect of GHR in sepsis. Sepsis was induced by CLP and mice were treated with vehicle (CLP) or with GHR (1 nmol/mouse) at 12, 18, 24, and 36 h after sepsis induction. Recombinant HMGB1 (100 μg/mouse) was added to GHR-treated animals at 18 h after CLP. Survival was monitored for 10 days. *, $p < 0.05$ vs CLP alone and vs HMGB1-treated animals (two-tailed Fisher's exact test). $n = 5$–10 mice/group.

with severe sepsis completely prevented the mortality caused by the cecal perforation (Fig. 3*A*). GHR treatment showed a wide therapeutic window, because even when the initiation of the treatment was delayed to 24 h after the induction of sepsis, GHR increased the survival from 27% to 75% (Fig. 3*A*). The delayed administration of GHR also attenuated the clinical signs of sepsis, including lethargy, diarrhea, huddling, and piloerection, and reduced the histopathological signs of the disease, such as massive accumulation of leukocytes in the peritoneum (Fig. 3*B*) and intestinal tract and in the gut and liver serosas, as well as segmental ischemia of the bowel with regions of hemorrhage, loss of intestinal crypts and intestinal wall perforation (data not shown). Previous studies and the present work (Fig. 1) showed that early administration of GHR prevent lethal endotoxemia by down-regulating a wide spectrum of inflammatory mediators. However, delayed administration of the neuropeptide in animals with severe sepsis did not significantly affect the systemic levels of proinflammatory (TNF-α, IL-1β, and IL-6) and anti-inflammatory (IL-10) cytokines (Fig. 3*C*). These data suggest that the therapeutic effect of GHR in established sepsis is mediated by the control of a late mediator other than the early inflammatory cytokines. Western blot analysis of the serum proteins showed increased HMGB1 levels in mice with sepsis (Fig. 3*D*). GHR treatment significantly reduced the circulating levels of HMGB1 (Fig. 3*D*). Moreover, administration of recombinant HMGB1 partially reversed the therapeutic effect GHR in CLP-induced sepsis (Fig. 3*E*). Taken together these results indicate that GHR rescues mice from septic death by down-regulating the release of HMGB1.

GHR improves bacterial clearance in vivo and shows bactericidal properties in vitro

CLP leads to a focal infection that subsequently becomes systemic and persistence of the local bacterial infection plays a critical role in sepsis mortality. Indeed, antibiotics can improve survival in murine sepsis induced by CLP (25). To evaluate whether GHR treatment influences the natural response against infection, we determine the bacterial levels at the primary peritoneal site of infection of septic mice. Contrary to the expected results, septic mice treated with GHR had lower peritoneal bacterial counts than septic mice treated with vehicle (Fig. 4*A*). This result suggests that GHR promotes bactericidal activities or that GHR is itself an antimicrobial peptide.

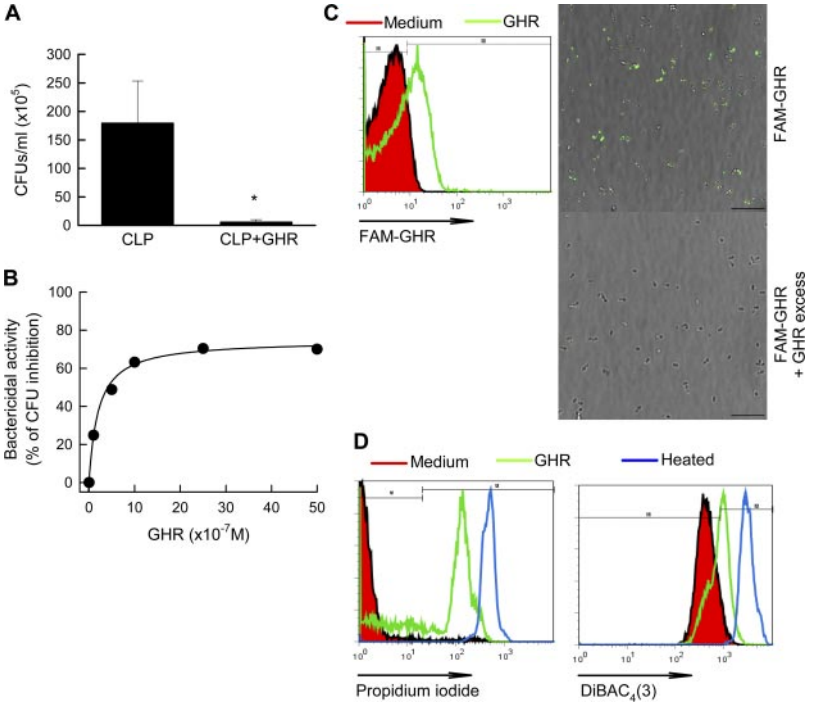

FIGURE 4. GHR improves bacterial clearance in vivo and shows bactericidal properties in vitro. *A*, Sepsis was induced by CLP, and mice were treated with GHR (1 nmol GHR/mouse at 12 and 18 h after CLP). Peritoneal lavage was obtained 24 h after surgical procedure, plated and incubated at 37°C, and the number of CFU/ml was counted. Data are mean ± SEM. $n = 16$/group. *, $p < 0.0005$ vs CLP alone (Mann-Whitney *U* test). *B*, Bactericidal activities of GHR against *E. coli* (DH5α). Each point represents the mean of triplicate viable counts determined as described in *Materials and Methods*, and expressed as 100 - the percentage of number of CFU/ml in control samples without neuropeptide. *, $p < 0.001$ (Student's *t* test). *C*, GHR binding to *E. coli*. Bacteria were incubated with medium (red histogram) or FAM-GHR (1 μM, green histogram) for 30 min and analyzed by flow cytometry and visualized by confocal microscopy. In binding competition assays, the addition of an excess of unlabeled GHR (9 μM) significantly reduced the number of FAM-GHR$^+$ cells while it increased the fluorescence background in the medium (bottom micrograph). Results are representative of three experiments. Bars in microscopy correspond to 10 μm. *D*, GHR treatment results in disruption of bacterial membrane. *E. coli* were cultured with medium (red histogram) or GHR (5 μM, green histogram) for 1 h. The membrane integrity and potential were indirectly determined by flow cytometric analysis of the incorporation of propidium iodide and DiBAC$_4$(3), respectively. *E. coli* heated at 80°C for 5 min (blue histogram) were used as controls of bactericidal activity (99.5% cells were positive for propidium iodide and DiBAC$_4$(3) staining). Results are representative of three experiments.

Antimicrobial peptides are integral components of the innate immune system and have a central role in host defense against infections. It has been recently described that some neuropeptides may participate in the immune defense against infections by directly killing bacteria (26). Because septic mice treated with GHR have decreased number of bacteria in the peritoneal fluid, we assessed the direct bactericidal activity of GHR in vitro, by incubating Gram-negative bacteria (*E. coli*) with different concentrations of GHR. GHR showed potent bactericidal properties against *E. coli* (Fig. 4*B*). This effect was dose dependent with a 75% of bacteria killed at 2.5 μM. The lack of recovery of bacterial viability after removal of GHR would indicate that the neuropeptide is bactericidal as opposed to bacteriostatic. We next investigated potential mechanisms involved in the bactericidal effect of GHR. It has been proposed that cationic antimicrobial peptides target the bacteria through electrostatic interactions with the microbial membrane, resulting in most cases in transmembrane pore formation and loss of membrane integrity that causes cell death. By using FAM-GHR, we observed that GHR was able to target the bacterial membrane (Fig. 4*C*). After binding, GHR seems to disrupt the membrane of the bacteria because a significant proportion of *E. coli* (69% of cells) treated with GHR incorporated propidium iodide, a small cationic molecule frequently used to monitor damage of microbial membrane integrity (Fig. 4*D*, *left panel*). This finding was confirmed by the fact that bacteria (54%) cultured with GHR showed significant increases in the uptake of DiBAC$_4$(3), a lipophilic anion sensitive to changes in membrane potential that enter into membranes only if their membrane potential has collapsed (Fig. 4*D*, *right panel*). These data collectively demonstrate that GHR acts as a bactericidal neuropeptide in vivo and in vitro, probably by disrupting the integrity of the cell membrane.

GHR inhibits the translocation and secretion of HMGB1 by activated macrophages

Macrophages are a major source of proinflammatory cytokines and secrete HMGB1 upon stimulation with LPS, and the HMGB1 release by macrophages is in agreement with that observed in serum of mice with sepsis (4, 8). To examine whether macrophages are direct cell targets of the inhibitory effect of GHR, we stimulate these cells with LPS in the presence or absence of the neuropeptide. GHR dose-dependently inhibited the release of several LPS-induced proinflammatory cytokines and chemokines and of NO (Fig. 5*A*). Thus, we examined the direct effect of GHR on the secretion of HMGB1 by LPS-stimulated macrophages. Unstimulated macrophages secreted very low levels of HMGB1, and LPS stimulation resulted in an increased secretion of HMGB1 (Fig. 5*B*). GHR significantly inhibited the release of HMGB1 induced by LPS. This effect was dose-dependent with a maximal effect at 10^{-7} M (Fig. 5*B*), a concentration that is within the physiological range (11). Although unstimulated macrophages displayed a strong staining for HMGB1 mostly restricted to the nucleus, LPS stimulation induced HMGB1 translocation from the nucleus to the cytoplasm before its secretion in 60% of the cells. GHR prevented the LPS-induced HMGB1 cytoplasmic translocation and retained

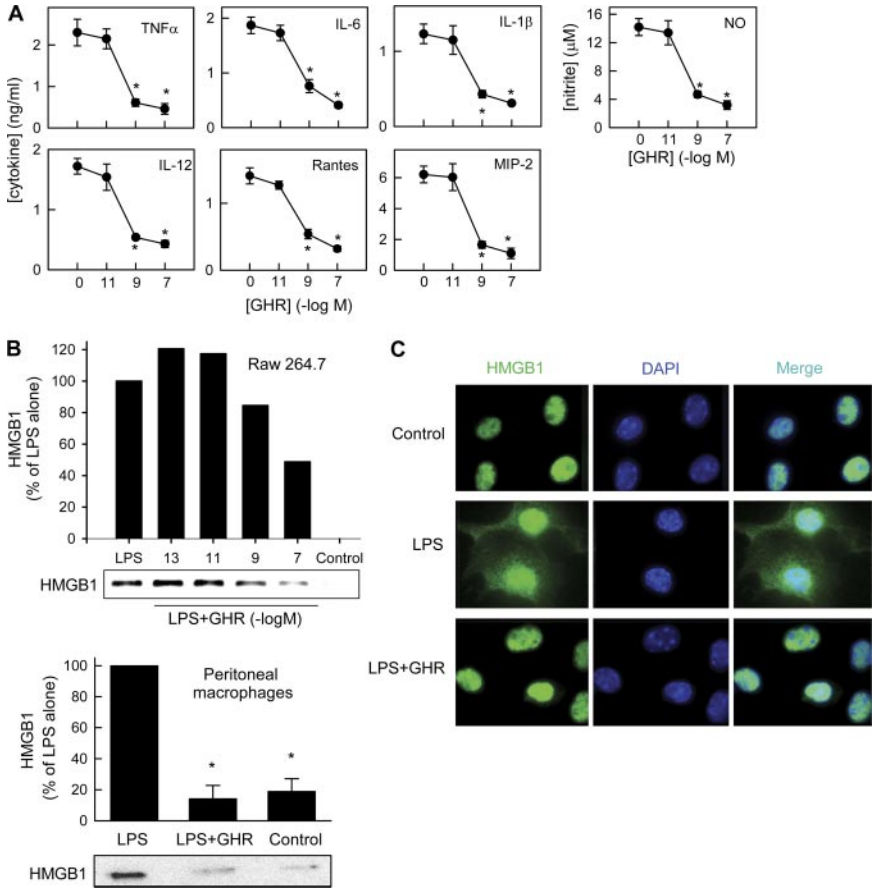

FIGURE 5. GHR inhibits the cytoplasmic translocation and secretion of HMGB1 in activated macrophages in vitro. *A*, GHR down-regulates the production of inflammatory factors by activated macrophages. Peritoneal macrophages were stimulated with LPS (1 μg/ml) in the absence or presence of different concentrations of GHR. At different time points (6 h for TNF-α and 24 h for IL-6, IL-12, IL-1β, RANTES, MIP-2, and NO), the levels of proinflammatory mediators in the culture supernatants were determined as described in the legend of the Fig. 1*C* ($n = 5$). $*$, $p < 0.001$, vs untreated control mice (Student's *t* test). *B*, GHR decreases HMGB1 secretion by activated macrophages. RAW 264.7 (*upper panel*) or peritoneal macrophages (*lower panel*) were stimulated with LPS (1 μg/ml) and different concentrations of GHR (10^{-7} M for peritoneal macrophages) for 24 h. HMGB1 content in culture supernatants was assayed by Western blotting and expressed as densitometric units relative to the LPS-treated condition on the same blot. Data are representative of three independent experiments. *C*, GHR inhibits cytoplasmic translocation of HMGB1 in macrophages. Peritoneal macrophages were cultured with medium (control), or stimulated with LPS (100 ng/ml) or with LPS plus GHR (10^{-7} M). After 12 h, macrophages were fixed-permeabilized, stained with DAPI (blue channel) and anti-HMGB1 Ab (green channel) and analyzed by fluorescent microscopy.

HMGB1 in the nucleus (only 20% of the cells showed translocation; Fig. 5*C*). A single administration of GHR was enough to signal macrophages and exert a long-term inhibition of HMGB1 release in vitro. Thus, GHR still down-regulated HMGB1 secretion when it was added 4 h after LPS stimulation (data not shown). These in vitro findings were relevant to the secretion of HMGB1 by macrophages during sepsis, because whereas peritoneal macrophages isolated from septic animals spontaneously produced high amounts of HMGB1 ex vivo, macrophages isolated from GHR-treated animals secreted much lower levels of HMGB1 (Fig. 6*A*). Furthermore, GHR efficiently blocked the in vivo translocation of HMGB1 from the nucleus to the cytoplasm (Fig. 6*B*). These results collectively demonstrate that GHR inhibits HMGB1 secretion by macrophages during sepsis.

GHR reduces serum HMGB1 during arthritis

HMGB1 has been proven to be a successful therapeutic target not only in severe sepsis, but also in experimental models of diverse infectious and inflammatory diseases. Among them, HMGB1 is elevated in serum and synovial tissue of patients with rheumatoid arthritis as well as in the joints of animals with arthritis. Indeed, in the mouse model of CIA, treatment with Abs to HMGB1 attenuates the severity of the disease. In addition, it has been described a protective effect of the GHR agonist growth hormone-releasing peptide 2 in arthritic rats (27). Thus, to further investigate the ability of GHR to down-regulate the release of HMGB1 during the course of an immune disorder, we induced CIA, treated the animals with GHR, and analyzed the levels of HMGB1 in the serum. GHR treatment of mice with established clinical signs of arthritis progressively decreased incidence and severity of CIA as compared with untreated mice, as assessed by clinical score (Fig. 7*A*).

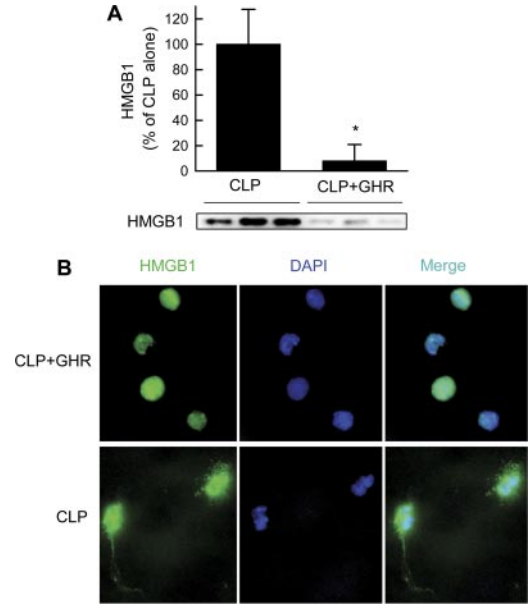

FIGURE 6. GHR deactivates peritoneal macrophages during sepsis. Mice were subjected to CLP and treated i.p. with GHR (1 nmol/mouse) 12 h later. Peritoneal lavage was obtained 18 h after sepsis induction. *A*, Peritoneal macrophages were isolated, cultured with medium at 10^6 cells/ml for 36 h, and the concentration of HMGB1 in the culture supernatants was determined by Western blotting and expressed as band densities relative to control samples (CLP alone) on the same blot. Data are mean $\pm$ SEM. $n = 8$/group. $*$, $p < 0.001$ vs CLP alone. *B*, Peritoneal macrophages were allowed to adhere for 2 h, fixed-permeabilized, stained with 4′,6-diamidino-2-phenylindole (blue channel) and anti-HMGB1 Ab (green channel) and analyzed by fluorescent microscopy.

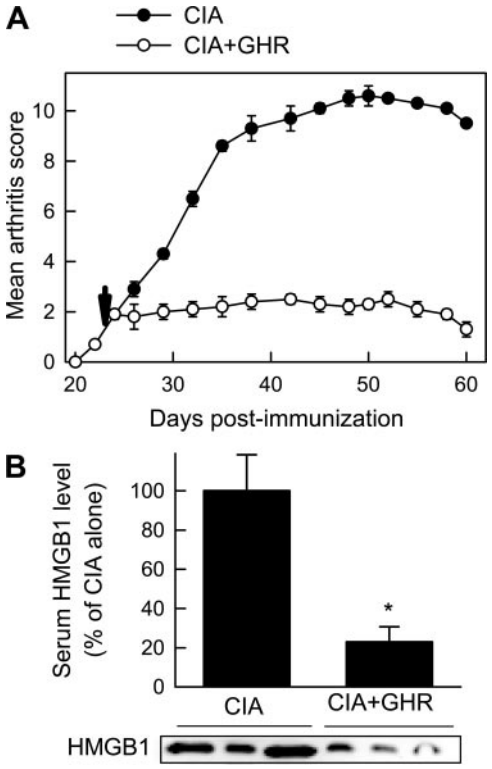

FIGURE 7. GHR treatment reduces the systemic levels of HMGB1 levels in arthritic mice. *A*, Severity of arthritis, assessed by clinical scoring in mice with established CIA injected i.p. (arrow) either with PBS (control) or with GHR (1 nmol/mouse) daily for 5 days. $n = 8–12$ mice/group. $p < 0.001$ vs control after day 28. *B*, Serum samples were obtained on the day 35 post immunization and assessed for the presence of HMGB1 by western blot and expressed as band densities relative to control samples (CIA alone) on the same blot. Data are mean ± SEM. $n = 5$/group. *, $p < 0.001$ vs CIA mice (Mann-Whitney *U* test).

Whereas untreated CIA mice show high levels of circulating HMGB1, GHR reduced the concentration of serum HMGB1 (Fig. 7*B*). These findings support the concept that GHR is an endogenous inhibitor of HMGB1 release and suggest that inhibition of HMGB1 could be a general mechanism of action of the neuropeptide.

Discussion

This work provides the evidence that GHR might represent a feasible therapeutic agent for the treatment of sepsis. GHR functions at several levels to regulate many crucial aspects of sepsis, such as bacterial clearance and the inflammatory response to infection. Thus, GHR has the potential to provide therapeutic advantage over agents that are directed against only one component of the disease.

Our data show that GHR is a potent immunomodulatory factor that directly inhibits the secretion of early and late inflammatory mediators of sepsis, with a special relevancy of down-regulation of HMGB1. Consistent with our results, during the past few years, evidence has been accumulated regarding the role of GHR in inflammation. GHR down-regulates the production of proinflammatory cytokines and chemokines by activated human PBMCs and coadministration of GHR with LPS to rats attenuated the plasma levels of TNF, IL-8, and MCP-1 (12, 13). Recently, Wu et al. (14, 17) have found that GHR down-regulates TNF and IL-6 and improves acute lung injury in CLP rats. These effects have been partially related to stimulation of the vagus nerve and to inhibition of the sympathetic nervous activity by GHR (14, 28). In addition, GHR improves the hemodynamic changes and glucose and lactate abnormalities (29) and tissue perfusion in septic rats (30). Although some of these mechanisms could contribute to the therapeutic action of this neuropeptide in sepsis, the evidence presented in this study strengthens the hypothesis of inhibition of a late mediator. We show that GHR is still protective against endotoxin lethality even if administered 12 h after a LD_{100} injection of LPS, and that GHR treatment can be delayed until 24 h after sepsis induction, several hours after the peak of early mediators. Indeed, administration of GHR 12 h after CLP does not modulate the serum levels of proinflammatory cytokines, but down-regulates the systemic levels of HMGB1. Furthermore, administration of recombinant HMGB1 to GHR-treated mice abolished the therapeutic effect of the neuropeptide on sepsis. In this sense, our study demonstrates that GHR is the most potent physiological inhibitor of HMGB1 release described to date in experimental sepsis. HMGB1 is a nuclear protein with cytokine activity that is involved in the pathogenesis of a wide range of immune-mediated diseases. Remarkably, numerous works has shown that HMGB1 is a necessary and sufficient late mediator of sepsis (reviewed in Ref. 3) and, therefore, it has been established as one of the major targets for its treatment. Because in many clinical cases hours pass before sepsis is diagnosed and specific treatment is implemented, targeting HMGB1 would expand the therapeutic window to a clinical relevant time. In any case, sepsis is a complex disease with different aetiologies and the physiology of HMGB1 is starting to be unraveled. In a recent work, Angus et al. (31) found that HMGB1 was elevated in both uncomplicated pneumonia and pneumonia with severe sepsis, although higher HMGB1 concentrations were associated with mortality.

The inhibitory effect of GHR on the release of HMGB1 in vitro and ex vivo by peritoneal macrophages resembled closely the profile observed in mice treated with GHR. Therefore, it is likely to be deactivation of resident and infiltrating macrophages the major mechanism in the therapeutic effect of the neuropeptide. Although activated macrophages release HMGB1 with a similar kinetics observed in serum of septic mice (8), other cells, including NK, pituicytes, enterocytes, and endothelial and dendritic cells also secrete HMGB1 upon stimulation (32–35). Although the contribution of these cells in the increase of circulating HMGB1 levels during sepsis have not yet been addressed, we cannot rule out the possibility that GHR exerts some effects on them in septic mice. In fact, GHR inhibits endothelial cell activation by TNF-α (13).

The ability of delayed administration of GHR to ameliorate ongoing disease fulfills an essential prerequisite for an anti-septic agent, as treatment starts after the onset of sepsis. In contrast, lysophosphatidylcholine and Abs to macrophage inhibitory factor loose their therapeutic effects if administration starts at 8–10 h after infection induction (36, 37). Similar to GHR, therapies based in neutralizing Abs against HMGB1 improve survival when started 24 h after CLP (8, 9, 38, 39). However, Ab-blocking strategies increase the formation of Ab-Ag complexes and clinical studies designed to block cytokines are disappointing. In this regard, inhibition of HMGB1 secretion might represent a therapeutic advantage compared with anti-HMGB1 Abs. Indeed, the therapeutic window shown by GHR in experimental sepsis has been uniquely achieved by specific inhibitors of HMGB1 (7, 8, 40). Thus, ethyl pyruvate and nicotine (7, 36) inhibit the secretion of HMGB1 and showed a similar therapeutic effect as GHR in severe sepsis. However, GHR is the first endogenous inhibitor of HMGB1 secretion shown to improve sepsis survival in a clinical relevant time frame.

Interestingly, patients with sepsis have also features consistent with immunosuppression, characterized by loss of lymphocytes

and dendritic cells, and manifested by an inability to clear the primary infection and by the development of secondary infections. In this regard, treatment strategies for severe sepsis are directed, at least in part, to eradicate the infection (41, 42). In the present study, we describe that GHR significantly diminishes peritoneal bacterial counts of septic animals and directly kills bacteria in vitro. Remarkably, the GHR concentrations found effective in both in vivo and in vitro antimicrobial studies are in the same range. The antimicrobial activity of GHR (with a LD_{50} of 0.2 μM) against *E. coli* is similar to that described for other neuropeptides, and it corresponds to the concentration of bactericidal neuropeptides found in certain microenvironments, such as in the proximity of nerve endings (26). Interestingly, GHR shares some important properties with natural antimicrobial peptides, including small size ($<$10 kDa), high positive charge, and amphipathic α-helix structures adopted upon interaction with membranes. Our data show that GHR initially binds to bacteria and alter the permeability and potential of the membrane. Therefore, GHR could interact with the negatively charged outer leaflet of the plasma membranes of bacteria and insert into the cell membrane. This would lead to a rapid loss of the cell homeostasis and eventually death of the pathogen by membrane disruption.

The lower bacterial load in the peritoneum of GHR-treated mice might mean lower stimulation for HMGB1 secretion and, therefore, contribute to the GHR-induced inhibition of circulating HMGB1. However, the fact that GHR improves survival and attenuates systemic HMGB1 levels in mice injected with LPS, and that GHR inhibits the secretion of HMGB1 by LPS-activated peritoneal macrophages in vitro, strongly supports that the neuropeptide directly inhibits HMGB1 release in our model. However, GHR could also modulate HMGB1-induced lethality through other indirect mechanisms. Endotoxin and HMGB1 seem to act synergistically in septic death, because coadministration of non-lethal doses of HMGB1 and LPS results in a mortality of 90% (4), and HMGB1 can bind CpG DNA leading to an augmentation of TLR9-dependent secretion of proinflammatory cytokines (43, 44), suggesting that some bacterial products could act as potential cofactors with HMGB1. Moreover, a recent study (45) has demonstrated that purified HMGB1 fails to induce the production of inflammatory cytokines by activated macrophages and that HGMB1 acquires proinflammatory activities through the binding to inflammatory mediators, such as IL-1β. In this context, the decreased bacterial levels observed at the peritoneal cavity of GHR-treated mice may also influence the HMGB1-induced lethality.

From a therapeutic point of view, it is important to consider that HMGB1 has been involved in the progression of various inflammatory and autoimmune diseases. In addition to sepsis, HMGB1 acts as a mediator of inflammation and organ damage in hepatic ischemia/reperfusion injury (3). Moreover, epithelial barrier disruption and bacterial infection are related to the pathogenesis of some inflammatory bowel disorders, and HMGB1 impairs intestinal barrier function and is a mediator of necrotizing enterocolitis (6, 46). In addition, HMGB1 administration to the joints causes arthritis and its blockade confers protection in this disorder (3). In this context, the GHR attenuation of lesions evoked by ischemia/reperfusion, improvement of the outcome in inflammatory bowel disease, and the reduction of arthritic signs (15, 26, 47, 48 and Fig. 7) could be explained, at least partially, by the inhibition of HMGB1 and the antibacterial activity of the neuropeptide. In fact, here we show that GHR attenuates the secretion of HMGB1 into the circulation of arthritic mice.

Of physiologic relevance is the observation that the secretion of GHR dramatically drops in certain pathologic inflammatory conditions, such as CLP, endotoxemia, rheumatoid arthritis, and *H. pylori* infection (17, 49–52). Furthermore, decreased endogenous GHR contribute to the increased incidence of atherosclerosis in patients with obesity (53). Therefore, it is tempting to speculate that during inflammatory processes, a reduction of the levels of GHR might exacerbate the ongoing inflammatory insult. Thus, GHR emerges as a natural antimicrobial and anti-inflammatory peptide, widely distributed in all body tissues and especially abundant in the proximity to physical barriers such as stomach, gut, and skin, where one of its primary functions could be related to the control of innate immunity and response against infections.

In summary, the present study provides the first evidence that GHR is an endogenous inhibitor of HMGB1 release and a bactericidal neuropeptide. In this way, our findings provide a new concept in the role of GHR in host defense and highlight this neuropeptide as a potential multistep therapeutic agent for human sepsis and other immune disorders.

Disclosures

The authors have no financial conflict of interest.

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Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders

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The induction of antigen-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. In addition to their classical role as sentinels of the immune-response-inducing T cell reactivity, dendritic cells (DCs) play an important role in maintaining peripheral tolerance through the induction/activation of regulatory T cells (Tr). The possibility to generate tolerogenic DCs opens new therapeutic perspectives in autoimmune/inflammatory diseases. Therefore, the characterization of the endogenous factors that contribute to the development of tolerogenic DCs is highly relevant. In this study, we report on the use of the known immunosuppressive neuropeptide, the vasoactive intestinal peptide, as a new approach to induce tolerogenic DCs with capacity to generate Tr cells, to restore tolerance *in vivo*, and to reduce the progression of rheumatoid arthritis and experimental autoimmune encephalomyelitis.

autoimmunity | regulatory T cell | tolerance

The immune system is faced with the daunting job of protecting the host from an array of pathogens, while maintaining tolerance to self-antigens (Ags). The induction of Ag-specific tolerance is essential to maintain immune homeostasis, to control autoreactive T cells, preventing the onset of autoimmune diseases, and to achieve tolerance toward transplants. Both thymic and peripheral mechanisms account for the ability of the immune system to induce tolerance. Attention has been focused recently on induction of active suppression by regulatory T cells (Tr) (1), and dendritic cells (DCs) have been shown to contribute to T cell tolerance (2, 3). The maturation/activation state of DCs might be the control point for the induction of peripheral tolerance, by promoting Tr differentiation. Thus, whereas mature DCs (mDCs) are potent Ag-presenting cells enhancing T cell immunity, immature DCs (iDCs) are involved in the induction of peripheral T cell tolerance under steady-state conditions (2–6). However, the clinical use of iDCs may not be suitable for the treatment of autoimmune diseases, because iDCs are likely to mature in inflammatory conditions (6), emphasizing the need to develop tolerogenic DCs with a strong potential to induce Tr. Immunosuppressive therapy, traditionally focused on lymphocytes, has been revolutionized by targeting the development and key functions of DC, and the generation of tolerogenic DCs in the laboratory has become the focus of new therapies (7).

Vasoactive intestinal peptide (VIP) is a neuropeptide released by both innervation and immune cells, particularly T helper (Th)2 cells, in response to Ag stimulation and under inflammatory/autoimmune conditions (8). VIP elicits a broad spectrum of biological functions, including immunomodulation, predominantly acting as a potent antiinflammatory factor and a suppressive agent for Th1 responses (9). Therefore, VIP has emerged as a promising therapeutic factor for the treatment of autoimmune/inflammatory diseases, including rheumatoid arthritis (RA), ulcerative colitis, uveoretinitis, and experimental autoimmune encephalomyelitis (EAE) (10–12). In this study, we investigated whether the presence of VIP during the early phases of DC differentiation induces the generation of regulatory DCs with the capacity to induce Tr and to prevent autoimmunity.

Materials and Methods

Cell Isolation and Cultures. Bone marrow (BM)-derived DCs (BM-DCs) were generated as described in ref. 13. Briefly, BM cells (2×10^6) obtained from BALB/c (H-2^d), C57BL/6 (H-2^b), or DBA/1 (H-2^q) mice were incubated in complete medium (RPMI medium 1640 supplemented with 100 units/ml penicillin/strectomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 10% heat-inactivated FCS) containing 20 ng/ml granulocyte macrophage colony-stimulating factor in the presence or absence of VIP (10^{-8} M). At day 6, nonadherent cells were collected (routinely containing 80–90% $CD11c^+$ cells) and stimulated for 48 h with LPS (1 μg/ml) to induce activation/maturation. In some experiments, DCs were pulsed with ovoalbumin (OVA), collagen II (CII), or myelin oligodendrocyte glycoprotein (MOG) (20 μg/ml) for 12 h. Allogeneic naïve CD4 T cells were purified from C57BL/6 mice by positive immunomagnetic selection (MACS, Miltenyi Biotec, Auburn, CA).

Flow Cytometry. Cells were incubated with various peridinin–chlorophyll–protein complex (PerCP)-, FITC- and phycoerythrin (PE)-labeled mAbs (BD Pharmingen), diluted at optimal concentration for immunostaining, fixed in 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). We used isotype-matched Abs as controls and IgG block (Sigma) to avoid the nonspecific binding to Fc-receptors.

Cytokine Assays. Cytokine contents in the culture supernatants were determined by specific sandwich ELISAs by using capture/biotinylated detection Abs from BD Pharmingen. For intracellular analysis of cytokines in restimulated CD4 T cells, 10^6 cells per ml were collected and stimulated with phorbol 12-myristate 13-acetate (1 ng/ml) plus ionomycin (20 ng/ml) for 8 h in the presence of monensin. Cells were stained with PerCP-anti-CD4 mAbs for 30 min at 4°C, washed, fixed/saponin-permeabilized with Cytofix/Cytoperm, stained with 0.5 μg per sample FITC- and PE-conjugated anticytokine-specific mAbs, and analyzed by flow cytometry. To distinguish between DC and T cell sources,

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Abbreviations: Ag, antigen; BM, bone marrow; DC, dedritic cell; BM-DC, BM-derived DC; CIA, collagen-induced arthritis; CII, collagen II; DLN, draining lymph nodes; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; iDC, immature DC; mDC, mature DC; MOG, myelin oligodendrocyte glycoprotein; OVA, ovalbumin; RA, rheumatoid arthritis; Tr, regulatory T cells; Th, T helper; VIP, vasoactive intestinal peptide

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intracellular cytokine analysis was done exclusively in the PerCP-labeled CD4 T cell population.

mRNA Analysis. Total RNA was isolated from CD4 T cells, and real-time PCR was used to determine Foxp3 and neuropilin mRNA expression, as described in refs. 14 and 15.

Mixed Leukocyte Reaction and Analysis of Tr Cell Function. Naïve CD4 T cells (2×10^5) were cultured with allogeneic $DC_{control}$ or DC_{VIP} at various T:DC ratios in the presence of IL-2 (100 units/ml) for 3 d. Cell proliferation was evaluated by using a cell-proliferation assay (BrdUrd) from Roche Diagnostics (Mannheim, Germany), and intracellular cytokine content was determined as described above. In some experiments, DCs (10^5) were cultured with purified allogeneic CD4 T cells (5×10^5). One week later, CD4 T cells were recovered by immuodepletion of $CD11c^+$ DCs and cultured in different numbers with syngeneic CD4 T cells (5×10^5) in the presence of allogeneic mDC (10^5), and the proliferative response was determined. Some cultures were performed in the presence of blocking anti-IL-10 (10 μg/ml) and/or anti-TGFβ1 (40 μg/ml) mAbs. To determine the cell-contact-dependence of the regulatory response, we placed responder CD4 T cells (5×10^5) with LPS-matured DC (10^5) in the bottom well of a Transwell system (Millipore) and syngeneic Tr_{VIP} (2×10^5) with allogeneic mDC (10^5) in the upper Transwell chamber. After 72 h, we measured the proliferative response of the bystander reactive CD4 T cells in the bottom well. To generate CII- and MOG-specific Tr cells, DBA1/J and C57BL/6 DCs (10^5) pulsed with CII or MOG, respectively, were cultured with syngeneic CD4 T cells (5×10^5) for 1week in the presence of CII or MOG (20 μg/ml).

Immunization Model. BALB/c mice were injected s.c. with different numbers (from 50 to 5×10^5) of cells of methylated BSA (mBSA)-pulsed $DC_{control}$ or DC_{VIP}, followed a week later by s.c. immunization with the Ags mBSA or OVA (60 μg) in complete Freund's adjuvant. Five days after Ag immunization, serum Ag-specific Ab, draining lymph nodes (DLN) T cell proliferative responses, and delayed type hypersensitivity (DTH) responses were measured. For the DTH responses, mice were injected i.d. with Ag (5 μg) or saline into the ears, and ear swelling was measured 24 h later by using a caliper. Ag-specific T cell proliferative responses were measured after *ex vivo* stimulation of DLN cells (4×10^5) with 10 μM Ag. Levels of mBSA-specific IgG in serum were determined by ELISA, as described in ref. 16.

Model for RA and EAE. RA was induced in DBA1/J mice by s.c. injection of CII, as described in ref. 10. Chronic EAE was induced in C57BL/6 mice by s.c. immunization with MOG_{35-55}, as described in ref. 17. Mice with established arthritis (with a clinical score of 2) were injected i.v. with different numbers of syngeneic CII-pulsed $DC_{control}$ or DC_{VIP} or with CII-specific $Tr_{control}$ or Tr_{VIP}. Mice with established EAE (with a clinical score of 1) were injected i.v. with different numbers of syngeneic MOG_{35-55}-pulsed $DC_{control}$ or DC_{VIP} or with MOG_{35-55}-specific $Tr_{control}$ or Tr_{VIP}. The clinical score was determined daily, based on joint inflammation for RA and tail/leg paralysis for EAE, as described in ref. 17. DLN cells were isolated at the peak of the diseases, stimulated with CII or MOG_{35-55} (20 μg/ml), and assayed for proliferation and cytokine production, as described above. The content of serum anti-CII or anti-MOG_{35-55} IgG antibodies was determined by ELISA, as described in refs. 10 and 18. To assess Ag-specificity, arthritic mice were injected with unpulsed, OVA-pulsed, or CII- or MOG_{35-55}-pulsed $DC_{control}$ or DC_{VIP} and immunized s.c. with OVA, CII, or MOG_{35-55} (150 μg of Ag in complete Freund's adjuvant) one week later. After 5 d, mice received 5 μg of Ag i.d. in the ear pinna, and the DTH response was determined, as described above. In some experiments, collagen-induced arthritis (CIA) and EAE mice received i.v. injections of neutralizing anti-IL-10 polyclonal Ab, neutralizing anti-TGFβ mAb, or preimmune rat IgG used as control Ig (500 μg of Ab per mouse) on alternate days up to 8 d after onset of disease.

Results and Discussion

The induction of Ag-specific tolerance is critical for the prevention of autoimmunity and maintenance of immune tolerance. In addition to their classical role as sentinels of the immune response inducing T cell reactivity, increasing evidence now indicates that DCs can induce specific T cell tolerance. Although underlying mechanisms are not fully elucidated, the capacity to induce Tr cells is an important property of tolerogenic/regulatory DCs. The generation of "designer" DCs with tolerogenic properties in the laboratory by using specific cytokines or immunologic and pharmacologic reagents is a desirable goal and represents the subject of intensive investigations. Because of its immunosuppressive action, VIP is a candidate for the induction of regulatory DCs with capacity to generate Tr. In a previous study, we showed that VIP treatment of activated DCs reduces their capacity to activate allogeneic and syngeneic T cells, an effect associated with the prevention of CD80/CD86 up-regulation (19). VIP treatment of iDC in the absence of activation resulted in DCs with increased capacity to induce Th2 responses (19). However, other immunomodulatory factors with capacity to induce tolerogenic DCs have been found to be effective when administered during the differentiation of DCs (6, 7). Therefore, we determined whether exposure to VIP during DC differentiation results in DC phenotypic and functional changes.

BM-DC Differentiated with VIP Induce Regulatory Tr1-Like Cells and Tolerance *in Vivo*. We first compared murine BM-derived DCs generated in the presence or absence of VIP in terms of surface markers and cytokine production. As previously described, BM cells cultured with granulocyte macrophage colony-stimulating factor for 6 d differentiate into iDCs (data not shown). Upon LPS stimulation, iDCs mature to DCs expressing high levels of DC markers (CD11c), MHC molecules (class I and class II), and costimulatory molecules (CD40, CD80, and CD86) (Fig. 1*a*, $DC_{control}$). However, DCs generated in the presence of VIP (DC_{VIP}) were resistant to the LPS-induced up-regulation of the costimulatory molecules (CD40, CD80, and CD86) (Fig. 1*a*). Upon

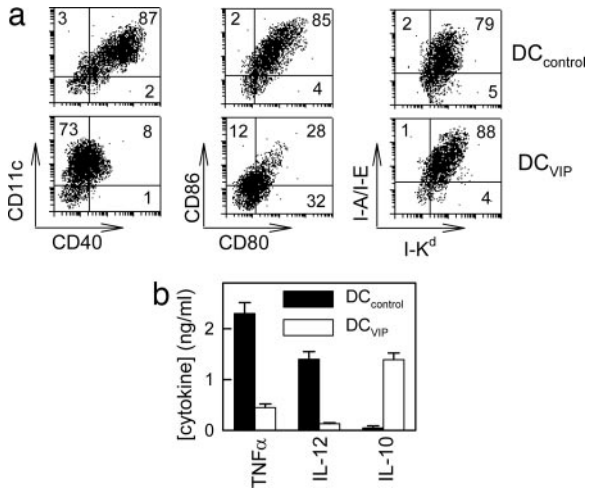

Fig. 1. VIP induces a stable ''semimature'' phenotype in BM-DCs. DCs were generated from mouse BM cells in the absence ($DC_{control}$) or presence (DC_{VIP}) of VIP and activated with LPS to induce DC maturation. (*a*) $DC_{control}$ and DC_{VIP} were double-labeled for different markers and analyzed by flow cytometry. Numbers represent the percentage of positive cells ($n = 4$). (*b*) Cytokine content in the DC supernatants was determined by ELISA ($n = 4$).

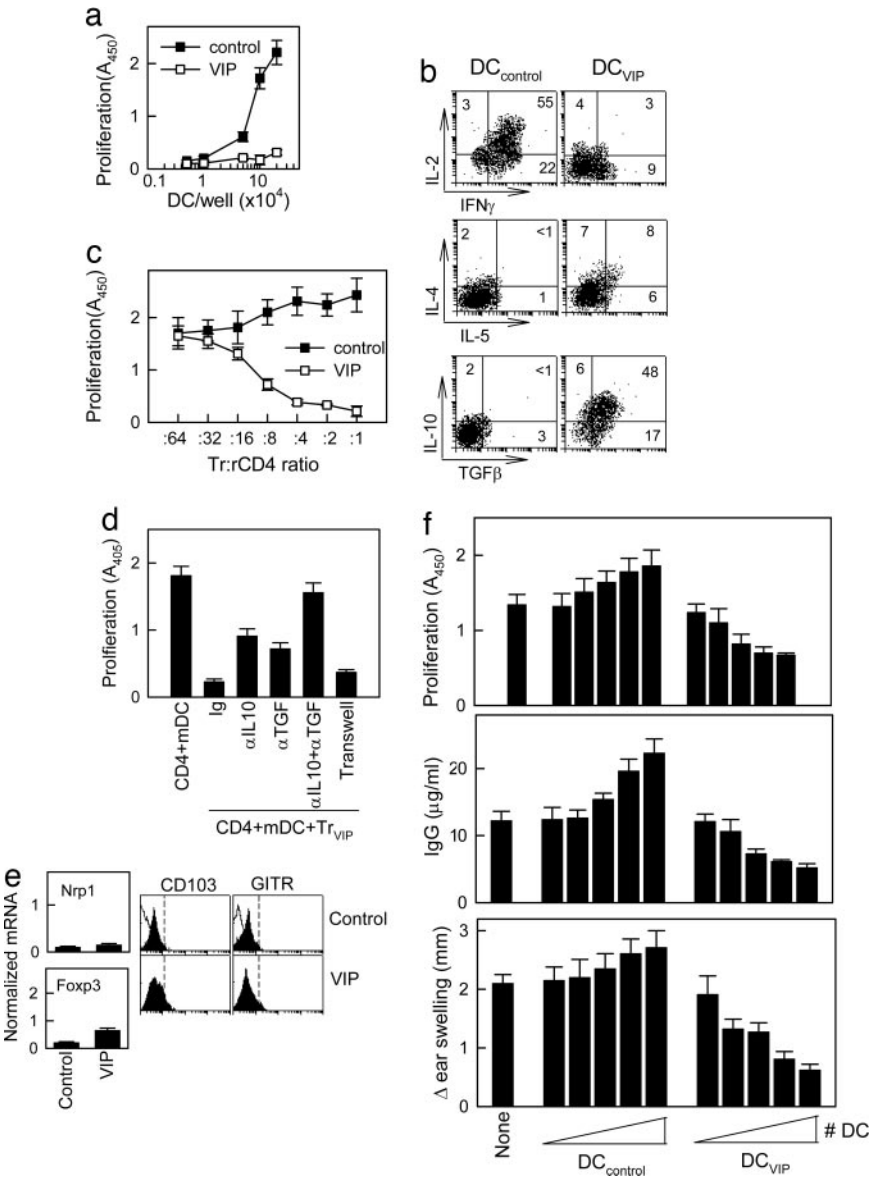

Fig. 2. Murine DCs differentiated in the presence of VIP induce regulatory Tr1 cells and tolerance *in vivo*. DCs were generated from mouse BM cells in the absence ($DC_{control}$) or presence (DC_{VIP}) of VIP and activated with LPS to induce DC maturation. (*a*) $DC_{control}$ or DC_{VIP} was added to allogeneic CD4 T cells (5×10^5), and the proliferative response was determined. $DC_{control}$ or DC_{VIP} without T cells did not proliferate. Each result is the mean ± SD of three experiments performed in duplicate. (*b*) Purified CD4 T cells were exposed to allogeneic $DC_{control}$ or DC_{VIP} and activated with phorbol 12-myristate 13-acetate plus ionomycin. Intracellular cytokines were determined in CD4-gated cells by flow cytometry. Numbers represent percentage of positive cells ($n = 5$). (*c*) Purified CD4 T cells were stimulated for 1 week with allogeneic $DC_{control}$ or DC_{VIP}. The resulting regulatory CD4 T cells (Tr) were incubated with syngeneic responder CD4 T cells (rCD4) in the presence of allogeneic mDCs, and the proliferative response was determined ($n = 4$). (*d*) Isolated CD4 T cells were cocultured with syngeneic Tr_{VIP} and allogeneic mDCs in the presence or absence of blocking anti-IL10 and/or anti-TGFβ. Additionally, CD4+mDCs were separated from Tr_{VIP}+mDC in a Transwell system. The proliferative response of responder CD4 T cells was determined ($n = 4$). (*e*) Sorted CD4 T cells generated with $DC_{control}$ or DC_{VIP} were analyzed for neuropilin 1 and Foxp3 mRNA expression by real-time RT-PCR and for surface CD103 and glucocorticoid-induced TNF receptor (GITR) expression by flow cytometry. Open histograms and dashed lines represent isotype controls. One representative experiment of two is shown. (*f*) Mice were injected s.c. with increasing numbers (from 50 to 5×10^5 cells) of Ag-pulsed $DC_{control}$ or DC_{VIP} 1 week before priming with Ag. Five days later, mice were tested for DLN Ag-specific T cell proliferation, serum antibody levels, and DTH responses. Mice injected with Ag alone (None) were used as controls. Results are the mean ± SD for each group ($n = 4$) tested separately and are representative of three experiments.

toll-like receptor activation, iDCs mature into cells capable of producing high levels of inflammatory cytokines. In contrast to $DC_{control}$, which produce TNF and IL-12, and low levels of IL-10, DC_{VIP} produce very low levels of proinflammatory cytokines (TNF and IL-12) but secrete significant levels of the antiinflammatory cytokine IL-10 (Fig. 1*b*). Taken together, these results indicate that the DCs generated in the presence of VIP are resistant to LPS-induced up-regulation of costimulatory molecules and produce IL-10. These characteristics are quite similar to those reported for tolerogenic DCs generated with other immunomodulatory factors, such as IL-10 or the activated form of vitamin D $1,25(OH)_2D_3$ (2–4, 20–24).

Tolerogenic DCs are poor stimulators of T cell proliferation and cytokine production (20, 25–28). To examine the capacity of the DC_{VIP} to stimulate T cells, we cocultured $DC_{control}$ or DC_{VIP} with alloreactive CD4 T cells. Priming with $DC_{control}$ results in a strong proliferation of allogeneic CD4 T cells, whereas DC_{VIP} induce only weak proliferation (Fig. 2*a*). In addition, CD4 T cells primed with DC_{VIP} reexposed to fresh LPS-stimulated allogeneic DCs (mDC) did not proliferate (data not shown), indicating that DC_{VIP} induces anergic T cells and/or Tr. Although Tr generated by exposure to regulatory/tolerogenic DCs do not proliferate in response to the Ag, they can release antiinflammatory cytokines, such as IL-10 and TGFβ. Therefore, we assessed the cytokine profile of T cells cocultured with DC_{VIP}. In contrast to T cells exposed to $DC_{control}$, which show a predominant Th1 cytokine profile, with high levels of IFNγ and IL-2, CD4 T cells primed with allogeneic DC_{VIP} exhibit a Tr1-like phenotype, characterized by IL-10 and TGFβ but not IL-2 and IFNγ production (Fig. 2*b*).

After TCR stimulation, Tr cells suppress the proliferation and IL-2 production of Ag-specific effector T cells. To determine whether T cells exposed to DC_{VIP} become functional Tr, we restimulated CD4 T cells with allogeneic mDCs in the presence of syngeneic CD4 T cells previously exposed to allogeneic $DC_{control}$ ($Tr_{control}$) or DC_{VIP} (Tr_{VIP}). Tr_{VIP} inhibit the proliferation of syngeneic responder CD4 cells in response to allogeneic mDCs in a dose-dependent manner, whereas $Tr_{control}$ are not suppressive (Fig. 2*c*). Similar results were obtained in respect to IL-2 production (data not shown). Therefore, the phenotype of Tr_{VIP} correlates with their regulatory T cell activity.

The observation that Tr_{VIP} produce high levels of the immunosuppressive cytokines IL-10 and TGFβ suggests that the inhibitory

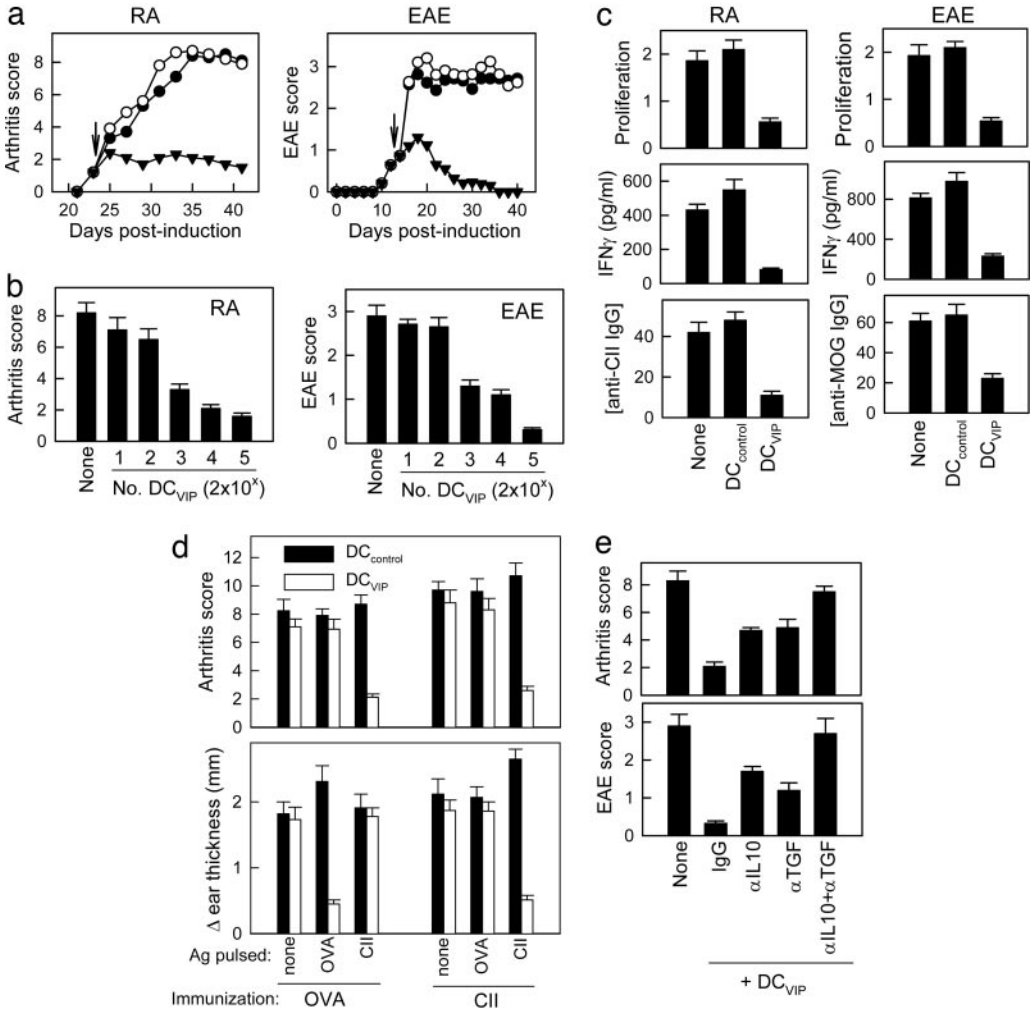

Fig. 3. Therapeutic effect of DC differentiated with VIP in RA and EAE. (*a*) DBA1/J mice (H-2^q) with established CIA or C57BL/6 mice (H-2^b) with established EAE were treated (arrows) with syngeneic CII-pulsed DCs or MOG-pulsed DCs, respectively, generated in the absence (DC$_{control}$, ○) or presence (DC$_{VIP}$, ▼ of VIP. Untreated CIA and EAE mice (none, ●) were used as controls. Clinical score was monitored ($n = 12$). (*b*) CII- and MOG-pulsed DC$_{VIP}$ were injected at different doses. (*c*) CII-induced proliferation and IFNγ production by spleen T cells, and the levels of anti-CII IgG in sera were determined in CIA mice injected with DC$_{control}$ or DC$_{VIP}$ ($n = 5$). (*d*) The effect of DC$_{VIP}$ is Ag-specific. Arthritic mice were treated with unpulsed, CII-pulsed, or OVA-pulsed DC$_{control}$ or DC$_{VIP}$ after disease onset. One week later, mice were immunized s.c. with OVA or CII and challenged i.d. in the ear pinna with the respective Ag 5 d later. Clinical score and DTH responses were determined 24 h later ($n = 5$). (*e*) Untreated CIA or EAE mice or animals injected with DC$_{VIP}$ and treated with control Ig, anti-IL10, anti-TGFβ, or a combination of both mAbs (10 mice per group).

effect of Tr$_{VIP}$ on responder CD4 T cell proliferation might be mediated through soluble factors produced. When Tr$_{VIP}$ and responder CD4 T cells were separated in transwell experiments by a semipermeable membrane that allows the free exchange of soluble factors but excludes direct cell contact of responder CD4 T cells and Tr$_{VIP}$, the proliferation of effector CD4 cells was still inhibited, indicating that soluble factors mediate the inhibitory effect (Fig. 2*d*). In regular cocultures, the addition of anti-TGFβ, or anti-IL-10 Abs reversed inhibition modestly. However, the addition of both anti-IL-10 and anti-TGFβ Abs reverses the inhibitory effect almost completely (Fig. 2*d*).

Several populations of CD4 Tr have been described and characterized, including the naturally occurring thymic-born CD4$^+$CD25$^+$ Tr and the induced peripheral Tr, consisting of IL-10-producing Tr1 and TGFβ-secreting Th3/Tr2 (29). Regulatory DCs do not participate in the generation of naturally occurring CD4$^+$CD25$^+$ Tr; however, they play an important role in the differentiation of peripherally induced Tr1 and Th3/Tr2 Tr (30–32). Although the CD4$^+$CD25$^+$ population is slightly increased in Tr$_{VIP}$, the fact that Tr$_{VIP}$ did not express significant levels of the CD4$^+$CD25$^+$ Tr markers Foxp3, neuropilin-1, glucocorticoid-induced TNF-receptor-family-related gene, and CD103 (Fig. 2*e*), argues against the possibility that DC$_{VIP}$ induce the generation of CD4$^+$CD25$^+$ Tr cells. There are no reports on the expression of neuropilin-1 in IL-10-induced Tr1 cells. However, in contrast to CD4$^+$CD25$^+$ Tr, and in agreement with our results, Tr1 cells generated by repetitive stimulation with IL-10-secreting regulatory DCs have been shown to express low levels of CD25 and Foxp3 (33).

Although the precise mechanisms remain unknown, several possibilities may account for the generation of Tr cells by DC$_{VIP}$. The activation of naïve CD4 T lymphocytes requires several signals delivered by mDCs and mediated through Ag/MHCII–TCR, CD80/CD86–CD28, and CD40–CD40L interactions. Costimulatory molecules, especially CD40, appear to be key determinants of the decision between tolerance and immunity (34). The characteristic phenotype of DC$_{VIP}$, i.e., high levels of MHC plus poor expression of costimulatory molecules, which will deliver stimulatory but not costimulatory signals, is in agreement with DC$_{VIP}$'s tolerance-inducing ability. In addition, the observation that DC$_{VIP}$ secrete IL-10 may be linked to the stability of DC$_{VIP}$'s tolerogenic-

like phenotype (20, 35–37). Previously, VIP has been reported to inhibit NF-κBp65 nuclear translocation, DNA-binding, and transactivating activity in macrophages (9), and we have recently found that both NF-κBp65 nuclear translocation and IkB phosphorylation are inhibited in DC_{VIP} (M.D., E.G.-R., and D.G., unpublished data). The connection among NF-κB transactivating activity, CD40 expression, and DC function (including TNF-α and IL-12 production) has been established in a number of recent studies. The association between tolerance, particularly tolerogenic DCs, and lack of CD40 expression or signaling has been demonstrated both *in vivo* and *in vitro* (28). Expression of CD40 depends on NF-κBp65 (38), and the inhibition of NF-κB in DCs leads to failure of CD40, CD80, and CD86 expression upon LPS-stimulation and to the generation of tolerogenic DCs (39). In addition, a recent study suggests that VIP treatment induced a decrease of toll-like receptors (TLR-2/4) expressions in DCs in a murine model of Crohn's disease by a mechanism that would involve a decrease of NF-κB activation (40). Therefore, we would like to propose that the mechanism by which VIP induces tolerogenic DCs involves the cAMP/PKA-mediated inhibition of IκB phosphorylation and NF-κBp65 nuclear translocation, leading to lack of CD40 expression, TLR-2/4 signaling, and inflammatory cytokine production.

Because DC_{VIP} appear to have a predominantly negative effect on Th1 cells, we determined the effect of DC_{VIP} in an *in vivo* model of DTH. Ag (methylated-BSA)-pulsed DC_{VIP} and $DC_{control}$ were administered i.v., followed a week later by s.c. antigenic immunization. We determined T cell proliferation in response to *ex vivo* restimulation, Ab production, and DTH after a secondary s.c. Ag administration (Fig. 2*f*). Mice that received $DC_{control}$ developed DTH reactions higher than controls (no DCs), whereas those receiving DC_{VIP} exhibited reduced DTH. In addition, DLN T cells from mice inoculated with $DC_{control}$ proliferated at higher levels than controls (no DCs), and, again, inoculation of DC_{VIP} resulted in a substantial reduction in T cell proliferation after *ex vivo* restimulation with the Ag. Similarly, mice inoculated with $DC_{control}$ produced high levels of anti-mBSA Abs, whereas those inoculated with DC_{VIP} had anti-mBSA Ab levels below control (no DCs) (Fig. 2*f*). These results indicate that DC_{VIP} induce tolerance *in vivo*. The induction of tolerance is restricted to the Ag presented by the inoculated DC, because we did not observe reduction in DTH in mice injected with mBSA-pulsed DC_{VIP} when we used an unrelated Ag (OVA) for immunization and *ex vivo* T cell restimulation (data not shown). These experiments suggest the possibility of using a VIP *in vitro* system to generate Ag-specific tolerogenic DCs, followed by *in vivo* administration of these cells to patients with autoimmune diseases.

Therapeutic Effect of DC_{VIP} in Autoimmunity. Several reports have recently proposed the possibility of using regulatory/tolerogenic DCs generated *ex vivo* as a therapeutic tool to prevent organ-specific autoimmune diseases (3, 7, 21). Interestingly, DC_{VIP} retained their T cell regulatory capacity *in vitro* and *in vivo* under inflammatory conditions. This observation is particularly relevant for conditions in which ongoing Ag presentation is associated with chronic inflammation, including autoimmune diseases. Therefore, we tested the therapeutic effect of DC_{VIP} in two murine models of RA and multiple sclerosis (MS). For RA, we used the CIA, an experimental disease model induced by immunization with CII, which shares a number of clinical, histologic, and immunological features with RA. For MS, we used the EAE model induced by $MOG_{35–55}$ in C57BL/6 mice that mirror different clinical characteristics of MS. Inoculation of $DC_{control}$ does not ameliorate arthritis (i.e., joint inflammation, cartilage destruction, and bone erosion) or EAE (i.e., tail and leg paralysis) (Fig. 3*a*). In contrast, administration of syngeneic DC_{VIP} after the onset of disease abrogates arthritis and EAE progression in a dose-dependent manner (Fig. 3 *a* and *b*). The therapeutic effect of DC_{VIP} was associated with the down-regulation of the autoimmune component of both diseases, because

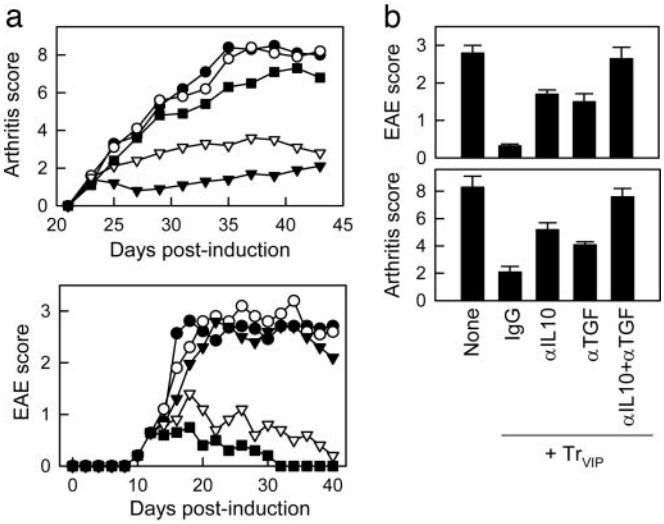

Fig. 4. DC_{VIP}-induced Tr prevent autoimmunity. (*a*) Therapeutic effect on arthritis. CIA (H-2^q) or EAE mice (H-2^b) with established disease were treated with syngeneic CII- and MOG-specific $Tr_{control}$ (○) or with different doses of CII- or MOG-specific Tr_{VIP} (10^6 cells, ▼; 5×10^5 cells, ▽; 5×10^4 cells, ■). Untreated mice (●) were used as CIA and EAE controls. Clinical score was determined (n = 10). (*b*) Untreated CIA/EAE mice or CIA/EAE mice injected with Tr_{VIP} and treated with control Ig, anti-IL10, anti-TGFβ, or anti-IL10 plus anti-TGFβ Abs. Clinical score was measured at the peak of the disease (n = 10).

DLN T cells from DC_{VIP}-treated mice showed weak proliferation and IFNγ production in response to the autoantigen (Fig. 3*c*). Furthermore, this inhibition of the Th1-type autoreactive response correlates with decreased levels of CII- and MOG-specific autoantibodies (Fig. 3*c*). The effect of DC_{VIP} was Ag-specific. Unpulsed or OVA-pulsed DC_{VIP} showed a weak therapeutic effect on arthritis, while reducing OVA-specific, but not CII-specific, DTH responses. In contrast, CII-pulsed DC_{VIP} inhibit arthritis and DTH response toward CII but not toward OVA (Fig. 3*d*). Similar Ag-dependence was observed in the EAE model (data not shown). These results indicate that DC_{VIP} generated *ex vivo* could prevent organ-specific autoimmune disorders in matched subjects, probably by inducing Ag-specific Tr cells, which suppress the ongoing autoreactive/inflammatory response. The participation of Tr cells in the therapeutic effect of DC_{VIP} correlated with the fact that DC_{VIP} induce *in vitro* the generation of IL-10/TGFβ-producing regulatory CD4 T cells (Fig. 2). Therefore, we further examined the role of Tr in the therapeutic effect of DC_{VIP} on both CIA and EAE. *In vivo* blockade experiments showed that treatment with anti-IL-10 or anti-TGFβ Abs significantly decrease disease amelioration, and treatment with both Abs abrogates the beneficial effects exerted by DC_{VIP} (Fig. 3*e*), suggesting the partial involvement of newly generated Tr cells in such action.

DC_{VIP}-Induced Tr Ameliorate Autoimmunity. In certain circumstances, successful suppression of an autoimmune response might require high numbers of Tr, and the *in vivo* administration of DC_{VIP} might not be sufficient for a complete and rapid suppression. Therefore, we decided to generate *in vitro* Ag-specific DC_{VIP}-induced Tr cells and to subsequently determine their suppressive capacity *in vivo* in both the CIA and EAE models. We generated CII- or MOG-specific Tr_{VIP} through stimulations of CD4 T cells with syngeneic CII- or MOG-pulsed DC_{VIP}. $Tr_{control}$ were generated in the same manner with $DC_{control}$. Treatment with Tr_{VIP}, but not $Tr_{control}$, of mice with established CIA or EAE prevented disease progression in a dose-dependent manner (Fig. 4*a*). This effect was mainly mediated through TGFβ and IL-10, because *in vivo* administration

of anti-IL10 and/or anti-TGFβ Abs abrogated the protective effect (Fig. 4*b*). In both models, the protective effect of Tr_{VIP} was Ag-specific, because OVA-specific Tr_{VIP} did not efficiently ameliorate arthritis or paralysis (data not shown). These results indicate that Ag-specific Tr1-like cells generated *in vitro* with DC_{VIP} can efficiently modulate pathogenic immune responses *in vivo*.

VIP has been previously found to ameliorate CIA and EAE, mainly by down-regulating the two components of both diseases, inflammation and Th1-mediated autoimmunity (ref. 10 and E.G.-R., A.F.-M., A.C., D.P., D.G., and M.D., unpublished results). The involvement of Tr cells in the therapeutic effect of VIP was demonstrated by the fact that CD4 T cells isolated by VIP-treated CIA or EAE mice showed an increased regulatory/suppressive activity against self-reactive Th1 cells. Phenotypic analysis of these Tr cells indicated that they consist of a mix of $Foxp3^+CD4^+CD25^+$ and IL-10^+Tr1-like cells (E.G.-R., A.C., A.F.-M., D.G., and M.D., unpublished results). In addition, by using a transgenic TCR murine model, we found that VIP induces the *in vivo* generation of Ag-specific tolerogenic IL-10-producing DCs with capacity to generate/activate Tr1-like cells (M.D., E.G.-R., and D.G., unpublished results). These findings validate the data obtained in this study, demonstrating that the pharmacological use of VIP in the treatment of autoimmunity is exerted partially through the induction of tolerogenic DCs and Tr1-like cells.

It has been proposed that tolerance induction by DCs requires maturation signals different from microbial or inflammatory stimuli. In steady-state conditions, VIP could represent one of the endogenous maturation signals driving the differentiation of tolerogenic DCs with a regulatory phenotype. VIP is secreted in the lymphoid microenvironment, mainly by Th2 cells, after Ag stimulation, and VIP levels are increased in immunopathologic conditions, such as autoimmunity and inflammation (8, 9). Therefore, DC_{VIP} may represent a population of DCs that have matured to display a stable tolerogenic phenotype. Under steady-state conditions, DC_{VIP} could be loaded with self- and commonly encountered Ags, and, after migration to the lymphoid organs, they could induce Tr1 differentiation and tolerance. Interestingly, in subjects with various autoimmune disorders, reduced serum VIP levels and increased VIP-specific autoantibodies have been reported (41).

Numerous strategies based on immunosuppressive agents, such as vitamin-D3, IL-10, TGFβ, glucocorticoids, and *N*-acetyl-L-cysteine, alone or in combinations, have been used to induce tolerogenic DCs (7). However, in the case of regulatory DCs induced with vitamin D analogs, it looks as if these regulatory DCs induce $CD4^+CD25^+$ Tr cells rather than Tr1-like cells (7). Our data demonstrate that VIP is very efficient at the induction of regulatory DCs, in comparison with current strategies, and we propose that the addition of VIP to cocktails of immunomodulatory agents will increase their effectiveness.

In conclusion, the possibility of generating tolerogenic DC_{VIP} opens therapeutic perspectives for the treatment of autoimmune/inflammatory diseases and in allogeneic transplantation. *In vitro* pulsing of tolerogenic DC_{VIP} with self-Ags, followed by *in vivo* injection, leads to the differentiation of Ag-specific Tr cells. Therefore, the inclusion of tolerogenic DC_{VIP} in future therapeutic regimens may minimize the dependence on nonspecific immunosuppressive drugs used currently for autoimmune disorders.

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EXTENDED REPORT

Therapeutic effect of cortistatin on experimental arthritis by downregulating inflammatory and Th1 responses

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Background: Rheumatoid arthritis is a chronic autoimmune disease of unknown aetiology characterised by chronic inflammation in the joints and subsequent destruction of the cartilage and bone.
Aim: To propose a new strategy for the treatment of arthritis based on the administration of cortistatin, a newly discovered neuropeptide with anti-inflammatory actions.
Methods: DBA/1J mice with collagen-induced arthritis were treated with cortistatin after the onset of disease, and the clinical score and joint histopathology were evaluated. Inflammatory response was determined by measuring the levels of various inflammatory mediators (cytokines and chemokines) in joints and serum. T helper cell type 1 (Th1)-mediated autoreactive response was evaluated by determining the proliferative response and cytokine profile of draining lymph node cells stimulated with collagen and by assaying the content of serum autoantibodies.
Results: Cortistatin treatment significantly reduced the severity of established collagen-induced arthritis, completely abrogating joint swelling and destruction of cartilage and bone. The therapeutic effect of cortistatin was associated with a striking reduction in the two deleterious components of the disease—that is, the Th1-driven autoimmune and inflammatory responses. Cortistatin downregulated the production of various inflammatory cytokines and chemokines, decreased the antigen-specific Th1-cell expansion, and induced the production of regulatory cytokines, such as interleukin 10 and transforming growth factor β1. Cortistatin exerted its effects on synovial cells through both somatostatin and ghrelin receptors, showing a higher effect than both peptides protecting against experimental arthritis.
Conclusion: This work provides a powerful rationale for the assessment of the efficacy of cortistatin as a novel therapeutic approach to the treatment of rheumatoid arthritis.

Rheumatoid arthritis (RA) is an autoimmune disease that leads to chronic inflammation in the joints and subsequent destruction of the cartilage and erosion of the bone. Although the contribution of T helper cell type 1 (Th1) responses in RA is not completely understood, several studies in animal models point to a pathogenic role for Th1-derived cytokines.[1 2] Th1 cells reactive to components of the joint infiltrate the synovium, release proinflammatory cytokines and chemokines, and promote macrophage and neutrophil infiltration and activation. Inflammatory mediators, such as cytokines and free radicals, produced by infiltrating inflammatory cells, play a critical role in joint damage.[2] The fact that the inflammatory process in RA is chronic suggests that immune regulation in the joints is disturbed. Available therapies based on immunosuppressive agents inhibit the inflammatory component of RA and have the potential to slow progressive clinical disability by delaying erosions and deformity.[3] However, they neither reduce the relapse rate nor delay disease onset, and because continued treatment is required to maintain a beneficial effect, they have multiple side effects.[4] This illustrates the need for novel therapeutic approaches to prevent the inflammatory and autoimmune components of the disease.

Cortistatin (CST) is a recently discovered cyclic neuropeptide related to somatostatin, which shares many of somatostatin's pharmacological and functional properties, including the depression of neuronal activity and inhibition of cell proliferation.[5] However, CST also has many properties distinct from somatostatin, such as slow-wave sleep induction and locomotor activity reduction.[5] Various human immune cells, including lymphocytes, monocytes, macrophages and dendritic cells, produce CST but not somatostatin, and its levels correlate with cell differentiation and activation state,[6 7] suggesting that CST might be a major endogenous regulatory factor in the immune system. Indeed, we have recently reported a new role of CST as a potent anti-inflammatory factor. CST prevents sepsis-induced mortality by inhibiting the production of inflammatory mediators by activated macrophages and decreasing the recruitment of neutrophils and monocytes to inflamed organs.[8] Therefore, the aim of this study is to investigate the potential therapeutic action of CST in an experimental model of RA. Here, we show that treatment with CST has great benefit at the clinical and pathological levels, as the therapeutic effect of CST was exerted at multiple levels, being associated with the downregulation of inflammatory and Th1-mediated autoimmune components of the disease.

METHODS

Arthritis induction and treatment

Animal experimental protocols were reviewed and approved by the ethics committee of the Spanish Council of Scientific Research. For the induction of collagen-induced arthritis (CIA), DBA/1J mice (7–10 weeks old, Jackson Laboratory, Bar Harbor, Maine, USA) were injected subcutaneously with 200 μg of collagen type II (CII) (Sigma, St Louis, Missouri, USA) emulsified in complete Freund's adjuvant (CFA) containing 200 μg of *Mycobacterium tuberculosis* H37 RA (Difco, Detroit, Michigan, USA). At day 21 after primary immunisation, mice were given subcutaneous booster doses of 100 μg of CII in CFA.

Abbreviations: CFA, complete Freund's adjuvant; CIA, collagen-induced arthritis; CII, collagen type II; CST, cortistatin; DLN, draining lymph nodes; IFN, interferon; IL, interleukin; PPD, purified protein derivative; RA, rheumatoid arthritis; sst, somatostatin receptors; Th1, T helper cell type 1; TGF, transforming growth factor; TNF, tumour necrosis factor

CST (American Peptides Company, Sunnyvale, California, USA) treatment consisted of intraperitoneal administration of 0.1, 1, 2, 5 or 10 nmol/mouse/day of rat $CST_{1–29}$ on five consecutive days starting at 25 days after immunisation, when all mice showed established arthritis (clinical score >2). These doses of CST were chosen on the basis of previous experiments with CST in a model of inflammatory bowel disease or with other anti-inflammatory neuropeptides in CIA.[9 10] To see whether a unique pulse of the neuropeptide was therapeutic, CST (2 nmol) was administered once at day 25. In each experiment, a control group of mice was injected intraperitoneally with phosphate-buffered saline (untreated). Because CST is structurally related to somatostatin and exerts some of its immunomodulatory effect through the receptor of the anti-inflammatory hormone ghrelin, in some experiments we injected ghrelin or somatostatin intraperitoneally (Sigma; 15 μg/mouse/day for five consecutive days after day 25). Clinical arthritis was assessed by two independent, blinded examiners as described,[9] by using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, moderate swelling and oedema; grade 3, extreme swelling and pronounced oedema; and grade 4, joint rigidity. Each limb was graded, giving a maximum possible score of 16 per animal. For histological analysis, the paws were randomly collected by two independent experimenters at day 45 after primary immunisation, fixed in 4% buffered formaldehyde, decalcified, paraffin-embedded, sectioned and stained with H&E or Masson–Goldner trichromic stain. Histopathological changes were scored in a blinded manner based on cell infiltration, cartilage destruction and bone erosion parameters as described.[11] For determination of cytokine in joints, protein extracts were isolated by homogenisation of joints (50 mg tissue/ml) in 50 mM Tris–HCl, pH 7.4, with 0.5 mM dithiothreitol, and proteinase inhibitor cocktail (10 μg/ml, Sigma). Serum samples were collected at the peak of disease (day 35) and the levels of anti-CII IgG, IgG1 and IgG2a antibodies were measured by ELISA as described.[9] Cytokine and chemokine levels in the serum and joint protein extracts prepared at the disease peak (day 35) were determined by specific sandwich ELISAs using capture/biotinylated detection antibodies from BD Pharmingen (San Diego, California, USA) according to the manufacturer's recommendations. Neutrophil infiltration in the joint was monitored by measuring myeloperoxidase activity in joint extracts isolated at day 35 after immunisation as described.[11]

Assessment of T cell autoreactive response

Because the T cell autoreactive response precedes the maximal clinical manifestations of the disease, single-cell suspensions (10^6 cells/ml) from draining lymph nodes (DLN) and the synovial membrane of knee joints were obtained at 30 days after immunisation. Cells were stimulated in complete medium (RPMI 1640 containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) with different concentrations of heat-inactivated CII for 48 h (for the determination of cytokine) or for 72 h (for proliferative response).[9] Cell proliferation was evaluated by using a cell proliferation assay with 5-bromo-2′-deoxy-uridine (Roche Diagnostics GmbH, Mannheim, Germany). Cytokine content in culture supernatants was determined by specific sandwich ELISAs as above. For intracellular analysis of cytokines, DLN and synovial cells were stimulated with inactivated CII (10 μg/ml) for 8 h, in the presence of monensin, and then stained with PerCP-anti-CD4 monoclonal antibodies at 4℃, washed, fixed/saponin permeabilised, stained with fluorescein isothiocyanate- and phycoerythrin-conjugated anti-cytokine-specific monoclonal antibodies (BD Pharmingen), and analysed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, California, USA). To distinguish between monocyte/macrophage and T cell sources, intracellular cytokine analysis was done exclusively in the PerCP-labelled CD4 T cell population. As a recall antigen control, 30 μg of purified protein derivative (PPD) was injected subcutaneously in the CII–CFA emulsion, and in vitro T cell function after culture stimulation with 10 μg/ml PPD was assessed as above.

Alternatively, synovial cells (10^6 cells/ml) isolated from CIA mice at day 30 after immunisation were stimulated with inactivated CII (10 μg/ml) in the absence or presence of different concentrations of CST, ghrelin or somatostatin, with or without 10^{-6} M cyclosomatostatin or [D-Lys^3]-growth hormone releasing peptide 6 (Lys-GHRP-6; Sigma). Cytokine levels were determined in supernatants after 48 h culture.

Data analysis

All values are expressed as mean (SD). The differences between groups were analysed using the Mann–Whitney U test and, if appropriate, the Kruskal–Wallis analysis of variance test.

RESULTS

CST decreases the severity of collagen-induced arthritis

CIA is a murine experimental disease model that shares a number of clinical, histological and immunological features with RA, and it is used as a model system to test potential therapeutic agents. CST treatment of mice with established clinical signs of arthritis progressively attenuated the severity of CIA and decreased the percentage of mice with arthritis, as compared with untreated mice (fig 1). Daily administration of 2 nmol of CST for five days offered the best therapeutic effect, although a single injection was enough to significantly ameliorate the pathological signs of arthritis (fig 1). The beneficial effect was dose-dependent (fig 1). Because we observed few differences between the 2 nmol and 10 nmol doses, all further experiments used five administrations of the 2 nmol dose on consecutive days. We saw no loss of the therapeutic effects 3 weeks after cessation of CST treatment (fig 1). In addition, throughout our study, we did not observe any overt toxicity or lethality caused by daily peptide injection. Histopathological analyses of joints showed that CST treatment completely abrogated CIA—characteristic chronic inflammation of synovial tissue (infiltration of inflammatory cells—lymphocytes, plasma cells, macrophages and neutrophils—into the joint cavity and periarticular soft tissue, pannus formation, cartilage destruction and bone erosion (fig 2). The CST-mediated inhibition of neutrophil infiltration was confirmed with decreased joint myeloperoxidase activity. In addition, CST treatment inhibited the osteoclast-inducing activity observed in the CIA mice with recruitment of osteoclast in basic multicellular units that produce focal subchondral bone erosion (fig 2, arrows).

CST inhibits inflammatory response in CIA

Next, we investigated the mechanisms underlying the decrease in the severity of CIA following CST treatment. Several pieces of evidence have shown the involvement of a wide array of cytokines and chemokines in joint inflammation and arthritis progression.[1 2] Because CST has been recently suggested to be a potent anti-inflammatory factor,[8] we evaluated its effect on the production of inflammatory mediators that are mechanistically linked to the severity of CIA. CST treatment significantly reduced protein expression of inflammatory cytokines (tumour necrosis factor (TNF)α, interferon (IFN)γ, interleukin (IL)6, IL1β and IL12) and chemokines (Rantes and MIP-2) in the joint of mice with arthritis (fig 3A). In addition, joints of CST-treated mice showed increased levels of the regulatory cytokine IL10 (fig 3A). The broad anti-inflammatory activity of CST in the inflamed joint

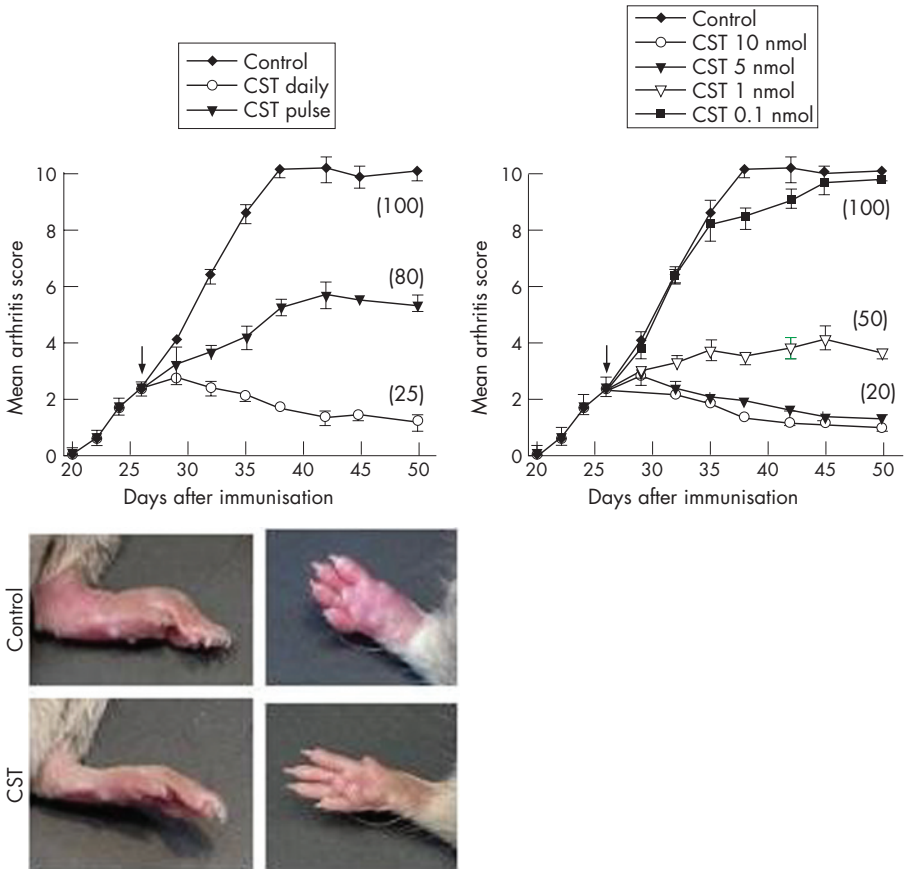

Figure 1 Cortistatin (CST) decreases the severity of collagen-induced arthritis. DBA1 mice with established CIA were injected intraperitoneally (arrow) either with phosphate-buffered saline (control) or with different doses of CST (2 nmol/mouse for left panel) daily for five days or with a single administration (pulse) on day 25. The severity of arthritis was assessed by clinical scoring. Numbers in parenthesis are the frequency of arthritis (percentage of mice with an arthritis score >2 at day 50). Images show representative examples of the paw swelling in mice of the different experimental groups. n = 8–16 mice per group. $p<0.001$ versus control for 1, 2, 5 and 10 nmol CST treatments after day 32.

was accompanied by downregulation of the systemic inflammatory response. CST decreased CIA-induced serum levels of the proinflammatory cytokines TNFα and IL1β (fig 3B).

CST downregulates Th1-mediated CII-specific response in CIA

Although macrophages and neutrophils are the major sources of inflammatory mediators, CD4 T cells play a key role in the initiation and perpetuation of CIA by producing IFNγ, a potent inducer of the inflammatory response. In fact, CIA is considered an archetypal example of Th1-type cell-mediated autoimmune disease.[2] Therefore, CST could ameliorate CIA by reducing autoreactive T cell responses and/or migration to the joints. We determined the proliferation and the cytokine profile of DLN cells isolated from CST-treated mice with arthritis in response to antigen (CII) in vitro. DLN cells obtained from CIA mice showed marked CII-specific proliferation and effector T cells producing high levels of Th1-type cytokines (IFNγ, IL2 and TNFα) and low levels of Th2-type cytokines (IL4 and IL10) (fig 4A). In contrast, DLN cells from CST-treated mice proliferated much less, produced low levels of Th1 cytokines and high amounts of suppressive cytokines (IL10 and transforming growth factor (TGF)β1); the Th2-type cytokine IL4 was not significantly affected (fig 4A). This effect was antigen-specific, because CST treatment did not affect proliferation and cytokine production by PPD-stimulated spleen cells from PPD/CFA-immunised CIA mice (not shown). This suggests that CST administration during CIA progression partially inhibits CII-specific Th1-cell clonal expansion. In order to distinguish whether the decrease in Th1 cytokine production induced by CST treatment is a consequence of either downregulation of cytokine release or inhibition of Th1 cell expansion, and to identify the source of IL10 (macrophages or CD4 T cells), we determined the intracellular expression of these cytokines by flow cytometry in sorted CD4 T cells. CST significantly decreased the number of TNFα/IFNγ-producing Th1 cells, and increased the number of IL10-producing CD4 T cells in DLN (fig 4B). Thus, CST administration to CIA mice regulates the expansion of autoreactive/inflammatory Th1 cells and presumably IL10-secreting T cells. We observed similar effects on synovial cells (fig 4C).

High levels of circulating antibodies directed against collagen-rich joint tissue invariably accompany the development of RA and CIA, and their production is a major factor in determining susceptibility to the disease.[12] CST administration resulted in reduced serum levels of CII-specific IgG, particularly autoreactive IgG2a antibodies (fig 4D), generally reflective of Th1 activity.[13] These data provide further evidence that CST administration during CIA reduces the Th1 autoreactive responses both in the joint and peripherally.

CST deactivates synovial cells through both somatostatin and ghrelin receptors

The decrease in inflammatory mediators observed in the CST-treated CIA mice (fig 3) could be a consequence of the diminished infiltration of inflammatory cells in the inflamed joints. However, CST inhibited the production of pro-inflammatory mediators by synovial cells isolated from CIA mice on in vitro CII restimulation (fig 5A). This suggests that, in addition to the reduction in inflammatory infiltration, CST administration could deactivate the inflammatory response of infiltrating/proliferating synovial cells.

CST shows a high homology with somatostatin, binds to somatostatin receptors (sst) and shares some functions with

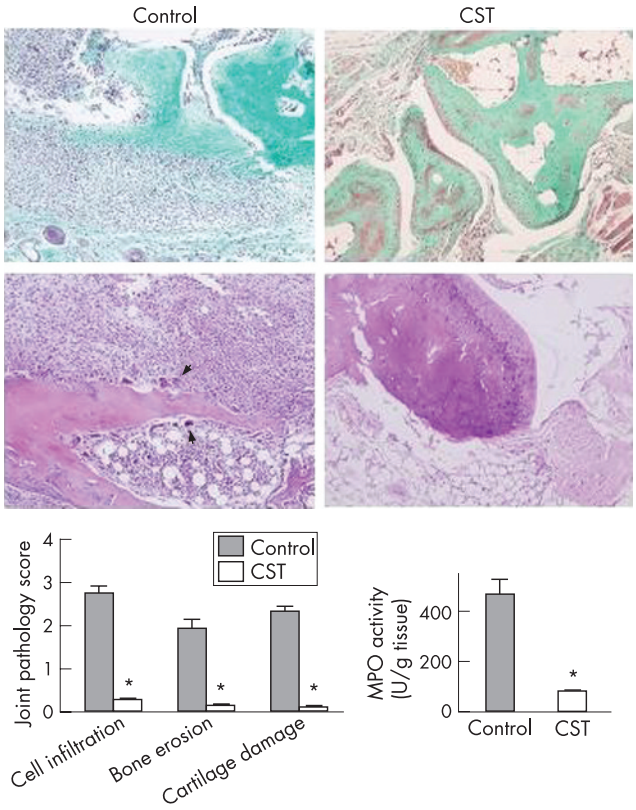

Figure 2 Cortistatin (CST) ameliorates the histopathology of collagen-induced arthritis (CIA). DBA1 mice with established CIA were injected intraperitoneally either with phosphate-buffered saline (control) or with CST (2 nmol/mouse) daily for five days after day 25 post immunisation. Histological analysis of trichrome-stained (upper) or H&E-stained (lower) sections of joints obtained at day 45 was performed. Arrows point to osteoclasts destroying bone. Scoring of inflammation, cartilage damage and bone erosion of paws from untreated (control) and CST-treated CIA mice is shown. Neutrophil infiltration in the joints was determined by measuring myeloperoxidase (MPO) activity in protein extracts isolated at day 35. *p<0.001 versus control.

somatostatin.[5] In addition to binding to sst in immune cells, CST, but not somatostatin, can also bind to other receptors, including the receptor for the growth-hormone secretagogue ghrelin,[14] a hormone recently described as a potent anti-inflammatory factor.[15 16] Therefore, next we compared the effect of the three peptides in synovial cell activation and CIA progression, and investigated the receptor involved in the effect of CST. CST showed higher inhibitory effect on the inflammatory response of synovial cells and on the severity of CIA than somatostatin (fig 5A,B). In addition, the sst-antagonist cyclosomatostatin partially reversed the effect of CST, although it fully blocked the effect of somatostatin (fig 5C). This suggests that CST could exert its effects through both sst-dependent and sst-independent mechanisms. Indeed, a ghrelin-receptor antagonist partially blocked the inhibitory effect of CST, but not of somatostatin, on synovial cell activation (fig 5C), pointing to ghrelin receptor as a putative candidate for the sst-independent mechanism. In this sense, ghrelin showed a similar potency to that of CST on CIA protection and synovial inflammation (fig 5B,C).

DISCUSSION

The initial stages of RA and CIA involve multiple steps, which can be divided into two main phases: initiation and establishment of autoimmunity, and later events associated with the evolving immune and inflammatory responses. The crucial process underlying the initiation of disease is the induction of autoimmunity to collagen-rich joint components; later events involve a destructive inflammatory process.[1 2] Progression of the autoimmune response involves the development of autoreactive Th1 cells, their entry into the joint tissues, and future recruitment of inflammatory cells through multiple mediators. Certain therapeutic approaches address the autoimmune component of CIA and RA, complementing existing anti-inflammatory therapies. In this study, we show that the neuropeptide CST provides a highly effective therapy for CIA. Its therapeutic effect is associated with a striking reduction in the two deleterious components of the disease—that is, the autoimmune and inflammatory responses. CST treatment decreased the presence of autoreactive Th1 cells in the periphery and the joint. In addition, CST strongly reduced the inflammatory response during CIA progression by down-regulating the production of several inflammatory mediators, such as various cytokines and chemokines in the joints. As a consequence, CST reduced the frequency of arthritis, ameliorated symptoms and avoided joint damage. From a therapeutic point of view, it is important to take into account the ability of delayed administration of CST to ameliorate ongoing disease, which is an essential prerequisite for an anti-arthritic agent, as treatment is started after the onset of arthritis in patients. The fact that we did not observe a loss in the beneficial effect of CST with time suggests that an initial treatment with CST could

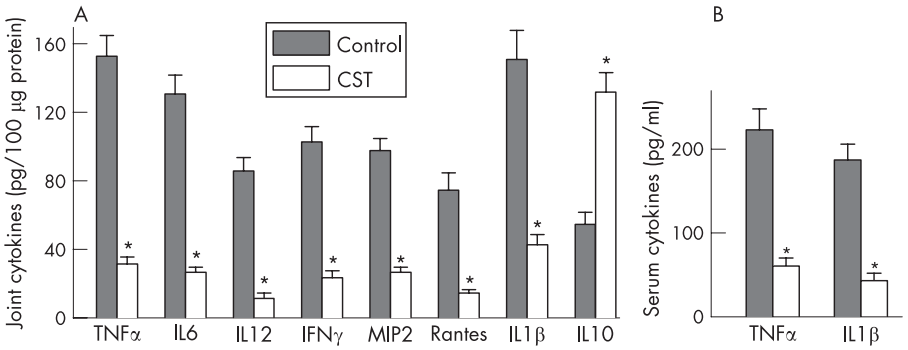

Figure 3 Cortistatin (CST) inhibits the inflammatory response in collagen-induced arthritis (CIA). DBA1 mice with established CIA were injected intraperitoneally either with phosphate-buffered saline (control) or with CST (2 nmol/mouse) daily for five days after day 25 post immunisation. Systemic and local expression of inflammatory mediators was assayed by ELISA in joint protein extracts (A) and sera (B) isolated at day 35 after immunisation. A paw from an unimmunised mouse was analysed simultaneously for the assessment of the basal response. n = 6–8 mice/group. *p<0.001 versus controls. IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor.

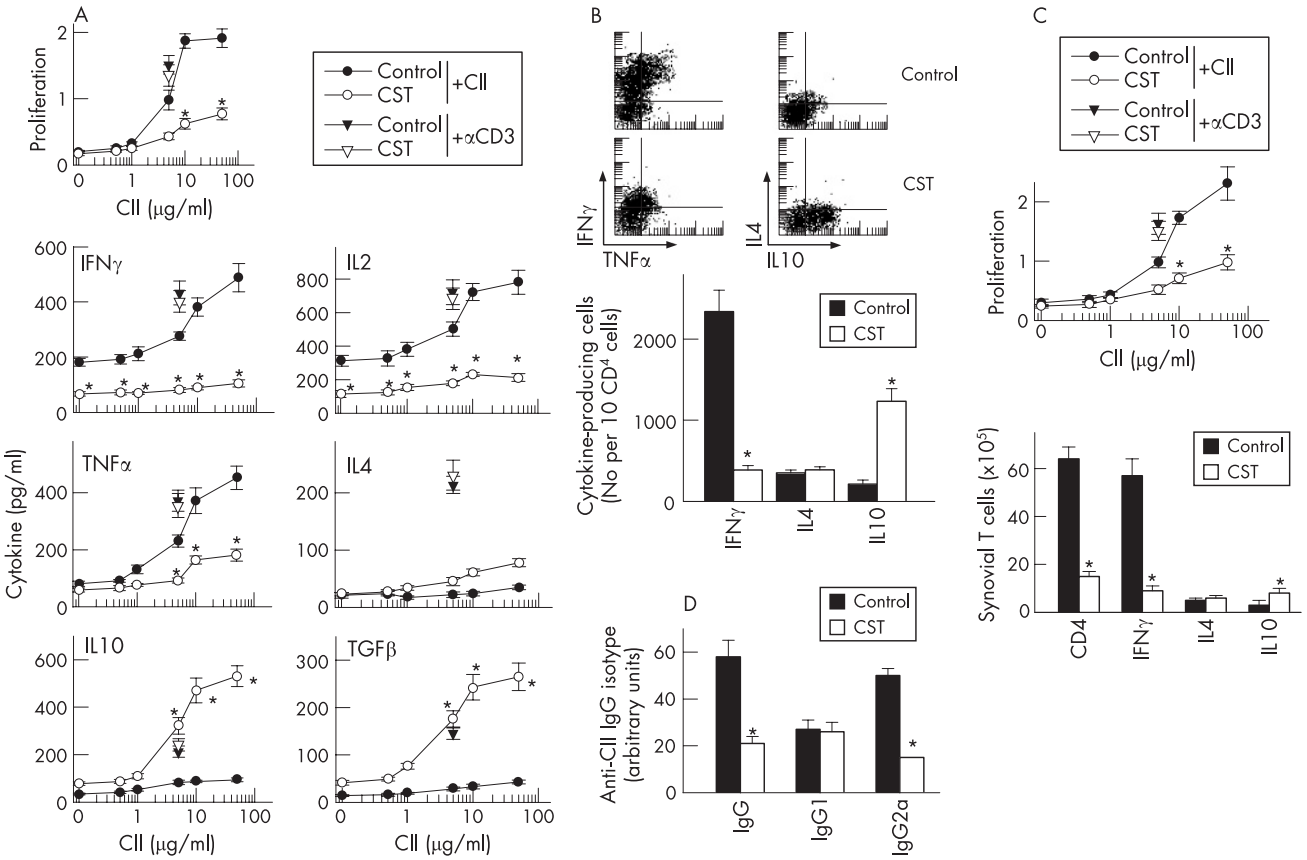

Figure 4 Cortistatin (CST) downregulates T helper cell type 1 (Th1)-mediated response in collagen-induced arthritis (CIA). DBA1 mice with established CIA were injected intraperitoneally either with with phosphate-buffered saline (control) or with CST (2 nmol/mouse) daily for five days after day 25 post immunisation. (A) Proliferative response and cytokine production of draining lymph nodes (DLN) cells isolated at day 30 from untreated (control) or CST-treated CIA mice were determined after in vitro stimulation with various concentrations of collagen type II (CII). Stimulation of DLN cells with anti-CD3 antibodies (?, for untreated CIA mice; ?, for CST-treated CIA mice) was used for assessment of nonspecific stimulation. A pool of three non-immunised DBA/1 DLN cell samples was used for assessment of the basal response. No proliferation or cytokine production by T cells was detectable in the presence of an unrelated antigen (OVA). n = 5 mice/group. (B) Number of CII-specific cytokine-producing T cells. DLN cell samples from untreated (control) or CST-treated CIA mice were restimulated in vitro with CII (10 μg/ml) and analysed for CD4 and intracellular cytokine expression by flow cytometry. Dot plots show representative double staining for interferon (IFN)γ/tumour necrosis factor (TNF)α or interleukin (IL)4/IL10 expression in gated CD4 T cells. The number of IFNγ-expressing, IL4-expressing and IL10-expressing T cells relative to 10^4 CD4 T cells is shown in the lower panel. Data shown represent pooled values from two independent experiments. (C) CII-specific proliferative response and the number of cytokine-producing CD4 T cells were determined in synovial membrane cells isolated from untreated (control) or CST-treated CIA mice and stimulated in vitro with CII (10 μg/ml) for 48 h. Data show the results of pooled synovial cells from three animals per group. (D) The levels of CII-specific IgG, IgG1 and IgG2a antibodies in sera collected at day 35 from untreated (control) or CST-treated CIA mice were determined by ELISA (8–12 mice/group). *$p<0.001$ versus controls. TGF, transforming growth factor.

induce remission of the disease. Therefore, in contrast to other treatments for RA with potential side effects, long-term treatment may not be required with CST.

The capacity of CST to regulate a wide spectrum of inflammatory mediators might offer a therapeutic advantage over other treatments directed against a single mediator, such as the new biological agents. Chemokines are responsible for the infiltration into the joint and activation of various leucocyte populations, which contribute to the pathology of CIA.[1 2 17] The fact that CST treatment reduced the expression of a plethora of chemokines could partially explain the absence of inflammatory infiltrates in the joint tissues of CST-treated mice, being especially relevant for chemokines such as MIP-2 (chemotactic for neutrophils) and Rantes (for macrophages and T cells), all involved in CIA pathogenesis.[17 18] In addition to regulating cell recruitment to the joints, CST also regulates the activation of inflammatory cells in the joints. Thus, CST downregulated the production of the proinflammatory/cytotoxic cytokines TNFα, IFNγ, IL6, IL1β and IL12 in the inflamed joint and increased the levels of the anti-inflammatory cytokines IL10 and TGFβ, which ameliorate the disease.[19 20] The decrease in inflammatory mediators could be the consequence of a diminished infiltration of inflammatory cells in the synovium. However, the fact that CST inhibited the production of proinflammatory mediators by synovial cells isolated from CIA mice on in vitro CII-specific response argues against this hypothesis. This suggests that, in addition to the reduction in inflammatory infiltration, CST deactivates the inflammatory response. A recent study demonstrated that CST acts as a macrophage-deactivating factor by downregulating the production of a wide range of inflammatory mediators,[8] suggesting that the deactivation of resident and infiltrating macrophages is a major mechanism involved in the anti-inflammatory action of CST in CIA.

CIA is also a Th1-mediated disease, and the bias towards Th1 cytokines (mainly IFNγ and TNFα) is crucial in the establishment of chronic inflammation in the joint.[1 2] Our findings shows that the administration of CST to mice with arthritis results in a decreased CII-specific Th1-mediated response. It appears that the inhibition of the Th1 response is caused by a direct action on synovial and DLN cells, as synovial and DLN

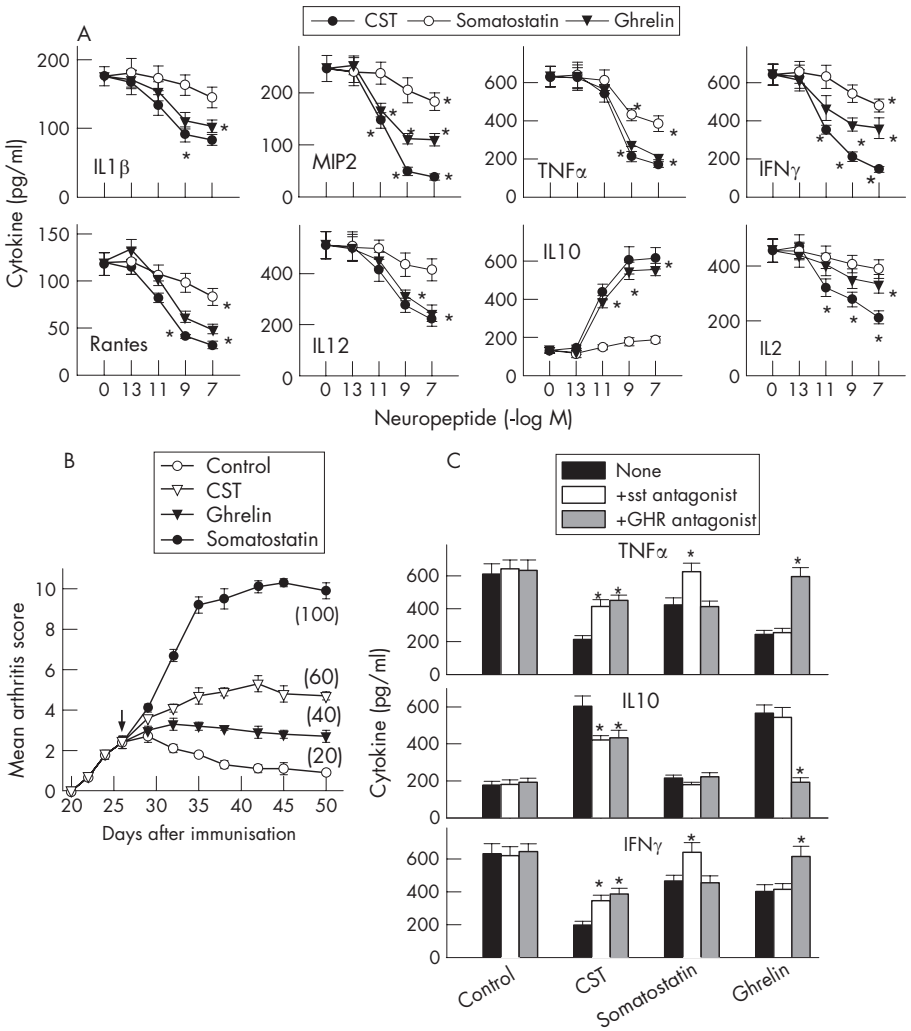

Figure 5 Cortistatin (CST) inhibits synovial cell activation through both somatostatin and ghrelin receptors. (A) Synovial membrane cells isolated from collagen-induced arthritis (CIA) mice at peak of disease were activated with collagen type II (CII) (10 μg/ml) in the absence or presence of different concentrations of CST, somatostatin or ghrelin, and the levels of chemokines and cytokines in the culture supernatants were determined by ELISA. n = 3 experiments performed in duplicate. *$p<0.001$ versus control. (B) The severity and frequency (parenthesis) of arthritis in mice with CIA treated intraperitoneally (arrow) for five consecutive days with phosphate-buffered saline (control), CST (5 nmol), somatostatin (5 nmol) or ghrelin (5 nmol). n = 8 mice/group. $p<0.001$ versus control after day 32. (C) Synovial membrane cells isolated from CIA mice were activated with CII (10 μg/ml) and treated with medium (control), CST (10^{-8} M), somatostatin (10^{-8} M) or ghrelin (10^{-8} M) in the absence (none, black bars) or presence of the somatostatin-receptor antagonist cyclosomatostatin (sst-antagonist, white bars; 10^{-6} M) or the ghrelin-receptor antagonist Lys-GHRP-6 (GHR-antagonist, grey bars; 10^{-6} M). Cytokine contents in the culture supernatants were determined by ELISA. n = 3 experiments performed in duplicate. *$p<0.001$ versus neuropeptide-treated cells in the absence of antagonist. IFN, interferon; IL, interleukin; MIP, macrophage inflammatory protein; TNF, tumour necrosis factor.

cells obtained from CST-treated animals are refractory to Th1 cell stimulation. In contrast to IFNγ and TNFα, CST increased the production of IL10 and TGFβ1. However, the fact that CST increased IL10, but not IL4, production in synovial and DLN CD4 T cells argues against a shift towards Th2 responses. IL10 has been recently recognised as a signature cytokine for a subset of CD4 T cells that exert regulatory functions and are involved in the restoration of the immune tolerance.[21]

CST shares receptors, and many structural and functional properties, with somatostatin. However, the lack of increased CST expression in somatostatin-deficient mice and the exclusive roles described for CST in nervous system[5 22 23] argue against a compensatory role of CST. Our work supports this hypothesis, as CST was significantly more efficient in protecting from CIA development than somatostatin. The superior potency of CST in reducing inflammation may reside in its capacity to activate different receptors and transduction pathways. Whereas somatostatin binds only to sst, CST can also activate other receptors, including the ghrelin receptor, associated with anti-inflammatory actions.[14 15] In this study, ghrelin was also therapeutic against CIA. Therefore, the possibility exists that CST is exerting its therapeutic effect on CIA at least partially through ghrelin receptor. Indeed, effects of CST on synovial inflammatory response were partially reversed by both somatostatin- and ghrelin-receptor antagonists. However, the participation of CST-specific receptors not yet identified cannot be ruled out. It is important to note that somatostatin has been extensively tested in human subjects, including patients with RA.[24 25] Therefore, based on its somatostatin-like structure, CST should be well tolerated in doses similar to those that are able to prevent CIA. Indeed, it has been reported that the clinical use of CST in humans has been without any toxic effects.[26]

In summary, this work identifies CST as a new immunomodulatory factor with the capacity to deactivate the inflammatory response in vivo at multiple levels and to maintain immune tolerance, and provides a powerful rationale for the assessment of the efficacy of CST as a novel therapeutic approach to the treatment of RA and other chronic autoimmune disorders.

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DISCUSIÓN

Los mamíferos responden ante las infecciones mediante la acción conjunta de la inmunidad innata y adaptativa. Si bien el proceso inflamatorio es esencial para una exitosa eliminación de los patógenos, respuestas descontroladas incrementan el riesgo de desencadenar procesos autoinmunes y pueden producir daños en los tejidos que pueden derivar en el fallo de los órganos y la muerte. Por lo tanto, el poder destructivo del sistema inmune precisa de mecanismos que regulen su actividad, desactivando o eliminando las células que participan en la respuesta inflamatoria, evitando que linfocitos T y B autorreactivos reaccionen contra el propio organismo y restableciendo la homeostasis. Las respuestas inmunes se encuentran reguladas por mediadores endógenos producidos principalmente por las mismas células del sistema inmune. La aparición de enfermedades inflamatorias y autoinmunes es consecuencia, al menos en parte, de un desbalance entre factores proinflamatorios y antiinflamatorios y/o células Th1/Th17 autorreactivas y Treg. De esta forma, los mecanismos que posee el propio organismo para restablecer la homeostasis pueden ser aprovechados en terapias contra desórdenes inmunológicos. En la presente tesis se identifican a los neuropéptidos AM, CST, GHR, UCN y VIP como agentes endógenos capaces de restablecer la homeostasis en condiciones de inflamación sistémica y sepsis. Además, se muestra que VIP y CST, mediante la generación de DC tolerogénicas y a través de la supresión de la respuesta Th1, respectivamente, son capaces de restablecer la homeostasis en condiciones de autoinmunidad, como son los modelos CIA y EAE.

1. SEPSIS y SHOCK SÉPTICO

La sepsis y el shock séptico afectan millones de pacientes cada año con un enorme coste para el sistema de salud. A pesar de mas de 3 décadas de investigación sobre esta enfermedad, la mortalidad aún varía entre un 30% y un 70% y tanto su incidencia como el número de muertes que provoca están aumentando (16, 235-237). Actualmente, existen unas pautas publicadas para el tratamiento de la sepsis severa y shock séptico que recomiendan la administración de fluido intravenoso, antibióticos de amplio espectro y, en pacientes apropiados, glucocorticoides, terapia intensiva de insulina o proteína C activada (31). Sin embargo, la alta incidencia y el alto porcentaje de muertes en pacientes con sepsis severa y shock séptico que todavía existe, muestran la necesidad de encontrar terapias adicionales. En este trabajo se muestra que el tratamiento con CST, GHR, AM, UCN o VIP protege contra la letalidad inducida por tres modelos murinos distintos de sepsis, i.e. endotoxemia, administración de *E. coli* y CLP.

En el caso de shock endotóxico, el aumento en la supervivencia de los ratones tratados con los neuropéptidos está asociado a una disminución en la concentración sistémica de mediadores proinflamatorios y factores involucrados en la coagulación, entre los que se incluyen TNF, IL-1, IL-6, NO y SAA, y por otro lado, se observa un aumento en la concentración sérica de la proteína antiinflamatorias IL-10 (197, 238-241).

Existen gran cantidad de resultados que confirman el papel antiinflamatorio de los péptidos CST, GHR AM, UCN y VIP y su uso terapéutico en desórdenes inflamatorios y autoinmunes.

La administración de AM a animales con sepsis o endotoxemia inhibe la hiperpermeabilidad vascular y previene la disminución local y sistémica de variables hemodinámicas como volumen minuto del corazón, resistencia periférica total, perfusión de órganos y entrega sistémica de oxígeno (242, 243). Además su administración atenúa el daño tisular y la disfunción en la barrera intestinal en CLP, y el daño agudo pulmonar inducido por LPS (244, 245). Recientemente, los efectos antiinflamatorios de AM en sepsis y shock séptico fueron confirmados sugiriendo que podrían estar mediados por el factor de transcripción PPAR-γ (246). Consecuentemente con la capacidad de regular la respuesta inflamatoria, ratones que carecen de un alelo del gen de AM producen mayor cantidad de TNF e IL1β y muestran mayor daño hepático en respuesta al LPS (247). También se observó que AM exógena disminuye la peritonitis y la gravedad de las ulceraciones en el colon inducidas por ácido acético (248, 249). Por otro lado, durante la colitis inducida por TNBS, la cual está caracterizada por una inflamación severa de tipo Th1 en el trato gastrointestinal, el tratamiento con AM reduce los signos clínicos e histopatológicos de la colitis y aumenta la supervivencia (250). Es más, la administración de AM a ratones con artritis inducida por CIA, eliminan la hinchazón de las articulaciones y la destrucción de hueso y cartílago (251). En ambos modelos, el efecto terapéutico se encuentra asociado a una inhibición de la respuesta Th1 e inflamatoria, incluyendo un amplio espectro de mediadores inflamatorios. Estos resultados fueron confirmados recientemente (252, 253).

Coherente con el papel antiinflamatorio de UCN, el pretratamiento de ratones con UCN y la posterior infección con *L. monocytogenes* aumenta la producción de IL-10 y disminuye la de IFNγ y TNF (254). También se ha visto que UCN induce apoptosis en macrófagos (232) y que la inhibición sobre la secreción de TNF por macrófagos parece estar mediada por la inducción de PGE2 y COX2 (255). Al igual que AM, la administración de UCN en ratones con colitis inducida por TNBS o con artritis inducida por CIA reduce los signos patológicos de ambas

enfermedades, inhibiendo tanto la respuesta inflamatoria como la del tipo Th1 (250, 256). Se ha demostrado que UCN ejerce funciones antiinflamatorias en el sistema nervioso, inhibiendo la respuesta inflamatoria y neurotóxica inducida por células de la microglía (257). El tratamiento con UCN suprime la encefalitis inducida por EAE (234) y revierte varios de los indicadores de daño en distintos modelos de neuroinflamación de la enfermedad de Parkinson (258-260).

Acorde con el efecto terapéutico en sepsis, el tratamiento con GHR o el agonista del receptor de GHR (GHRP-2) de animales con CLP o endotoxemia atenúa varios de los signos asociados al daño de órganos durante la sepsis, como son los niveles séricos de transaminasas, lactato y creatinina (222, 261). Además, la administración de GHR reduce el daño en los pulmones, incrementa el flujo sanguíneo pulmonar e inhibe la activación de NF-κB y la consecuente secreción de citoquinas proinflamatorias durante la sepsis (262, 263). El efecto antiinflamatorio de GHR también se ha observado en modelos de isquemia y reperfusión (I/R). La administración de GHR previene el daño en las neuronas corticales inducido por I/R (264) e inhibe la producción de citoquinas proinflamatorias y la infiltración de neutrófilos, disminuye la disfunción de la barrera intestinal, atenúa el daño de los órganos y aumenta la supervivencia (265). Al igual que en sepsis y artritis, HMGB1 también posee un papel central en la patología de I/R (266-269).

Por otro lado, existen resultados que sugieren que los efectos antiinflamatorios del tratamiento con GHR están mediados por la "vía antiinflamatoria colinérgica" (265, 270). Esta vía es un mecanismo neuronal capaz de inhibir la activación de macrófagos mediante la activación del sistema parasimpático, cuyo principal neurotransmisor es la acetilcolina. De hecho, la acetilcolina es capaz de desactivar los macrófagos de manera efectiva y la activación experimental de la "vía antiinflamatoria colinérgica" mediante la estimulación del nervio vago eferente disminuye las concentraciones séricas de TNF y HMGB1 en endotoxemia y CLP. Wang y col. mostraron que la vagotomía de los animales antes de inducirles isquemia o sepsis elimina los efectos beneficiosos del tratamiento con GHR (265, 270), sugiriendo que el tratamiento con GHR requiere el nervio vago intacto para inhibir la secreción de citoquinas proinflamatorios. De hecho, no observaron efecto directo de GHR en la secreción de TNF o IL-6 por células de Kupffer o macrófagos peritoneales de rata (270). Por el contrario, y de manera similar a nuestros resultados, Robinson y col. describieron que GHR exógena inhibe la activación de NF-κB y la consecuente producción de IL-1β y TNF en macrófagos murinos estimulados con LPS, al mismo tiempo que activa p38 MAPK y aumenta la secreción de IL-10 (271). Es más, Dixit *et al.* han

mostrado que GHR, tanto exógena como endógena, tiene efecto antiinflamatorio directo sobre linfocitos T y monocitos humanos (201, 272).

En distintas condiciones de inflamación crónica, los niveles de estos neuropéptidos se encuentran elevados, hecho que podría ser interpretado como una respuesta del huésped para intentar retornar el organismo a la homeostasis. De esta forma, se ha detectado en humanos que las concentraciones plasmáticas de AM se encuentran elevadas en pacientes con sepsis severa o shock séptico (273-275). Análogamente, la expresión de AM se encontró elevada en suero y distintos tejidos de animales con endotoxemia o sepsis inducida por CLP (276-278). Pacientes con AR, muestran una mayor concentración de UCN en el fluido sinovial, de manera que el pico de concentración de UCN coincide con la etapa inflamatoria activa de la AR (279, 280). En individuos con gastritis por *H. pylori*, los niveles de UCN son superiores en las biopsias gástricas con respecto a pacientes controles (281). Por último, la expresión de UCN se encuentra incrementada en las mucosas de individuos con enfermedad intestinal crónica (282, 283). Los niveles de GHR se han detectado asociados a distintos desordenes inmunológicos. Durante la sepsis intra-abdominal post-operación, por ejemplo, los niveles plasmáticos de GHR se encontraron elevados y positivamente correlacionados con los niveles de las citoquinas TNF e IL-6 (284). De manera similar, la administración de LPS en humanos induce un rápido incremento de GHR plasmática cuyo pico aparece 30 min después del pico de TNF (285). En casos de inflamación crónica, los datos parecen ser contradictorios. En humanos con enfermad inflamatoria intestinal (colitis ulcerosa y enfermedad de Crohn) los niveles en suero de GHR se encuentran significativamente elevados (286). Análogamente, la concentración de GHR está aumentada en pacientes celíacos, pero revierte a los valores normales durante la dieta libre de gluten (287). Por el contrario, en humanos y ratas con AR la concentración plasmática de GHR resultó menor que en individuos normales (288). A su vez, los niveles plasmáticos y gástricos de GHR son menores en pacientes positivos para *H. Pylori*, de manera que el grado de atrofia producido por la infección se correlaciona negativamente con la concentración de GHR en plasma (289). Sin embargo, estos estudios sólo tienen en cuenta la concentración total de GHR, i.e. GHR acilada y GHR desacilada. Pero se ha visto que sólo la GHR acilada es capaz de unir a su receptor y ejercer funciones biológicas (198). De hecho, en pacientes con gastritis atrófica crónica mientras que los niveles de GHR plasmáticas son similares al de los pacientes normales, la concentración plasmática de GHR acilada es significativamente superior (290). De igual manera, los niveles periféricos de VIP también se encuentran elevados en condiciones de inflamación (197, 291, 292). Es más, se ha descrito una expresión alterada de los receptores de VIP en células T relacionada con una inmunidad Th1 en pacientes con EM y AR (184-186).

En contraste a la acción de los neuropéptidos, los intentos para combatir la inflamación en pacientes con shock séptico basados en drogas no selectivas como corticosteroides y antiinflamatorios no esteroideos fallaron en aumentar la supervivencia de pacientes (293, 294). La segunda generación de drogas para el tratamiento de la sepsis y shock séptico tuvo como objetivo bloquear a algún mediador inflamatorio. El tratamiento preventivo con anticuerpos neutralizantes contra TNF, por ejemplo, previene completamente la muerte por endotoxemia o infusión de *E. coli* (295, 296). Sin embargo, los ensayos clínicos cuyo objetivo fue el bloqueo de moléculas como TNFα, IL-1, PAF, moléculas de adhesión, radicales libres del oxígeno, o NO no mostraron una clara mejoría en la supervivencia de pacientes (28). Esta divergencia entre los modelos experimentales y la realidad clínica puede deberse a varias razones. En primer lugar, mientras que en los modelos animales la secuencia de eventos está claramente definida y por lo tanto resulta relativamente simple interrumpir la cascada inflamatoria bloqueando alguno de sus componentes, en humanos existe una gran variabilidad entre los pacientes que reside no sólo en las diferencias genotípicas y fenotípicas, sino también en los distintos estímulos iniciales que provocaron la enfermedad. Como consecuencia, la cascada de eventos en pacientes con sepsis o shock séptico resulta substancialmente más compleja, lo que explicaría, por un lado que el sistema inmune responda sólo parcialmente frente a terapias dirigidas a mediadores individuales y por otro, que el momento de la intervención resulte crucial en la eficacia terapéutica. En este sentido, el amplio espectro de acción de estos neuropéptidos sobre la cascada inflamatoria y de coagulación, sumado a la capacidad de retener su efecto terapéutico aún cuando sean administrados a tiempos tardíos, representa una clara ventaja terapéutica sobre aquellas terapias dirigidas hacia un único componente de la enfermedad.

Por otro lado, los resultados contradictorios entre modelos experimentales y ensayos clínicos también podrían deberse a que los modelos de endotoxemia e infusión de bacterias vivas recapitulan en buena medida el shock séptico, pero no la sepsis severa (30). Cabe recalcar que la definición de sepsis se basa en signos clínicos y es demasiado amplia y común a grupos heterogéneos de pacientes que no tienen necesariamente el mismo desorden, y aunque actualmente todavía no ha sido ampliamente aceptado, los síndromes clínicos de la sepsis severa y el shock séptico podrían ser enfermedades distintas tanto desde el punto de vista clínico como inmunológico y bioquímico (17). No todos los pacientes con sepsis severa desarrollan shock séptico, ni todos los pacientes que desarrollan shock séptico lo hacen luego de un período de sepsis severa. La divergencia entre estos patrones clínicos podría estar basada en el hecho de que estos dos síndromes sean enfermedades diferentes, causadas por mediadores distintos (52). Así,

los niveles de TNF observados en CLP y humanos con sepsis son generalmente muy bajos comparados con los que se encuentran después de la infusión de LPS. De hecho, el bloqueo de TNF, aunque beneficioso en endotoxemia, resulta perjudicial en CLP. Por el contrario, tanto humanos con sepsis como ratones con CLP presentan altos niveles de HMGB1 sérica. HMGB1 extracelular está involucrada en la patogénesis de diversas enfermedades de origen inflamatorio y autoinmune, y en particular, distintos datos experimentales sugieren que posee un papel central como mediador tardío en el desarrollo y/o mortalidad de la sepsis severa, surgiendo como potencial blanco de terapias (37). El tratamiento con CST, AM, GHR, UCN o VIP protege a los ratones de la muerte no sólo por endotoxemia o sino también por sepsis inducida por CLP. En el caso de GHR, UCN y VIP, el efecto terapéutico durante la sepsis ya establecida está asociado a la inhibición de la secreción sérica de HMGB1, mientras que las concentraciones sistémicas de las citoquinas proinflamatorias TNF, IL-1, IFNγ e IL-6 y de la citoquina antiinflamatoria IL-10 no se ven modificadas por el tratamiento. Es más, la administración de HMGB1 recombinante revierte el efecto terapéutico de los neuropéptidos. En conjunto, estos datos indican que al menos parte de los efectos beneficiosos de GHR, UCN y VIP en sepsis se deben a la disminución de los niveles circulantes de HMGB1. Se ha demostrado que la inhibición específica de la actividad extracelular de HMGB1 mediante anticuerpos bloqueantes o antagonistas (49) rescata de la muerte por sepsis aún cuando son administrados a tiempos tardíos. Sin embargo, desde un punto de vista terapéutico, y como se discutió para el caso de otros mediadores proinflamatorios, la neutralización de un único mediador podría no resultar del todo eficiente en humanos. De manera similar a GHR, UCN y VIP, también se han reportado otras terapias capaces de inhibir la secreción de HMGB1 a la circulación y aumentar la supervivencia de ratones con sepsis (297-302). Sin embargo, estos neuropéptidos son los primeros inhibidores endógenos de la secreción de HMGB1 capaces de proteger contra la muerte inducida por sepsis en una ventana clínicamente relevante.

Si bien existen varios tipos celulares que al ser estimulados secretan HMGB1, la cinética de secreción de HMGB1 por macrófagos estimulados con LPS se correlaciona con la cinética de aparición de HMGB1 en el suero de los ratones con sepsis inducida mediante CLP, por lo que es probable que sean estas células una de las principales fuentes de HMGB1 sistémica durante la sepsis. Monocitos y macrófagos activados acetilan HMGB1 en la señal de localización nuclear, lo que impide su transporte al núcleo y lleva a la acumulación de HMGB1 en vesículas citoplasmáticas y su subsecuente secreción (40, 303). GHR, UCN y VIP inhiben la secreción activa de HMGB1 por macrófagos activados, interfiriendo en la translocación del núcleo al citoplasma. De manera similar a los neuropéptidos, otros inhibidores de la secreción activa de

HMGB1 previenen su translocación citoplasmática (297, 300, 302, 304-306). Recientemente se ha demostrado que el neuropéptido PACAP, que posee una alta homología con VIP y con el cual comparte gran cantidad de sus efectos antiinflamatorios (307), además de inhibir la secreción de HMGB1, inhibe la producción de TNF inducida por HMGB1 extracelular in vitro e in vivo (308). Teniendo en cuenta que estos neuropéptidos comparten entre sí gran parte de sus mecanismos de acción (309), cabría esperar que GHR, UCN o VIP también sean capaces de interferir con la actividad inmunoestimuladora de HMGB1 extracelular. Sin embargo, el hecho de que la administración de HMGB1 recombinante revierta el efecto terapéutico de los 3 neuropéptidos, sugiere que éstos interfieren con la secrecion, pero no con los efectos adversos de HMGB1 extracelular.

Originalmente, Wang *et al* describieron la presencia de HMGB1 en el suero de pacientes con sepsis, en contraste con su total ausencia en pacientes sanos. En este trabajo seminal, los niveles del HMGB1 sérica se muestran superiores en los pacientes que sucumbieron a la sepsis comparado con los supervivientes (48). De manera similar, más tarde se encontró que niveles de HMGB1 en suero se correlacionan de manera positiva con el desarrollo de coagulación intravascular diseminada, falla de órganos y muerte de los pacientes (310). Por el contrario, otros estudios en pacientes con infecciones o sepsis mostraron resultados cualitativamente diferentes. Por ejemplo, se detectaron los niveles circulantes de HMGB1 persistentemente elevados tanto en pacientes con sepsis como con shock séptico, aunque no se encontró correlación entre la concentración de HMGB1 y la gravedad de la enfermedad (311). Recientemente, en un estudio con pacientes con neumonía adquirida en la comunidad (la causa más común de sepsis severa), se encontró que la concentración de HMGB1 en plasma se mantuvo elevada durante el curso de hospitalización sin diferencias entre aquellos pacientes que desarrollaron o no sepsis severa, aunque levemente superior en los pacientes que no sobrevivieron. Llamativamente, la mitad de los supervivientes mostraron altas concentraciones de HMGB1 aun después de resolver la enfermedad (312).

Por lo tanto, aún cuando HMGB1 es un importante mediador de la sepsis en roedores y los niveles circulantes de HMGB1 están elevados en sepsis humana, parecería haber, en el mejor de los casos, sólo una débil correlación entre los niveles sistémicos de HMGB1 y la prognosis clínica. Sin embargo, habría que tener en cuenta que todos los datos clínicos respecto a los niveles de HMGB1 en suero o plasma de pacientes fueron obtenidos midiendo los niveles de la proteína inmunorreactiva por ELISA o Western blot. Pero los niveles de HMGB1 inmunorreactiva podrían diferir de los niveles de HMGB1 biológicamente activa. En primer lugar, HMGB1 es capaz de interactuar con productos bacterianos y otros componentes del suero

para formar complejos (313, 314). Es más, mientras que los niveles circulantes de HMGB1 no se correlacionan con la gravedad de la enfermedad cuando las muestras son analizadas en sueros no fraccionados (311, 312), sí se observa una correlación positiva entre la letalidad de la sepsis y los niveles circulantes de HMGB1 cuando las muestras son fraccionadas por ultrafiltración descartando los complejos superiores a 100 kD (48). De hecho, se ha descrito que trombomodulina puede unirse a HMGB1 previniendo la señalización de HMGB1 por su receptor RAGE (315). También se demostró que HMGB1 no sólo interactúa con LPS, facilitando su unión a CD14 y aumentando la producción de TNF mediada por LPS en monocitos humanos (316), sino que además la administración conjunta de HMGB1 en concentraciones no letales junto con una LD_{10} de LPS incrementa la mortalidad a un 90% (48). HMGB1 también interactúa con fragmentos de DNA que poseen motivos CpG, permitiendo el *crosslinking* entre el TLR9 y el receptor de HMGB1 RAGE y aumentando la intensidad de señal de los motivos CpG (317, 318). HMGB1 extracelular puede interactuar con proteínas séricas como IgG1, y en particular, puede unir citoquinas como IL-1 y aumentar su actividad pro-inflamatoria (319). Por otro lado, HMGB1 es susceptible de sufrir varias modificaciones post-traduccionales. Recientemente se demostró que ROS liberadas por la mitocondria durante la muerte dependiente de caspasas pueden oxidar HMGB1, eliminando su propiedad inmunoestimuladora (320). Además, con el fin de ser activamente secretada, HMGB1 puede ser acetilada, fosforilada, metilada y ribosilada (303, 321-323), aunque hasta la fecha se desconoce si estas modificaciones modulan su actividad extracelular. De esta forma, la actividad de HMGB1 puede depender por un lado, de la contribución relativa de otros factores que varían durante el curso de la sepsis, como son la presencia de infecciones, citoquinas proinflamatorias y otros factores séricos como trombomodulina y anticuerpos, mientras que por otro lado, también puede estar influenciada por el modo en que HMGB1 es liberada al medio extracelular, i.e. apoptosis, necrosis o secreción activa.

Durante el transcurso de la sepsis, la exacerbada respuesta inmune inicial es seguida por un período de inmunosupresión que está caracterizado por una carencia en la respuesta de los neutrófilos, un desvío de la respuesta Th1 hacia Th2 y una marcada disminución en los niveles de linfocitos B, linfocitos T $CD4^+$ y DC foliculares (18). Como resultado, los pacientes no sólo son incapaces de resolver la infección primaria, sino que además tienden a desarrollar infecciones secundarias nosocomiales (21). En este aspecto, si bien cabría esperar que el tratamiento con agentes antiinflamatorios como GHR, UCN o VIP afecte negativamente la respuesta contra la infección bacteriana, observamos por un lado, que UCN y VIP muestran una tendencia a disminuir el número de bacterias en el peritoneo de los ratones con CLP (Figura 1.

Anexo resultados). Por otro lado, el tratamiento con GHR disminuye cerca del 90% el número de bacterias en el peritoneo.

La bajada en la cantidad de bacterias peritoneales que se observa en el tratamiento con GHR se correlaciona con la habilidad de este neuropéptido para matar *E. coli* in vitro. Es más, la concentración en la que GHR muestra su máxima actividad bactericida (2,5 μM) coincide con la concentración a la que el neuropéptido fue administrado i.p. en los ratones con sepsis. En conjunto, estos resultados indican que la menor cantidad de bacterias en el peritoneo de los ratones tratados con GHR se debe a un efecto bactericida directo del neuropéptido. De hecho, además de poseer la habilidad de modular respuestas inmunes e inflamatorias de una manera dependiente de receptores, un significativo porcentaje de péptidos, entre los que se incluyen varios neuropéptidos, también tienen la capacidad de dañar o desestabilizar directamente la membrana de bacterias, virus o parásitos, siendo ambas funciones parte integral de la inmunidad innata (324). Los péptidos antimicrobianos forman parte de los mecanismos de defensa innatos de prácticamente todos los seres vivos. En líneas generales, los péptidos antimicrobianos son relativamente cortos (entre 12 y 100 aminoácidos), poseen una carga neta positiva, son anfipáticos y suelen derivar de péptidos precursores a partir de uno o más pasos proteolíticos (325). Hasta la fecha, se han descrito cientos de estos péptidos, lo cual indica la importancia que tienen en el sistema inmune.

La mayoría de los péptidos antimicrobianos caracterizados hasta la fecha poseen una carga positiva neta que varía de +2 a +9 y que permite la interacción electroestática inicial con las membranas fofolipidídicas cargadas negativamente de las bacterias. Las superficies celulares bacterianas poseen gran cantidad de componentes aniónicos, incluyendo el LPS y lípidos aniónicos en las Gram negativas y el ácido lipoteicoico en las Gram positivas. Una vez que atraviesan la pared extracelular, los péptidos deben interaccionar con la membrana citoplasmática bacteriana, que a diferencia de las membranas citoplasmáticas de eucariotas, son ricas en fosfolípidos aniónicos que le confieren una carga neta negativa, permitiendo de esta forma que los péptidos catiónicos se unan selectivamente a membranas bacterianas (326). Otro factor que podría contribuir a la atracción electrostática entre los péptidos catiónicos y las bacterias es el hecho de que el potencial de membrana de los procariotas sea aproximadamente un 50% mayor que el de los mamíferos. De hecho se cree que el potencial quimiosmótico podría actuar concentrando los péptidos catiónicos en las superficies microbianas (326). En el caso de las bacterias Gram negativas, los péptidos catiónicos se insertan en la membrana externa mediante un proceso que involucra interacciones electrostáticas, hidrofóbicas y probablemente el *prefolding* de los péptidos en estructuras asociadas a la membrana; esto desestabiliza la

estructura membranosa externa y la permeabiliza a otras moléculas de péptidos. El resultado final es que los péptidos alcanzan la membrana citoplasmática, donde penetran en la interfase entre las partes hidrofílicas e hidrofóbicas de la membrana. Como resultado final los péptidos pueden formar un canal transiente en la membrana, disolver la membrana, o translocarse al citoplasma y una vez ahí unirse a su blanco. De esta forma, uno de los principales modos de acción de los péptidos catiónicos antimicrobianos consiste en desestabilizar la membrana provocando su despolarización, la pérdida de gradientes de iones y metabolitos y la interrupción de procesos esenciales como la respiración (327).

GHR, es un péptido de 28 aminoácidos octanoilado con un peso molecular de aproximadamente 3 kD y una carga neta de +8 a pH 7. In vitro, GHR es capaz de unirse a *E. coli* y despolarizar y permeabilizar su membrana (238)(328), probablemente interfiriendo con sus funciones esenciales y provocando la muerte del patógeno. Por otro lado, existen algunos trabajos que postulan que la simple perturbación de la membrana por los péptidos antimicrobianos puede no ser suficiente para matar eficientemente a las bacterias (329, 330) y que, por lo tanto, la perturbación de la membrana y la muerte del patógeno pueden ser eventos independientes que ocurren individualmente o complementariamente a otros mecanismos de acción de los péptidos antimicrobianos (326). Algunos estudios sugieren que la interferencia con algún proceso intracelular por parte de los péptidos puede contribuir o incluso ser necesario para ejercer sus propiedades bactericidas (331-333). De hecho, se ha sugerido que la muerte mediada por péptidos antimicrobianos puede ocurrir como resultado de varios mecanismos de acción independientes o cooperativos. Incluso, los péptidos podrían matar la misma especie mediante más de un mecanismo dependiendo de factores como fase de crecimiento, localización tisular y la presencia o ausencia de otros mecanismos inmunes (326).

Los ratones con CLP desarrollan una peritonitis polimicrobiana, por lo que en su cavidad peritoneal no sólo hay gran variedad de bacterias, sino también distintas células del sistema inmune. Así, la composición del fluido intraperitoneal es sumamente compleja, convirtiendo la situación in vivo substancialmente más compleja que las condiciones en la que se analizó la actividad antimicrobiana de GHR in vitro. Si bien aún queda por determinar si GHR también mata otros tipos de bacterias in vitro, los péptidos antimicrobianos pueden interactuar simultáneamente con patógenos microbianos en una amplia variedad de condiciones, incluyendo las mezclas complejas del medio extracelular o fagolisosomas, por lo que los péptidos antimicrobianos pueden interactuar entre sí, con microorganismos, y con moléculas del huésped en estos sitios. Es más, algunos estudios sugieren que una interacción heteróloga entre péptidos podría ser importante para una actividad antimicrobiana global (334, 335). En particular, la

potencia bactericida de GHR es dependiente del pH y de la concentración $MgCl_2$. De hecho, a medida que disminuye el pH del medio, aumenta la capacidad bactericida de GHR (figura 2 Anexo resultados). Esto podría tener cierta relevancia fisiológica en distintos escenarios. En primer lugar, el estómago es el principal órgano productor de GHR, el cual además de poseer un pH notablemente bajo es susceptible de sufrir infecciones bacterianas, principalmente con *H. Pylori* (336). Por otro lado, una de las características del foco inflamatorio es la acidosis local, producida en parte por los ácidos grasos que surgen como productos secundarios del metabolismo bacteriano (337). Por último, además de ser secretados al medio extracelular, los péptidos antimicrobianos pueden ser liberados en los fagolisosomas que contengan bacterias fagocitadas, y donde el valor de pH es cercano a 5 (338).

Al igual que GHR, otros neuropéptidos tienen la capacidad de matar patógenos. De hecho, estos neuropéptidos poseen varias de las características biofísicas propias de los péptidos antimicrobianos, i.e. son productos proteolíticos de precursores proteicos más grandes, son pequeños, poseen carga neta positiva y son anfipáticos. Se ha descrito que AM muestra actividad bactericida contra *E. coli* y *S. aureus*, matando a un 50% de las bacterias a una concertación de 0,038 μM (339, 340). También se ha demostrado que VIP posee actividades antimicrobianas contra patógenos como *E. coli*, *P. aeruginosa*, *C. albicans* y *S. Mutans* (341). Por el contrario, no se han observado propiedades bactericidas con el péptido UCN.

En conjunto, estos trabajos muestran que los péptidos CST, AM, GHR, UCN y VIP, poseen la capacidad de regular la secreción por macrófagos de una amplia gama de mediadores proinflamatorios, entre los que se incluyen mediadores críticos de enfermedades inflamatorias sistémicas. Consecuentemente, la terapia con CST, AM, GHR, UCN o VIP protege contra la muerte séptica inducida por tres modelos distintos y aún cuando la terapia comience a tiempos tardíos en condiciones en las que la enfermedad ya está establecida. Estas características de los neuropéptidos, la de regular una plétora de mediadores involucrados en la sepsis severa y el shock endotóxico en diversas situaciones y al mismo tiempo regular la carga bacteriana, resultan de vital importancia a la hora de trasladarlos a la clínica, ya que en humanos existe una gran variación entre los pacientes y los tiempos a los que puedan comenzar las terapias.

2. Autoinmunidad

Gran cantidad de enfermedades inflamatorias son consideradas autoinmunes en origen y se conocen varios de sus respectivos autoantígenos (342). Una vez iniciado, el fenómeno de autoinmunidad puede destruir selectivamente los tejidos diana. En la EM, las células T atacan al

sistema nervioso central y en la AR las células T atacan los tejidos de las articulaciones. Otras enfermedades inflamatorias crónicas, como la enfermedad inflamatoria intestinal y alergias, podrían representar un fallo en los mecanismos de tolerancia hacia proteínas medioambientales normalmente inocuas y microorganismos no patogénicos. Las DC poseen el doble papel de primar la respuesta inmune y de inducir tolerancia. La habilidad de estas células para detectar y traducir estímulos ambientales dicta el destino de los linfocitos T y en última instancia determina la característica de la respuesta inmune. Los enfoques terapéuticos y experimentales actuales para la inducción de tolerancia están basados en la utilización de agentes no específicos de antígenos, los cuales interfieren con la función de todos los linfocitos T. Por el contrario, la fisiología de las DC abre la posibilidad de manipular la autoinmunidad de una manera antígeno-específica.

La presencia de VIP durante la primera etapa de diferenciación de DC a partir de células de médula ósea de ratón genera DC incapaces de madurar con estímulos inflamatorios. Estas DC (DCvip) exhiben un fenotipo tolerogénico, caracterizado por alta expresión de MHC II junto con baja expresión de las moléculas coestimuladoras CD40, CD80 y CD86, baja producción de citoquinas Th1 (IL-12 y TNF) y producción elevada de IL-10. Estas DCvip son incapaces de primar la respuesta T alogénica. La estimulación de linfocitos T CD4 alogénicos con las DCvip induce células que muestran las características propias de células Treg del tipo Tr1, i.e. altos niveles de producción de IL-10 y TGFβ y bajos niveles de IFNγ, IL-2 e IL-4, baja capacidad proliferativa intrínseca, y supresión de la proliferación y activación de otras células T $CD4^+$ específica de antígeno. La capacidad para inducir Tr1 específicas de antígeno por DCvip es retenida in vivo, suprimiendo la respuesta de hipersensibilidad retardada, y exhibiendo un potente efecto terapéutico en dos modelos distintos de autoinmunidad como CIA y EAE.

Durante la presentación antigénica, el estado de activación de las DC define la diferenciación de los linfocitos T y por lo tanto, el tipo de respuesta. Evidencias originales sugirieron que las DC inmaduras inducen tolerancia mientras que DC maduras inducen la diferenciación de células Th1, Th2 o Th17 (343, 344) . Las DC inmaduras en estado basal son capaces de inducir tolerancia mediante depleción o anergia de linfocitos T, o incluso mediante la generación de Treg (99). Así, originalmente se propuso el uso de DC inmaduras en enfermedades autoinmunes. Sin embargo, es probable que las DC inmaduras se conviertan en DC maduras in vivo una vez que entren en circulación y migren hacia los tejidos dañados donde existe un estado de inflamación crónica (345). En este sentido, la capacidad de las DCvip para inducir tolerancia mediante la generación de células Tr1 aún en condiciones de inflamación, resulta particularmente relevante para el tratamiento de enfermedades autoinmunes, rechazos de

transplantes y enfermedad del injerto contra el huésped (GVHD). De hecho, las DCvip no sólo previenen la progresión de enfermedades autoinmunes como CIA y EAE, sino también la enfermedad inflamatoria intestinal y GVHD (346, 347). A diferencia con lo observado en CIA y EAE, en la enfermedad inflamatoria intestinal, en la cual la inflamación es el componente predominante, el efecto de las DCvip parece ser independiente del antígeno y más bien está relacionado con una inhibición directa de la inflamación por la producción de IL-10 (347). De manera similar, se demostró que DC tolerogénicas regulan la inflamación sistémica durante endotoxemia y peritonitis (348).

Las DC tolerogénicas pueden ser utilizadas no sólo para controlar respuestas contra antígenos propios, sino también para controlar respuestas contra moléculas ajenas al huésped que pueden ser introducidas accidentalmente, como es el caso de las alergias, o deliberadamente, como es el caso de los aloantígenos de células u órganos donantes. Se han demostrado los efectos beneficiosos de DC tolerogénicas en alergias (349) y transplantes alogénicos, como el transplante alogénico de médula ósea (120). El transplante de médula ósea es un tratamiento necesario en varias enfermedades hematopoyéticas donde, luego de irradiación o quimioterapia, el sistema inmune del huésped es reconstituido con células de médula ósea. En el caso de las leucemias, si bien las células T donantes son responsables de la eliminación de tumores, también pueden iniciar al mismo tiempo GVHD, que es la mayor causa de morbilidad y mortalidad en pacientes recipientes de transplantes de médula ósea. De esta forma, con el fin de evitar GVHD sin afectar la respuesta contra el tumor se han desarrollado protocolos que usan DC tolerogénicas en modelos de transferencia de médula ósea (120, 350). De igual manera, las DCvip disminuyen la respuesta alogénica específica del haplotipo de las células T $CD4^+$ en ratones recipientes, induciendo la generación de Treg en el injerto, y evitando GVHD (346). Las DCvip no impiden la erradicación del tumor por el transplante, presumiblemente porque no afectan la respuesta citotóxica de las células T $CD8^+$ del injerto contra las células leucémicas. Al igual que en el tratamiento de CIA y EAE, el papel de las Treg en el efecto terapéutico de las DCvip ha sido confirmado mediante la reversión parcial por anticuerpos bloqueantes contra IL-10 y TGFβ y por la generación de Treg ex vivo por DCvip con efecto preventivo sobre GVHD (346).

Si bien no se conocen del todo los mecanismos por los que VIP induce DC tolerogénicas, estudios con agonistas y antagonistas específicos de los receptores sugieren que el efecto está mediado por VPAC1 (351), el cual está acoplado a adenilato ciclasa. De hecho, la inhibición de PKA revierte la inducción de DCvip. Además, la ausencia de maduración de las DCvip en respuesta a un estímulo inflamatorio está asociada a la inhibición de la la fosforilación de IκB y

la consecuente translocación de NF-κB al núcleo (351). Se ha demostrado que factores NF-κB juegan un papel importante en la generación y maduración de las DC (352-356). La actividad transactivadora de NF-κB se ha relacionado con la expresión de varias moléculas coestimuladoras como CD40, CD80 y CD86 y con la producción de TNFα e IL-12 por DC (357, 358). DC pulsadas con antígeno, en las que la función de NF-κB está inhibida, inducen Treg con la capacidad de transferir tolerancia a animales receptores primados y de una manera dependiente de IL-10 (359). Esta capacidad para inhibir la transactivación de NF-κB también ha sido descrita para otros agentes que generan DC tolerogénicas como corticosteroides, análogos de vitamina D3 y otras sustancias inmunosupresoras (360).

Distintos mecanismos podrían estar involucrados en la generación de Tr1 por DCvip. El fenotipo de las DCvip, que comprende una alta expresión de MHC II junto con una baja expresión de moléculas coestimuladoras, permite que las DCvip presenten péptidos a las células T (señal 1) sin coestímulo (señal 2). De manera similar DC inmaduras pueden generar Tr1 singénicas y alogénicas (111, 112). Así, durante el contacto DCvip-célula T CD4 la entrega de señal 1 en ausencia de señal 2 podría ser uno de los factores en la generación de células Tr1. De hecho, aún cuando las DC inmaduras in vitro tienen una escasa habilidad para capturar y presentar antígenos debido a sus bajos niveles de MHC II y a no ser capaces de procesar antígenos para formar complejos de superficie MHC II-péptido (361), las DCvip no sólo expresan altos niveles de MHC II en superficie, sino que también son capaces de adquirir antígenos y de inducir Tr1 de manera antígeno-específica tanto in vivo como in vitro.

Sin embargo, la simple presencia de señal 1 en ausencia de señal 2 probablemente no sea suficiente para explicar la inducción de Tr1 por DCvip. Las DC estimuladas con TNF, por ejemplo, expresan altos niveles de MHC II y CD40, CD80 y CD86, pero in vivo inducen células T CD4 productoras de IL-10 y protegen a ratones de EAE (362). De hecho, la expresión de moléculas coestimuladoras por DC no sólo no implica inmunogenicidad (363), si no que además puede ser incluso necesaria para la inducción de tolerancia (364). Por último, la presentación de antígenos por DC en estado basal no necesariamente resulta en inactivación de las células T (365). Por otro lado, el denominador común entre DC no inmunogénicas, i.e. las DC tolerogénicas y las DC en estado basal, más que la baja expresión de moléculas coestimuladoras, parecería ser la producción de bajos niveles de citoquinas proinflamatorias como IL-12 y TNF (señal 3). De hecho, la producción de estas citoquinas resulta necesaria para una completa diferenciación de células T efectoras (97). Así, las DCvip no sólo no producen IL-12 ni TNF en respuesta al LPS, sino que además secretan grandes cantidades de IL-10. Esta IL-10 puede actuar de manera autocrina sobre las DC o de manera paracrina sobre las células T. El tratamiento de

DC con IL-10 disminuye su potencial inmunogénico, inhibiendo el aumento de expresión de moléculas coestimuladoras y la producción de citoquinas inflamatorias (366-368). A su vez, la exposición de DC a IL-10 durante su diferenciación las convierte en DC tolerogénicas capaces de inducir Treg (118, 119). Por otro lado, la IL-10 tiene efectos directos sobre los linfocitos T, induciendo anergia y suprimiendo su respuesta (369), o incluso puede diferenciarlos a Treg (71, 76, 370). Por lo tanto, es probable que la inducción de Treg por las DCvip se deba a que en la interacción DCvip-célula T, la célula T recibe una fuerte señal 1, junto con una débil señal 2, y como señal 3, IL-10, pero no TNF ni IL-12.

De manera similar a las DC generadas en presencia de IL-10 y TNF (118), las DCvip poseen un fenotipo $CD11c^{low}CD45RB^{high}$ (351). Las DC con fenotipo $CD11c^{low}CD45RB^{high}$ y capacidad para inducir células Tr1 han sido identificadas in vivo y, de hecho, la administración de VIP a ratones transgénicos para el TCR incrementa el porcentaje de esta población en el bazo (118, 351).

En células humanas, la presencia de VIP durante la diferenciación de DC a partir de monocitos de sangre periférica genera una población de DC con características similares a las observadas en DC de ratón. Así, las DCvip humanas en presencia de estímulos inflamatorios incrementan la expresión de MHC II, pero no la de las moléculas coestimuladoras CD40, CD80, CD83 y CD86, a la vez que secretan IL-10, pero no TNF, IL-6 ni IL-12 (371). Las DCvip humanas no sólo poseen la capacidad de generar Treg $CD4^+$con características similares a las Tr1, sino que además son capaces de generar células $CD8^+$ con fenotipo regulador caracterizado por una reducida actividad lítica y una baja capacidad proliferativa al mismo tiempo que poseen actividad supresora frente a células Th1 singénicas. Esta actividad reguladora parece residir en una población de células T $CTLA4^+CD8^+CD28^-$ productoras de IL-10, la cual ha sido asociada con un fenotipo Treg $CD8^+$ (372-374).

Las DC comprenden una variedad de poblaciones con diferentes fenotipos y funciones, las cuales están relacionadas con su estado de diferenciación, su localización en tejidos específicos y sus interacciones con antígenos y células inmunes (375). Existen resultados que sugieren que ciertas DC especializadas tendrían propiedades tolerogénicas intrínsecas y que, a su vez, la función de las DC estaría dictada por el microambiente particular en el que se encuentre durante su desarrollo o activación. Además, según el presente paradigma de diferenciación de DC, el *pool* de DC periféricas es mantenido principalmente por progenitores de la médula ósea que circulan por la sangre y migran a los tejidos periféricos donde se terminan de diferenciar a DC. De esta forma, el destino final de los precursores de DC va a estar dictado por las características particulares del microambiente que encuentre al extravasar desde la sangre (376).

Teniendo en cuenta que fibras nerviosas con VIP han sido identificadas tanto en medula ósea como en la piel, tracto gastrointestinal y órganos linfoides secundarios y que células del sistema inmune también secretan VIP (164), cabría la posibilidad de que VIP forme parte de determinados microambientes jugando un papel en la generación de DC tolerogénicas. Así, en condiciones normales, el VIP liberado o bien de las innervaciones o bien por células inmunes podría formar parte de los mecanismos de tolerancia periférica mediante la generación de DC tolerogénicas. En particular, tejidos como los presentes en el intestino u órganos inmunoprivilegiados, como el CNS, placenta, testículos o globo ocular, las respuestas inflamatorias e inmunes se encuentran finamente reguladas con el fin de evitar daño tisular. Las DC intestinales se encuentran en continuo contacto con antígenos del lumen, como alimentos y flora intestinal, y de hecho, están claramente involucradas en la generación de un estado general de tolerancia frente la flora intestinal (377). DC de las placas de Peyer, por ejemplo, al ser activadas producen grandes cantidades de IL-10 y son capaces de diferenciar células T $CD4^+$ en productoras de IL-10 e IL-4 (378). Además, DC presentes en el intestino y tejidos linfoides asociados son capaces de promover el desarrollo de Treg $Foxp3^+$ en la periferia (67-69). VIP se encuentra particularmente elevado en la mucosa intestinal, el parenquima nervioso, el humor acuoso ocular y en la interfase materno-fetal (164), apoyando la hipótesis de VIP como agente tolerogénico en condiciones basales.

Además de tener la habilidad de generar células Tr1 mediante la diferenciación de DC tolerogénicas, varios trabajos muestran que in vivo, la administración de VIP también posee la capacidad de aumentar la población de células Treg $CD4^+CD25^+FoxP3^+$ (379-381). El tratamiento de ratones con EAE o CIA con VIP resulta en un aumento del número de células T $CD4^+CD25^+$ en los ganglios linfáticos drenantes, cerebro y articulaciones. Es más, la transferencia adoptiva de células T $CD4^+$ provenientes de ratones tratados con VIP no transfiere la enfermedad y el efecto terapéutico de VIP puede ser revertido por anticuerpos bloqueantes contra CTLA4, IL-10 y TGF-β1, demostrado el papel de las Treg en el efecto beneficioso de la administración de VIP (379-381). Así, las Treg inducidas por el tratamiento con VIP en ratones con artritis o EAE median su acción supresora a través los factores solubles IL-10 y TGF-β1 y por una manera dependiente de contacto que reside en CTLA4. Esto sugiere que in vivo, VIP es capaz de generar no sólo las Tr1, probablemente a través de la diferenciación de DC tolerogénicas, sino que también posee la habilidad de inducir o expandir Treg $CD4^+CD25^+FoxP3^+$.

La administración de CST a ratones con artritis retarda la aparición, disminuye la frecuencia y reduce la severidad de la enfermedad. El efecto terapéutico de CST modula las dos

fases de la enfermedad. Así, el tratamiento con CST afecta los eventos tempranos que están asociados al inicio y establecimiento de la respuesta autoinmune contra los componentes de los tejidos propios, a la vez que disminuye la respuesta inflamatoria destructiva que está asociada con las fases más tardías de la enfermedad. CST reduce el desarrollo de células autorreactivas Th1, su migración al órgano diana, y la producción de citoquinas proinflamatorias y quimioquinas, con la subsecuente inhibición del reclutamiento de macrófagos y neutrófilos. Esto resulta en una disminución en la producción de mediadores inflamatorios involucrados en la destrucción (citoquinas, NO, radicales libres y metaloproteasas de la matriz) por células residentes e infiltrantes (sinoviocitos). Además, la inhibición de la respuesta Th1 por CST ocasiona un menor título de autoanticuerpos IgG2a, los cuales activan neutrófilos y la cascada de complemento, contribuyendo a la destrucción del tejido. Resultados similares se encontraron en el tratamiento de colitis con CST (382). En ambos modelos, se observa un aumento en la población de linfocitos T $CD4^+$ productores de IL-10, sugiriendo que CST, al igual que VIP, es capaz de inducir/expandir la población de Treg. Es más, recientemente se ha mostrado que la administración de CST prolonga el tiempo de supervivencia de transplantes de piel alogénicos e incrementa la proporción de Treg $CD4^+CD25^+FoxP3^+$ (383). Análogamente, se ha descrito que UCN y AM aumentan la población de Treg $CD4^+CD25^+FoxP3^+$ y de linfocitos productores de IL-10 y TGFβ cuando son administrados a ratones con CIA (251, 256)

3. Potencial terapéutico

Una estrategia potencial para su uso en terapias es la aplicación directa a pacientes, ya que además de su acción de amplio espectro, la estructura molecular anfipática sumada al pequeño tamaño de estos neuropéptidos los convierte en compuestos atractivos para el tratamiento de la inflamación excesiva. Esto se debe a que estas características les confieren excelentes propiedades de permeabilidad que les permiten un rápido acceso a los sitios de inflamación donde pueden alcanzar altas concentraciones locales. Esto resulta crucial para los desórdenes neuroinflamatorios, en los que la barrera hematoencefálica suele estar comprometida.

Sin embargo, aún cuando estos neuropéptidos muestran un gran potencial terapéutico, hay que considerar que todos estos estudios han sido realizados en modelos animales y la extrapolación a las enfermedades humanas debe ser cauta. En humanos, las respuestas pueden variar dependiendo de las dosis utilizadas y la expresión de receptores específicos en los distintos tipos celulares, no sólo en aquellos que participan en la respuesta inmune. De hecho, habría que considerar la posibilidad de efectos secundarios como consecuencia de los efectos de

los neuropéptidos en sistemas distintos al inmune, dado que son capaces de producir hipotensión, disminución de movimiento gástrico, desórdenes endocrinos, diarrea y alteraciones en el ritmo cardíaco y en la memoria. No obstante, varios de estos neuropéptidos ya han sido testados en humanos para el tratamiento de distintos desordenes sin efectos adversos aparentes (384-390). Esto sugiere que podrían ser bien tolerados en humanos a dosis similares a las utilizadas en los presentes trabajos para el tratamiento de enfermedades inmunológicas en animales. Por lo tanto, en contraste con las drogas antiinflamatoriass existentes, estos neuropéptidos no estarían asociados con efectos secundarios adversos probablemente por ser compuestos fisiológicos, intrínsecamente no tóxicos. La alta afinidad por sus receptores específicos, los convierte en moléculas potentes para llevar a cabo sus acciones, permitiendo que sean utilizados a bajas concentraciones. Además, los neuropéptidos son eliminados rápidamente del cuerpo mediante mecanismos naturales de detoxificación hepática y excreción renal. Es más, dado que otras citoquinas, neuropéptidos y hormonas son capaces de balancear sus efectos, la homeostasis de los tejidos no debería ser perturbada excesivamente. Por último, al ser péptidos pequeños, su síntesis in vitro resulta relativamente simple y permite realizarles modificaciones si es necesario.

A pesar de todas estas ventajas, el traslado del tratamiento con neuropéptidos a terapias clínicas viables tiene ciertos obstáculos. El principal reside en que, como consecuencia de su conformación estructural, los neuropéptidos son altamente inestables y al mismo tiempo son extremadamente sensibles a la degradación por peptidasas presentes en la mayoría de los tejidos. Por esta razón, varias estrategias han sido desarrolladas con el fin de aumentar la vida media de estos péptidos. Para incrementar su estabilidad, se han descrito modificaciones o sustituciones de ciertos aminoácidos que en algunos casos convierten su estructura cíclica (391, 392). Por otro lado, se han desarrollado estrategias para aumentar la eficiencia con la que los neuropéptidos alcanzan los tejidos o células dianas a la vez que se disminuye su degradación, como es el caso de la inserción de VIP en micelas o nanopartículas o terapia génica (393-400). También se ha combinado el tratamiento de neuropéptidos con inhibidores de endopeptidasas o proteínas séricas específicas de unión a los neuropéptidos con el fin de estabilizarlos (246). Otras estrategias intentan aumentar la señalización combinando los neuropéptidos con inhibidores de fosfodiesterasas, ya que la mayoría de los neuropéptidos antiinflamatorios señalizan a través de la vía AMPc/PKA (401-403).

Además de la administración directa, el desarrollo de Treg por los neuropéptidos in vivo o ex vivo representa otra potencial estrategia de terapia clínica. En los últimos años, se ha centrado un esfuerzo considerable en el uso Treg antígeno-específicas generadas ex vivo para el tratamiento de enfermedades autoinmunes, trasplantes y desórdenes asmáticos (404-406). Las

posibilidades de trasladar el uso de Treg al escenario clínico se encuentran limitada, no sólo por el bajo número de estas células en circulación, sino que este inconveniente es probable que esté exacerbado en pacientes con desórdenes autoinmunes, en los que las Treg suelen ser deficientes o defectivas (404-406). En este sentido, la capacidad de las DC tolerogénicas inducidas con VIP y pulsadas con antígenos propios para inducir Treg antígeno-específicas resulta una atractiva estrategia para la expansión/generación de Treg antígeno-específicas ya sea ex vivo o in vivo, ya que por cada DC se van a generar al menos varias Treg.

En suma, estos resultados indican que los neuropéptidos actúan de una manera pleiotrópica, afectando distintos componentes de la respuesta inmune. Por un lado, afectan la respuesta inflamatoria, siendo los macrófagos la diana principal. Por otro lado, afectan la respuesta adaptativa, modulando la respuesta Th1, ya sea directamente, o mediante la generación de DC tolerogénicas. Estas características convierten a estos neuropéptidos antiinflamatorios en atractivos agentes para el tratamiento de enfermedades de base inflamatoria y/o autoinmune, como sepsis, shock séptico, AR, EM, enfermedad de Crohn o transplantes.

CONCLUSIONES

1. Cortistatina, Adrenomedulina, Urocortina y Grelina protegen contra la muerte inducida por endotoxemia o *Escherichia coli* y disminuyen la secreción de citoquinas y quimioquinas inflamatorias in vivo y por macrófagos estimulados con LPS.
2. La administración de Cortistatina, Adrenomedulina, Urocortina, Grelina o VIP protege contra la muerte inducida por CLP y en el caso de urocortina, grelina y VIP, disminuye los niveles séricos de la proteína HMGB1 y su secreción por macrófagos activados.
3. Grelina muestra propiedades bactericidas en sepsis e in vitro
4. La presencia de VIP durante la diferenciación de células dendríticas induce células dendriticas con capacidad para generar células T productoras de IL-10 y TGFβ, de restablecer tolerancia in vivo y de reducir la progresión de artritis reumatoide y encefalomielitis autoinmune experimental.
5. La administración de cortistatina o grelina reduce la gravedad de la artritis inducida por colágeno. En el caso de cortistatina, la reducción de los signos clínicos se encuentra asociada a una inhibición de la respuesta inflamatoria y Th1 con un concomitante aumento de la población de células T productoras de IL-10. En el caso de la grelina, se observa una disminución en los niveles séricos de HMGB1.

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ANEXO RESULTADOS

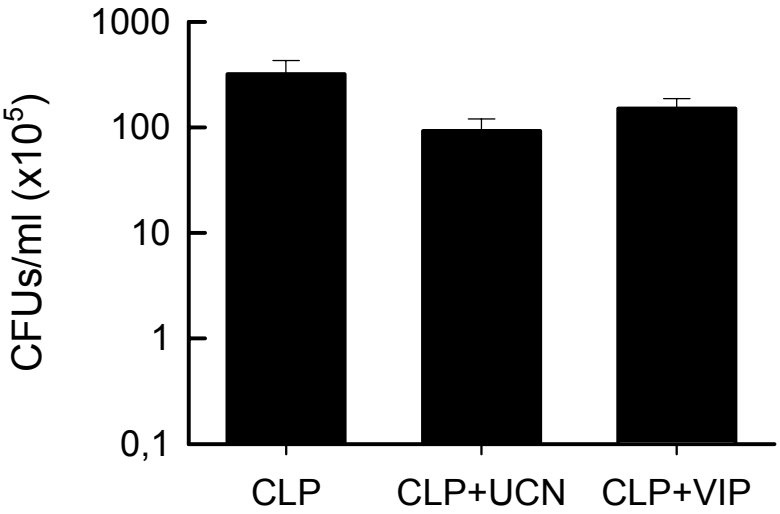


Figura 1. Número de bacterias en el peritoneo de ratones con sepsis. La sepsis fue inducida por CLP y los ratones tratados con UCN o VIP (1nmol UCN o VIP/ratón a las 12 y 18h después de CLP). Los lavados peritoneales fueron obtenidos a las 24h, plaqueados, incubados a 37ºC y el número de unidades formadoras de colonias (CFU) contado. Los datos son la media ± S.E.M. n = 10 por grupo.

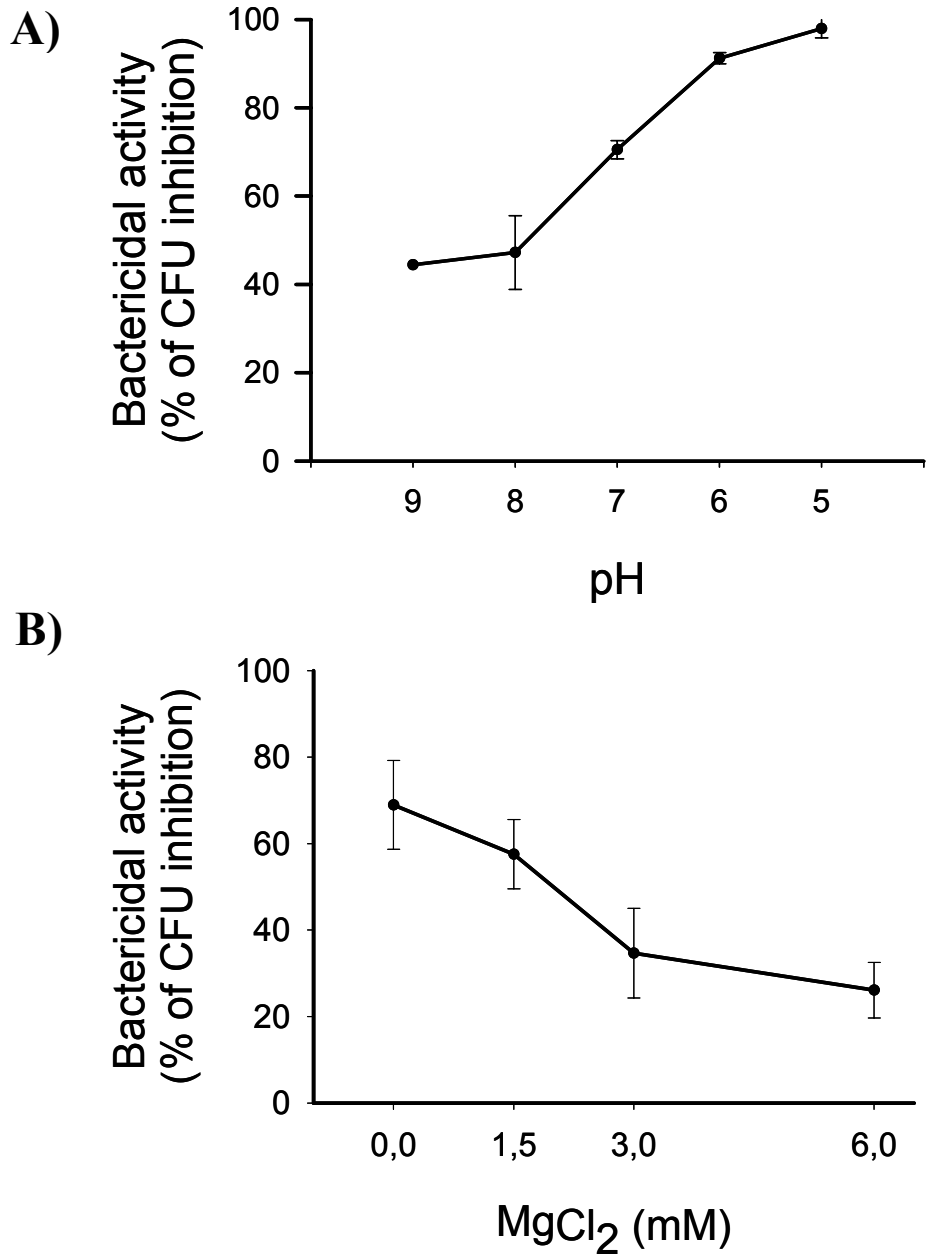
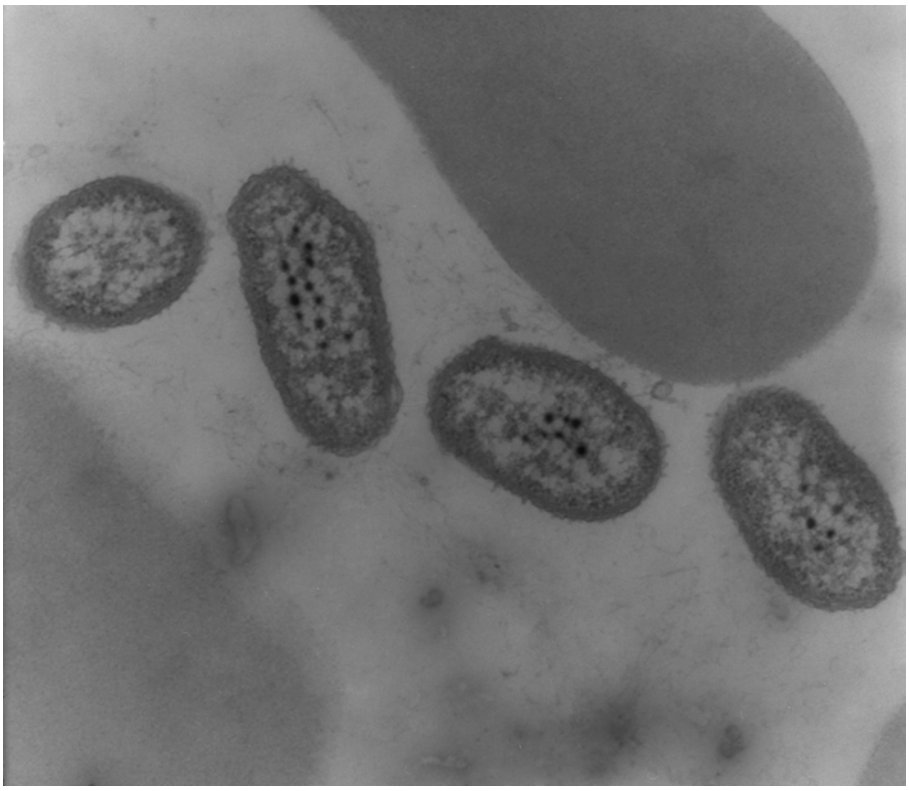


Figura 2. Propiedades bactericidas de GHR. *E. coli* (DH5α) fue incubada con 5μM de GHR como se describió antes (227) en distintos valores de pH **(A)** o distintas concentraciones de $MgCl_2$ **(B)**. Cada punto representa la media de experimentos realizados 3 veces por duplicado y expresados como 100-el porcentaje del número de CFU/ml en las muestras control ± S.E.M.

A)



B)

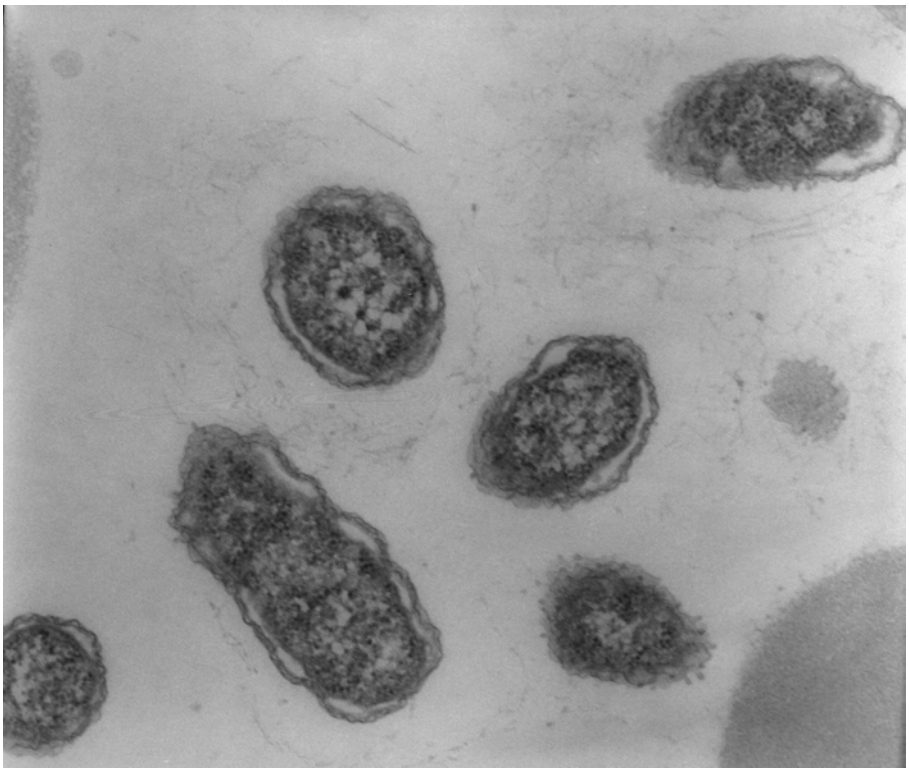


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Figura 3. Microscopía electrónica de *E. coli* tratadas con GHR. *E. coli* (DH5α) fueron incubadas con PBS **(A)** o con 5μM de GHR **(B)** por distitnos tiempos (0,5-6h), fijadas y analizadas por microscopía electrónica.

ANEXO OTRAS PUBLICACIONES

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