Universidad de Oviedo

Programa de Doctorado "Investigación en Cáncer"

"Participación de los receptores de quimiocinas CCR1 y CCR2 en el dolor inflamatorio y neoplásico en ratones"

Tesis doctoral

María Llorián Salvador

Mayo 2016

Universidad de Oviedo

Programa de Doctorado "Investigación en Cáncer"

"Participación de los receptores de quimiocinas CCR1 y CCR2 en el dolor inflamatorio y neoplásico en ratones"

Tesis doctoral

María Llorián Salvador

Mayo 2016

UNIVERSIDAD DE OVIEDO
Vicerrectorado
de Internacionalización y Postgrado

CENTRO INTERNACIONAL DE POSTGRADO
CAMPUS DE EXCELENCIA INTERNACIONAL

RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Participación de los receptores de quimiocinas CCR1 y CCR2 en dolor inflamatorio y neoplásico en ratones	Inglés:

2.- Autor	
Nombre: MARIA LLORIAN SALVADOR	DNI/Pasaporte/NIE:
Programa de Doctorado: Investigación en Cáncer	
Órgano responsable: INSTITUTO UNIVERSITARIO DE ONCOLOGÍA	

RESUMEN (en español)

La familia de las quimiocinas está constituida por un grupo de proteínas de peso molecular relativamente bajo clasificadas por la presencia canónica de aminoácidos cisteína en su extremo amino-terminal. En base a ello se consideran las quimiocinas de la serie CC, CXC, XC y CX3C donde X es un aminoácido que puede variar. La función biológica de estos péptidos se relaciona fundamentalmente con la capacidad para atraer células inmunes hacia tejidos dañados, pero además de este papel, también se reconoce su participación en la producción y modulación de la sensación dolorosa. A nivel experimental, numerosos estudios han descrito su participación en el dolor producido por daño neuropático (White y Wilson, 2008; Biber y Bodekke, 2014; Knerlich-Lukoskus et al., 2015). Sin embargo, existen menos trabajos que hayan evaluado su implicación en dolor inflamatorio y en dolor neoplásico (Pevida et al., 2012;2014).

En esta tesis, estructurada en 3 artículos, se explora la posible participación de unas quimiocinas de la serie CC, CCL2, CCL3 y CCL5, que se unen a los receptores CCR2 (la CCL2) y CCR1 (la CCL3 y la CCL5) en las respuestas nociceptivas medidas en ratones que presentan procesos inflamatorios o neoplásicos localizados en una extremidad posterior. En ellos se ha valorado el efecto antihiperalgésico y antialodínico derivado del bloqueo de los receptores CCR2 y CCR1 o de la inhibición de las quimiocinas implicadas en su producción. Como modelo de inflamación se empleó la administración de carragenina, para inducir una inflamación aguda , y de coadyuvante de Freund (CFA) para inflamación crónica. Por otra parte, en lo que respecta al dolor en el contexto del cáncer, se estudió en un modelo nuevo de dolor neoplásico derivado de la inoculación intratibial de células de cáncer de próstata RM1, del que se realizó una caracterización de su desarrollo macroscópico así como de las alteraciones histopatológicas y de respuesta a estímulos nociceptivos previamente al estudio de la participación de quimiocinas.

El primero de los aspectos estudiados se refiere al bloqueo de la hiperalgesia inflamatoria mediante la inhibición de los efectos derivados de la unión de la quimiocina CCL2 a su receptor. La administración sistémica aguda del antagonista del

receptor CCR2 RS504393 revirtió la hiperalgesia, pero no la alodinia, que aparece en ratones con inflamación aguda y crónica. Este efecto antihiperalgésico derivado del antagonismo de CCR2 no parece debido a un posible efecto antiinflamatorio ya que las dosis antihiperalgésicas no modificaron la evolución del proceso inflamatorio. Además, la administración intraplantar de pequeñas dosis de RS504393 también antagonizó la hiperalgesia inflamatoria, sugiriendo que el efecto de este fármaco ocurre a través de receptores expresados en el tejido inflamado. Mediante ensayos de ELISA se detectó un aumento de expresión de CCL2 en los tejidos inflamados, que con técnicas de inmunohistoquímica se localizó especialmente en macrófagos. En consonancia con estos resultados, la administración intraplantar de un anticuerpo anti-CCL2 evitó la hiperalgesia térmica del mismo modo que hizo el antagonista de CCR2, RS504393, lo cual indica la participación de esta quimiocina expresada a nivel local en la hiperalgesia inflamatoria.

En segundo lugar se estudió la posible implicación en el dolor inflamatorio de otras dos quimiocinas, también de la serie CC, CCL3 y CCL5, cuya participación en el procesamiento nociceptivo tras su unión al receptor CCR1 es menos conocido que en el caso de la CCL2. La administración sistémica aguda del antagonista de receptores CCR1, J113863, revirtió la hiperalgesia térmica medida tanto en ratones con inflamación aguda como crónica. Sin embargo, este fármaco sólo inhibió la alodinia mecánica debida a la inflamación crónica. Al igual que tras el bloqueo de CCR2, el grado de inflamación producida por carragenina o CFA no se modificó al administrar de forma aguda dosis analgésicas de J113863, descartando que la acción de este antagonista pudiera deberse a un posible efecto antiinflamatorio. Debido a que tanto la CCL3 como la CCL5 son agonistas endógenos del CCR1, se midieron sus niveles y su expresión tanto en la médula espinal como localmente en la zona inflamada. La expresión del mRNA de CCL3 así como de la propia proteína CCL3 medidas mediante PCR cuantitativa y ELISA respectivamente, aumentó en las patas de ratones inflamadas con carragenina o CFA, y se localizó principalmente en macrófagos y neutrófilos. Sin embargo, la inflamación no produjo cambios locales de los niveles de CCL5 ni modificó la expresión de CCL3 o de CCL5 en la médula espinal. De acuerdo con estos datos, la administración de un anticuerpo anti-CCL3 inhibió la hiperalgesia inflamatoria lo que indica que esta quimiocina juega un papel hipernociceptivo en el tejido inflamado.
Finalmente, se estudió la participación de las quimiocinas CCL2, CCL3 y CCL5 en un modelo de dolor neoplásico experimental debido a la inoculación de células procedentes de cáncer de próstata RM1 en ratones inmunocompetentes. El inóculo de 1000 células RM1 en la cavidad medular de la tibia provocó el desarrollo de tumores óseos con un patrón mixto osteoblástico/osteoclástico así como la activación de la astroglía, pero no de la microglía, en la médula espinal. Además, el crecimiento tumoral produjo hiperalgesia y alodinia a partir del cuarto día tras la inoculación, alcanzando su máximo entre los días 12 y 16. Estos síntomas nociceptivos fueron inhibidos tras la administración sistémica aguda de morfina así como del bisfosfonato

ácido zoledrónico. La eficacia de estos dos fármacos ampliamente utilizados en clínica junto con las características histopatológicas y su evolución confieren utilidad a este modelo como herramienta experimental para el estudio del dolor neoplásico. En lo concerniente a la participación de quimiocinas, se demostró que las células RM1 en cultivo pueden liberar CCL2 y CCL5, pero no CCL3. En los homogeneizados procedentes del tejido tumoral se detectó el aumento de la concentración de CCL2 pero no de las otras quimiocinas y ello puede explicar la inhibición de la hiperalgesia térmica producida tras la administración del antagonista de receptores CCR2, RS504393, en ratones con tumor.

El conjunto de resultados presentados en este trabajo indican el posible potencial analgésico de estrategias farmacológicas relacionadas con la inhibición de la acción de determinadas quimiocinas de la serie CC en patologías relacionadas con inflamación o cáncer.

RESUMEN (en Inglés)

Chemokines constitute a protein family with a low molecular weight classified in basis of the location of the canonical N-terminal cysteine residues. Thus, chemokines are divided into CC, CXC, CX3C and XC families, where X is a variable amino acid. The biological function of these peptides is not only associated with the ability to mediate immune cell trafficking to sites of inflammation, but also with the modulation of nociceptive responses. Experimentally, several studies have described the involvement of chemokines in neuropathic pain (White and Wilson, 2008; Biber y Bodekke, 2014; Knerlich-Lukoschus and Held-Feindt, 2015). Nonetheless, only some few studies have assessed their role in inflammatory (Lewis et al., 2014) and cancer pain (Pevida et al., 2012; 2014).

The present work, which is divided into in three papers, explores the possible involvement of some CC chemokines, namely CCL2, CCL3 and CCL5, in inflammatory and cancer pain behaviors. The antihyperalgesic and antiallodynic effect derived from either the blockade of CCR1 and CCR2 chemokine receptor or the neutralisation of the chemokines able to activate them has been explored. Inflammation was evoked by the administration of carrageenan and complete Freund´s adjuvant (CFA) to induce acute and chronic inflammatory responses, respectively. With regard to cancer induced-pain, the involvement of these chemokines has been assessed in a new model of bone cancer-induced pain derived from the presence of prostate tumoral cells.

The first study is related to the inhibition of inflammatory hyperalgesia evoked by the blockade of CCL2/CCR2. The acute systemic administration of the CCR2 antagonist RS504393 inhibited thermal hyperalgesia, but not allodynia, in mice with acute or

chronic inflammation. The antihyperalgesic effect derived from the CCR2 blockade seems unrelated to a putative anti-inflammatory effect since the administration of antihyperalgesic doses did not modify macroscopic or microscopic signs of inflammation. Moreover, the intraplantar administration of low doses of RS504393 also counteracted the inflammatory hyperalgesia, thus suggesting that the effect of the drug occurs through receptors expressed in inflamed tissues. An augmentation of CCL2 concentration measured by ELISA was detected in homogenates of inflamed tissues. This increase was mainly located in macrophages, as detected by immunohistochemical methods. In accordance with these results, the intraplantar administration of an anti-CCL2 antibody inhibited thermal hyperalgesia in the same way as the CCR2 antagonist did, thus suggesting the involvement of local CCL2 in the development of thermal inflammatory hyperalgesia.

Secondly, it has been evaluated the possible involvement in inflammatory pain of CCL3 and CCL5, other CC chemokines whose participation in nociceptive processing is not as well-known as it occurs with CCL2. The acute systemic administration of the CCR1 antagonist J113863 inhibited thermal hyperalgesia in both carrageenan and CFA inflamed mice. However, mechanical allodynia was reversed by J113863 only in chronically inflamed mice. As occurs with the CCR2 blockade, the acute administration of J113863 did not ameliorate the inflammatory process, thus discarding the possibility that an anti-inflammatory activity underlies the antinociceptive responses derived from the antagonism of CCR1. Considering that both CCL3 and CCL5 are CCR1 endogenous agonists, their levels were measured in spinal cord and inflamed tissues. An augmentation of CCL3 mRNA and its corresponding protein were measured by PCR and ELISA, respectively, both in acutely and in chronically inflamed paws. By immunohistochemical assays it was observed that CCL3 was mainly expressed in macrophages and neutrophils. Besides, paw levels of CCL5 as well as spinal cord levels of CCL3 and CCL5 remained unaltered in both models of inflammation. Accordingly, the inhibition of inflammatory hyperalgesia secondary to the intraplantar administration of an anti-CCL3 antibody indicates that CCL3 plays a hypernociceptive role in inflamed tissues.

Lastly, we have studied the involvement of the chemokines CCL2, CCL3 and CCL5 in an experimental model of bone cancer-induced pain based on the inoculation of prostate cancer cells RM1 in immunocompetent mice. The inoculation of 1000 RM1 cells in the tibial medullar cavity evoked the development of osseous tumors with a mixed osteoblastic/osteoclastic pattern. Moreover, we have observed by immunohistochemical assays astroglial activation in the spinal cord of mice inoculated with RM1 cells. Tumoral growth evoked hyperalgesia and allodynia from the 4th day after cell inoculation, and reached the maximum between the 12th and the 16th day. These nociceptive symptoms were inhibited by the acute administration of either

morphine or zoledronic acid.

The efficacy of these two widely used drugs in the clinical management of neoplastic pain together with the histopathological features and the development of the tumour supports the utility of this model as a tool to study bone cancer-induced pain. With regard to chemokine involvement, RM1 cells in culture release CCL2 and CCL5 but not CCL3. However, only CCL2 was augmented in the tumoral tissue. This fact supports the antihyperalgesic efficacy secondary to the systemic administration of the CCR2 antagonist RS504393 in mice intratibially inoculated with RM1 cells.

Altogether, the present results point towards the analgesic potential derived from the use of pharmacological approaches related to the inhibition of particular CC chemokines in painful inflammatory and cancer diseases.

SR/A. PRESIDENTE/A DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN Investigación en Cáncer

Índice de abreviaturas

AINE: aintiinflamatorio no esteroideo

Akt: Protein cinasa B

AMPA: ácido α-amino-3-hidroxil-5-metil-4-isoxazol-propionato

AMPc: adenosín monofosfato cíclico

ASIC: acid-sensing ion channel

CaMKII: cinasas dependiente de Ca^{2+}/calmodulina II

CB1, CB2: receptores cannabinoides tipo 1 y 2

CFA: Complete Freund's Adjuvant, Adyuvante Completo de Freund

CGRP: Calcitonin gen-related peptide, proteína relacionada con el gen de la calcitonina

COX: ciclooxigenasa

COX-1, COX-2: ciclooxigenasa tipo 2

DAG: diacilglicerol

DEG/ENaC: familia de las degenerinas y de los canales de Na^+ epiteliales

DRG: ganglios de la raíz dorsal

ERK: cinasas reguladas por señales extracelulares

FMRI: imagen por resonancia magnética funcional

GABA: ácido gamma aminobutírico

$GABA_A$, $GABA_B$: receptores GABA tipo A y B

GDNF: Glial cell-derived neurotrophic factor, factor neurotrópico derivado de células gliales

GFAP: glial fibrillary acidic protein, proteína glial fibrilar ácida

GPRC: G-protein coupled receptors, receptores acoplados a proteínas G

HCN: hyperpolarization-activated, cation nonselective

IASP: International Association for the Study of Pain, Asociación internacional para el Estudio del Dolor

Iba-1: ionized calcium binding adaptor molecule 1

IFN- γ: interferon γ

IL-1β: interleucina 1β

IP_3K: cinasa del inositol trifosfato

KCNK o K2P: two-pore domain potassium channels

LPS: lipopolisacárido

MAPK: proteína cinasa activada por mitógenos

mGluR: receptor metabotrópico de glutamato

MIA: monosodium iodoacetate

NGF: factor de crecimiento nervioso

NMDA: N-metil-D-aspartato

nNOS: neural nitric oxide synthase, isoforma neural de la oxido nítrico sintetasa

OPG: osteoprotegerina

P2X2, P2X3, P2X4: receptor purinérgico P2X2, P2X3, P2X4

PET: tomografia por emisión de positrones

PKA: proteína quinasa A

PKC: proteína cinasa C

PGE_2: prostaglandina E_2

PLC: fosfolipasa C

PMN: polimorfonucleares

RUNX1: runt-related transcription factor 1

SNC: sistema nervioso central

TLR: Toll-like receptors

TNF-α: factor de necrosis tumoral

TrkA: receptor de la tirosina quinasa A

TRP: transient receptor potential, receptor de potencial transitorio

TRPA: transient receptor potential anquirina

TRPC: receptor de potencial transitorio canónico

TRPM: receptor de potencial transitorio melastatina

TRPMI: receptor de potencial transitorio mucopilina

TRPP: receptor de potencial transitorio policistina

TRPV1-4: receptor de potencial transitorio vanilloide 1-4

WDR: neuronas convergentes o de amplio rango dinámico (wide dynamic range)

Índice

1.Introducción

1.1. CONCEPTO DE DOLOR. PROCESAMIENTO DE LAS SEÑALES NOCICEPTIVAS

Según la Asociación Internacional para el Estudio del Dolor (IASP), el dolor es "una experiencia sensorial y emocional desagradable asociada a una lesión hística real o potencial, o que se describe en términos de dicho daño". Esta definición refleja que la experiencia dolorosa no se limita únicamente a una percepción sensorial, sino que abarca también componentes cognitivos y emocionales, que aumentan la complejidad y subjetividad de este proceso, que puede ser percibido de manera diferente por cada persona y que puede ocurrir sin que exista daño en el tejido. El concepto de nocicepción, en cambio, es más puramente fisiológico y se refiere al conjunto de procesos implicados en la detección y procesamiento de los estímulos que contribuyen a la experiencia dolorosa (Loeser y Treede, 2008). Por ese motivo, en lo que respecta a la experimentación animal, lo que realmente se suele estudiar son aspectos relativos a la transmisión nociceptiva ya que la cuantificación del dolor como tal resulta extremadamente dificultosa.

1.1.1. La transmisión de señales nociceptivas en la periferia

La percepción del dolor comprende, al menos, cuatro elementos básicos: la transducción, que consiste en la transformación de estímulos nociceptivos en potenciales de acción nerviosos, la transmisión a través de las fibras nociceptivas de dicha señal, la plasticidad o modulación del mensaje nociceptivo que tiene lugar sobre todo en el sistema nervioso central (SNC) y, por último, la integración de las señales nociceptivas en los centros cerebrales.

La captación, transducción y transmisión de las señales nociceptivas comienza en los nociceptores, una población heterogénea de neuronas periféricas capaces de detectar estímulos térmicos, mecánicos o químicos (Basbaum y Jessell, 2000) y convertirlos en potenciales de acción. Son neuronas pseudounipolares en las que se distingue un terminal periférico que capta el estímulo externo e inicia los potenciales de acción y un axón que los conduce, el cuerpo o soma, localizado en los ganglios de la raíz dorsal (Dorsal Root Ganglia; DRG) o, en su caso, en el ganglio del trigémino y un terminal central que hará sinapsis con una neurona nociceptiva de segundo orden localizada en el asta dorsal de la médula espinal o en el núcleo caudal del trigémino (Woolf y Ma, 2007; Basbaum et al., 2009; Dubin y Patapoutian, 2010).

Los nociceptores se pueden clasificar en dos grandes grupos en función de su grado de mielinización y

de la sección de sus axones: Aδ y C. Los de tipo Aδ, que responden a estímulos mecánicos, químicos y a altas temperaturas, están formados por fibras mielinizadas de tamaño medio (0,4-4µm) (Djouhri y Lawson, 2004) y son, por tanto, de conducción rápida (5-30 m/s), median el dolor agudo y bien localizado, también denominado rápido o "primer dolor". Dentro de las fibras tipo A, mielinizadas, se incluyen las fibras tipo Aβ, que tienen un diámetro superior a las Aδ, y responden a estímulos mecánicos inocuos o de baja intensidad. No se comportan generalmente como nociceptores salvo bajo determinadas situaciones de daño tisular, en las que, como se comentará más adelante, son capaces de activar neuronas nociceptivas espinales dando lugar al fenómeno conocido como alodinia.

Los nociceptores de tipo C, en cambio, son un grupo heterogéneo de nociceptores polimodales ya que pueden ser activadas por estímulos térmicos, mecánicos y/o químicos (Schmelz et al 2003**;** Dubin y Patapoutian, 2010). Sus axones son fibras no mielinizadas, de diámetro pequeño (0,3-1,5µm) y conducción lenta (0,5-2m/s), relacionándose así con el dolor lento, peor localizado o "segundo dolor". A este grupo pertenecen la mayoría de las neuronas nociceptivas primarias que se encuentran en el sistema nervioso periférico (Woolf y Ma, 2007). A su vez, pueden dividirse en base a sus propiedades neuroquímicas en peptidérgicas y no peptidérgicas. Las primeras liberan neuropéptidos como la sustancia P o el péptido relacionado con el gen de la calcitonina (Calcitonin Gene-Related Peptide, CGRP) y expresan el receptor acoplado a tirosina cinasa de tipo A (TrkA), que responde al factor de crecimiento nervioso (Neural Growth Factor, NGF). Las fibras no peptidérgicas expresan el receptor neurotrófico derivado de las células gliales (Glial cell-Derived Neurotrophic Factor, GDNF), el receptor purinérgico P2X3 y la proteína RUNX1 y además presentan marcaje positivo frente a la isolectina B4 (Snider y McMahon, 1998; Braz et al., 2005; Woolf y Ma, 2007).

Cuando los nociceptores reciben estímulos externos de características apropiadas, se inicia el proceso de transducción. Para que el nociceptor sea activado es necesaria una despolarización de la membrana que alcance el potencial umbral y así inicie el potencial de acción, que conducirá la información hasta la médula. En esta transformación participan varias clases de proteínas presentes en la superficie de los nociceptores: receptores ionotrópicos, receptores metabotrópicos acoplados a proteínas G (G-Protein Coupled Receptors, GPCR) y receptores con actividad tirosina cinasa (Gold y Gebhart, 2010).

Los **receptores ionotrópicos** son canales iónicos de membrana permeables a Na^+ y/o Ca^{2+} que se activan indistintamente ante diferentes tipos de estímulos, sin que exista una selectividad clara entre un tipo de receptor ionotrópico y una modalidad de estímulo concreta. La superfamilia más conocida de receptores

ionotrópicos son los canales TRP (Transient Receptor Potential, receptor de potencial transitorio). A este grupo pertenecen más de 30 receptores descritos en mamíferos y están agrupados en 6 familias: vanilloide (TRPV), melastatina (TRPM), anquirina (TRPA), policistina (TRPP), mucolipina (TRPMI) y canónica (TRPC).

El primer TRP identificado y caracterizado fue el TRPV1 (Transient Receptor Potential Vanilloid 1) (Caterina et al., 1997). Este canal iónico se expresa en neuronas del DRG y del trigémino, en terminales nerviosos espinales y periféricos y en diversos tipos celulares no neuronales (Pedersen, 2005; Brito et al., 2014). Los TRPV1 son activados por estímulos térmicos superiores a 43°C y capsaicina, compuesto químico picante que se encuentra en las guindillas. Además, la acidificación del medio (pH<5.9), característico de los tejidos inflamados, no sólo activa también los canales, sino que aumenta la sensibilidad de éstos a la capsaicina y al calor (Caterina y Julius, 2001). En ratones deficientes de este receptor se ha demostrado una disminución en la sensación térmica, avalando la participación de este canal en el dolor evocado por calor (Caterina et al., 2000; Julius, 2013). Sin embargo, hay estudios recientes apuntan al posible papel que los receptores TRPV1 podrían tener también en la transducción de estímulos mecánicos (Walder et al., 2012; Kim et al., 2012). Curiosamente, a pesar de ser un receptor excitador, actualmente la única molécula aprobada para uso clínico es un agonista, la capsaicina, puesto que el estímulo repetido del receptor conlleva su desensibilización, haciendo al nociceptor menos sensible al estímulo doloroso.

A partir del descubrimiento de los receptores TRPV1, se identificaron otros miembros de la familia TRP, como el TRPV2, que se activa con temperaturas superiores a los 52°C, o los TRPV3 y TRPV4, activados con temperaturas moderadas (>31°C y >25°C respectivamente). Además, tanto los TRPV2 como los TRPV4 juegan un papel importante en la mecanotransducción al ser igualmente activados ante cambios a estímulos osmóticos y mecánicos (Premkumar y Abooj, 2013; Cohen y Moiseenkova-Bell, 2014). Otros canales pertenecientes a la familia TRP son el TRPM8 y TRPA1, sensibles a bajas temperaturas (<25°C y <18°C respectivamente), al mentol y, en el caso de TRPA1, a otras sustancias químicas como la formalina o el aceite de mostaza (Bautista et al., 2007; Julius, 2013; Premkumar y Abooj, 2013).

Los receptores ASIC (Acid-Sensing Ion Channel) pertenecen a la familia de las degenerinas y de los canales epiteliales de Na^{+} (DEG/ENaC), responden a descensos de pH del medio extracelular como los que tienen lugar durante los procesos inflamatorios, como consecuencia de un daño tisular o estrés metabólico. Destaca el ASIC3, que además de estar expresado en nociceptores, también se encuentra en

fibras que inervan el músculo cardiaco y esquelético (Basbaum et al., 2009; Delaunay et al., 2013). A pesar de que numerosos estudios demuestran su implicación en nocicepción, estudios con ratones transgénicos han generado resultados contradictorios, lo cual refleja que el papel de los receptores ASIC en la transmisión nociceptiva aún no ha sido esclarecido (Wemmie et al., 2013; Kellemberger y Shild, 2015).

Otro tipo de receptores ionotrópicos ampliamente estudiado son los activados por nucleótidos, entre los que cabe destacar el P2X2, P2X3, P2X4 y P2X7 en relación a la transmisión nociceptiva (Zhou et al., 2010; Burnstock 2013; Franceschini and Adinolfi 2014; Burnstock 2015). La activación de estos canales se produce por la unión del ATP que liberan las células dañadas al medio extracelular en respuesta a una estimulación mecánica o a la presencia de inflamación. Se ha demostrado la participación de receptores purinérgicos en la transducción de estímulos térmicos, mecánicos y en el desarrollo de hiperalgesia (González-Rodríguez et al., 2009; Burnstock, 2013; Yaksh et al., 2015)

Por último, se debe mencionar la familia de los canales de potasio KCNK o K2P (two-pore domain potassium channels), entre los que destacan KCNK2, KCNK4 y KCNK18. Este grupo de canales desempeña un papel crucial en el potencial de reposo de la membrana y la excitabilidad en neuronas somatosensoriales (Alloui et al., 2006). Se expresan en nociceptores de tipo C y se ha descrito su activación frente a estímulos térmicos y mecánicos (Alloui et al., 2006; Basbaum et al., 2009; Du y Gamper, 2013; Mathie y Veale, 2015). Además, a diferencia de otros canales iónicos como los TRP o los P2X, la actividad de los canales KCNK puede ser inhibida por diversos estímulos (Nöel et al., 2011; Mathie y Veale, 2015). Los ratones carentes de estos receptores mostraron alteraciones en la percepción de estímulos térmicos y mecánicos (Du y Gamper, 2013; Mathie y Veale, 2015).

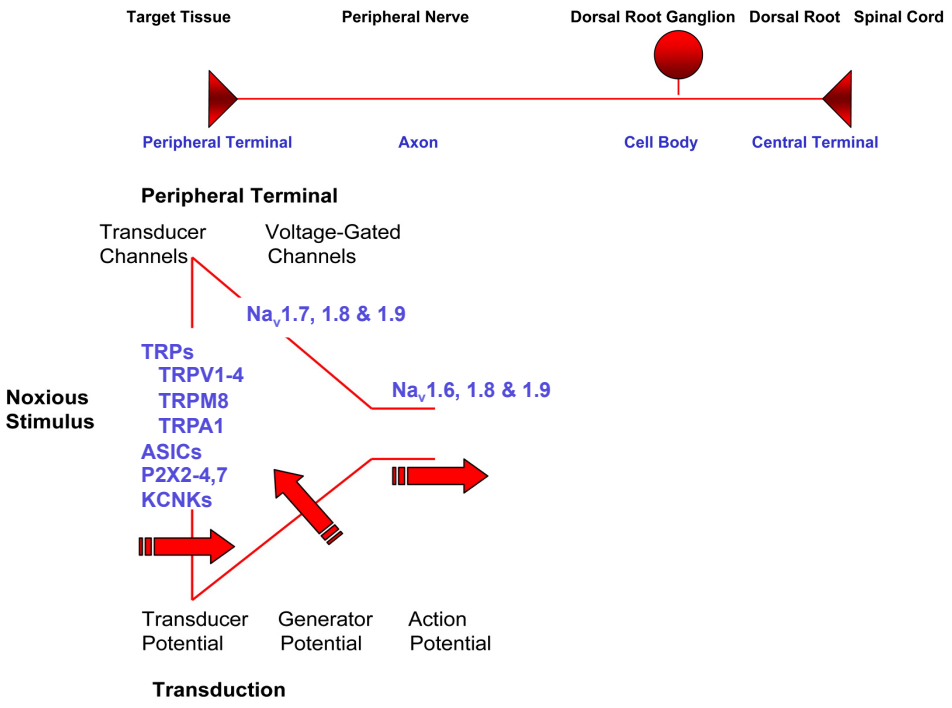

Figura 1. En la parte superior, representación esquemática de los componentes del nociceptor. En la parte inferior, canales iónicos presentes en el terminal periférico del nociceptor encargados de la transducción de la señal nociceptiva. Modificada de Woolf y Ma, 2007.

Como se ha mencionado anteriormente, al margen de los canales iónicos, en la activación de los nociceptores también participan **receptores metabotrópicos**, que en base a su estructura proteica pueden dividirse en dos grandes grupos. Uno lo constituyen los receptores acoplados a proteínas G (GPCR) y los receptores con actividad tirosina cinasa. La activación de estos receptores se realiza a través de factores liberados tanto desde los propios nociceptores como desde células no neuronales procedentes de tejidos lesionados o de su entorno (basófilos, mastocitos, macrófagos, neutrófilos, queratinocitos, fibroblastos…) (Basbaum et al., 2009; Ellis y Bennett, 2013; de Miguel et al., 2014). Estos factores constituyen un conjunto amplio de moléculas entre las que se incluyen péptidos como la sustancia P, la bradicinina, CGRP, neurotrofinas, citocinas, quimiocinas, moléculas lipídicas como las prostaglandinas, tromboxanos y leucotrienos así como aminas como pueden ser la histamina o la serotonina (Rittner et al., 2005; Ellis y Bennet, 2013). Estos factores, actúan a través de receptores de membrana acoplados a enzimas efectoras, como la adenilato ciclasa o la fosfolipasa C, que regulan la producción de segundos mensajeros, como el AMPc, el diacilglicerol, el inositol trifosfato o el ácido araquidónico. Es frecuente que, al final de estas cascadas de transducción, se produzca la fosforilación de diferentes canales de membrana, dando lugar a la disminución de su umbral de apertura, lo que

conduce a la *sensibilización periférica*, responsable de la *hiperalgesia primaria* (Basbaum et al., 2009; Reichling et al., 2013; Yaksh et al., 2015).

Entre los GPCR de mayor interés respecto a la sensibilización de nociceptores, los principales son los acoplados a proteínas G_s y G_q. Las primeras, producen el aumento de los niveles de AMPc, provocando la activación de la proteína cinasa A (PKA). Por esta vía actúan prostaglandinas, como la prostaglandina E_2 (PGE_2) que, a través de este mecanismo, produce la sensibilización de los receptores TRPV1 (Moriyama et al., 2005). Algunos mediadores como la bradicinina o distintas citocinas pueden actuar a través del aumento de la liberación de prostaglandinas. Otros sensibilizadores que actúan a través de la vía PKA son la noradrenalina, la serotonina y el neuropéptido CGRP (Masi et al., 2007; Linley et al., 2010; McCorvy et al., 2015). Sobre receptores acoplados a G_q actúan mediadores como la bradicinina, la histamina, la serotonina o las varias quimiocinas. Secundariamente a la estimulación de estos receptores, se activa la fosfolipasa C (PLC), que provoca la liberación de Ca^{2+} intracelular y la activación de proteína cinasa C (PKC), capaz de sensibilizar los TRPV1 y los TRPA1 (Than et al., 2013).

Por último, los receptores con actividad tirosina cinasa se activan por la unión de péptidos inflamatorios, como las neurotrofinas y citocinas. Estos mediadores aumentan drásticamente en situaciones de daño tisular o lesión y desencadenan un incremento de la excitabilidad de los nociceptores. Entre las neurotrofinas más ampliamente estudiadas está el NGF, que actúa a través de los receptores tirosina cinasa TrkA expresados en neuronas C peptidérgicas. Esta unión activa una cascada de señalización que incluye la PLC, la cinasa activada por mitógenos (MAPK) y la cinasa del inositol trifosfato (IP_3K). Además, el NGF puede también promover el aumento en la expresión de varias moléculas que participan en la nocicepción como la sustancia P, bradicinina, CGRP, receptores TRPV1, receptores purinérgicos tipo P2X y canales Nav 1.8 (Basbaum et al., 2009). Adicionalmente, algunas citocinas proinflamatorias como la interleucina 1β (IL-1β), interleucina 6 y el factor de necrosis tumoral (Tumor necrosis factor, TNF) promueven la liberación de otros mediadores como NGF o prostaglandinas, favoreciendo así el desarrollo de hiperalgesia (Basbaum et al., 2009; Mizumura y Murase, 2015).

En lo que respecta a la **conducción** del potencial de acción hasta la médula espinal, cabe señalar el papel fundamental que desempeñan los canales de sodio dependientes de voltaje (Na_V), aunque también participan los canales de potasio (K_V), los de calcio y los canales catiónicos no selectivos activados por hiperpolarización (HCN, hyperpolarization-activated cation nonselective). En mamíferos se conocen nueve isoformas diferentes de canales Na_V, denominados $Na_V1.1$ a $Na_V1.9$ (Alexander et al., 2015),

aunque no todos están relacionados con la transmisión nociceptiva. Los canales $Na_V1.1$, 1.6 y 1.7 son sensibles a tetrodotoxina mientras que los canales $Na_V1.8$ y 1.9 son resistentes a la misma (Basbaum et al., 2009; Bagal et al., 2014). En animales de experimentación se ha observado un aumento de la expresión de diferentes subtipos de canales de sodio en distintos modelos de dolor (Basbaum et al., 2009; Bagal et al., 2014).

Recientemente se ha conseguido obtener nociceptores funcionales a partir de la reprogramación de fibroblastos, lo que podría constituir una herramienta muy útil para el estudio de los mecanismos del proceso nociceptivo *in vitro* y servir de ayuda para el diseño y evaluación de posibles tratamientos para dolor (Wainger et al., 2015).

1.1.2 Transmisión de señales nociceptivas en la médula espinal

En función del tipo de fibra nociceptiva, los terminales aferentes penetrarán en distintas láminas de la médula en donde establecerán sinapsis con neuronas espinales. En función de las aferencias, la médula contiene neuronas nociceptivas específicas que responden sólo a estímulos nociceptivos de alto umbral que se encuentran localizadas en las láminas I y II (D´Mello y Dickenson, 2008; Todd, 2010) así como neuronas convergentes o de rango dinámico amplio (wide dynamic range, WDR), que están localizadas principalmente en la lámina V que transmiten información nociceptiva y táctil (Todd, 2010).

En las sinapsis espinales participan numerosos neurotransmisores y péptidos, que pueden ser excitadores o inhibidores. El glutamato es el neurotransmisor excitador más abundante y actúa a través de tres tipos de receptores: los receptores ionotrópicos AMPA (α-amino-3-hidroxil-5-metil-4-isoxazol-propionato) y NMDA (N-metil-D-aspartato) así como receptores metabotrópicos acoplados a proteínas G (mGluR). Ante un estímulo nocivo breve, el glutamato y en menor medida el aspartato, se unen a los receptores AMPA generando una despolarización rápida de la membrana de la neurona postsináptica a través del aumento del Na^+ intracelular. En esta situación, los receptores NMDA apenas participan debido al bloqueo de su poro por el cationes Mg^{2+} (Paoletti, 2011). Sin embargo, si el estímulo nociceptivo es tónico, como ocurre en condiciones patológicas, se produce una despolarización mantenida de la membrana, lo que provoca el desplazamiento de Mg^{+2}, permitiendo así la activación del receptor y la posterior entrada de Ca^{+2}, aumentando de esta manera la excitabilidad de la neurona nociceptiva. Por último, los receptores metabotrópicos acoplados a proteínas G_q están localizados en neuronas postsinápticas de la médula espinal y en las células gliales. Estos receptores intervienen en la

transmisión glutamatérgica lenta mediante la producción de segundos mensajeros y su participación en la transmisión nociceptiva está adquiriendo una relevancia creciente (Osikowicz et al., 2013).

En presencia de daño tisular o lesión persistente, la estimulación continuada de los nociceptores da lugar a la liberación mantenida de neurotransmisores excitadores en la médula espinal, lo que conlleva una despolarización sostenida de membrana. La activación de receptores NMDA secundaria al desplazamiento del catión Mg^{2+} (Paoletti et al., 2011) da lugar a un aumento de Ca^{2+} citosólico y a la activación de distintas enzimas como la isoforma neuronal de la óxido nítrico sintetasa (neural Nitric Oxide Synthase, nNOS,) (Budai et al., 1995), la cinasa dependiente de Ca^{2+}/calmodulina II (CaMKII) (Malinow et al., 1989) y las cinasas reguladas por señales extracelulares (Extracellular signal-Regulated Kinase, ERK) (Ji et al., 1999). Todo ello se traduce en cambios tanto postranscripcionales como de expresión génica que modifican la apertura de receptores NMDA y AMPA o la activación de proteínas que promueven cambios en el tráfico de receptores a la membrana plasmática. Por otra parte, se pueden producir aumentos en la expresión de c-Fos o de la ciclooxigenasa de tipo 2 (COX-2), además de atenuarse la función postsináptica inhibidora glicinérgica y gabaérgica (Zeinhoffer, 2008; Voscopoulos y Lema, 2010), como se comentará más adelante. Esta situación de hiperexcitabilidad neuronal da lugar a un estado de hipersensibilidad clave en los estados de dolor crónico (Zeilhofer, 2008; Voscopoulos y Lema, 2010), que se conoce como *sensibilización central* (D'Mello y Dickenson, 2008). Este fenómeno contribuye también al establecimiento de *hiperalgesia secundaria*, es decir, a la expansión de las sensaciones nociceptivas a zonas no dañadas situadas alrededor de la lesión original ya que las fibras aferentes Aβ que en condiciones no patológicas no son capaces de estimular neuronas convergentes espinales, sí podrían hacerlo ahora dando lugar al fenómeno conocido como alodinia, definida por la IASP como una respuesta nociceptiva desencadenada tras la aplicación de un estímulo no nociceptivo (Loeser y Trade, 2008; Basbaum et al., 2009).

Al margen de los aminoácidos, en la sinapsis nociceptiva espinal hay muy diversos péptidos implicados, como el CGRP, el neuropéptido Y, la galanina o la sustancia P. Estos no desempeñan un papel tan inmediato en la transmisión de señales dolorosas. La sustancia P, que actúa a través de receptores NK1 acoplados a proteínas G_q, es el más estudiado y, como la mayoría de los otros péptidos, produce una despolarización más lenta que la ocasionada a través de la activación de canales (Giordano, 2005).

Además de los aspectos relacionados con la neurotrasmisión excitadora, la otra parte importante de la

transmisión nociceptiva espinal es la relacionada con la inhibición. Aunque en ella participan aminoácidos como la glicina y el ácido gamma-aminobutírico (GABA), desde el punto de vista de la farmacología la acción más importante es la de los opioides. Éstos previnenen la excitabilidad y propagación de potenciales de acción al estimular los receptores opioides de tipo mu, delta y kappa (Grace et al., 2015). Su efecto puede tener lugar a nivel presináptico mediante la supresión de la entrada de Ca^{+2} y la consiguiente inhibición en la liberación de neurotransmisores, o a nivel postsináptico, provocando la hiperpolarización de la neurona a través de la inhibición de la apertura de canales de Ca^{+2} y la apertura de canales de K^{+} (Zöllner y Stein, 2007).

Otros transmisores clave en la inhibición de estímulos nociceptivos junto con los opioides serian los cannabinoides, que actúan sobre los receptores cannabinoides tipo 1 y 2 (CB1 y CB2), presentes tanto en la periferia como en la médula espinal y en diferentes áreas cerebrales (Guindon y Hohmann, 2009; Curto-Reyes et al., 2010; Yaksh et al., 2015; Woodhams et al., 2015). La estimulación de estos receptores favorece su acoplamiento a proteinas G_i con la consiguiente inhibición de la adenilato ciclasa, que promueve la hiperpolarización y reducción de la liberación de neurotransmisores en el espacio sináptico suprimiendo de esta forma la transmisión de estímulos nociceptivos (Guindon y Hohmann, 2009; Davis, 2014; Woodhams et al., 2015).

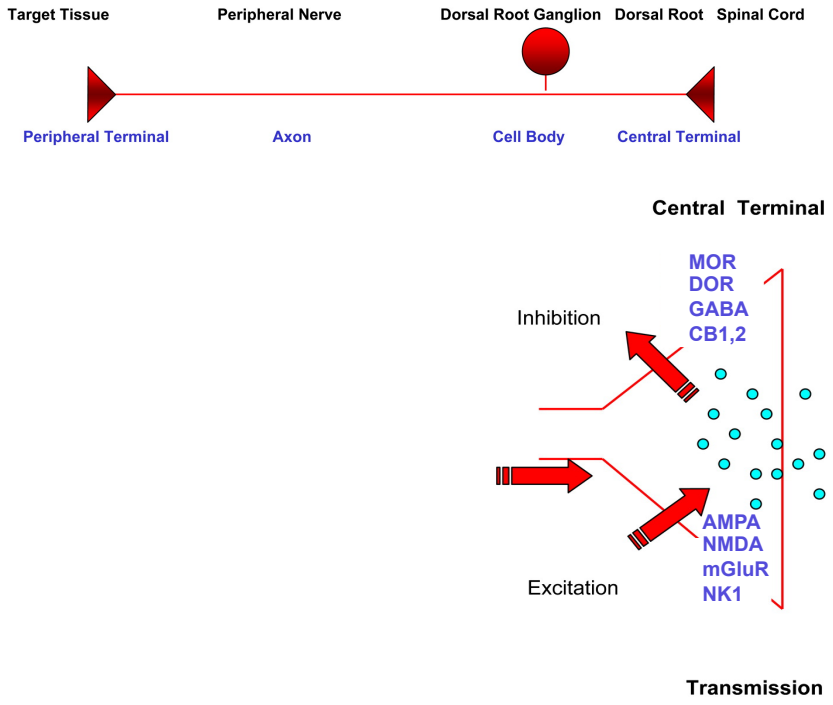

Figura 2. En la parte superior, representación esquemática de los componentes del nociceptor .En la parte inferior, receptores presentes en el terminal central del nociceptor. Modificada de Woolf y Ma, 2007.

1.1.2.1. Participación de las células gliales en la transmisión nociceptiva espinal

La glía está formada por un conjunto de células inmunes residentes en el SNC cuyas funciones más relevantes estaban clásicamente relegadas al sustento y la defensa de la población neuronal. En la última década, se ha dirigido una gran atención al estudio de las funciones de la glía en médula espinal, concretamente de la microglía y los astrocitos, mostrando la función crucial de la microglía tanto en condiciones fisiológicas (Salter y Beggs, 2014; Arnoux et al., 2015) como patológicas, especialmente en el campo del dolor (Basbaum et al., 2009; Ji et al., 2013; Alfonso Romero-Sandoval y Sweitzer, 2015; Tsuda, 2016).

La células gliales se activan tras diversos tipos de daños y lesiones que conducen a estados dolorosos, como pueden ser lesiones espinales, neuropatías periféricas, diversos estados inflamatorios o cáncer óseo (Honoré et al., 2000; Hains et al., 2006; Ji et al., 2013; Pevida et al., 2013; Pevida et al., 2014a). Aunque la activación de la astroglía en este contexto resulta mucho más general y evidente, existen datos contradictorios relativos a la activación de la microglía en algunos modelos de dolor, como en algunos modelos de cáncer óseo (Honoré et al., 2000; Hald et al., 2009; Ji et al., 2013; Pevida et al., 2014a; Ducorneau et al., 2014) o neuropatía por quimioterápicos (Zheng et al., 2011; Zhang et al., 2012a; Pevida et al., 2013). Una vez activadas, las células gliales presentan cambios morfológicos (hipertrofia y aumento de la densidad celular) y sobreexpresión de proteínas marcadores gliales, como Iba-1 (ionized calcium binding adaptor molecule-1) en el caso de la microglía y GFAP (glial fibrillary acidic protein) en el de los astrocitos. Además, se produce la regulación al alza de receptores expresados en las células gliales como son los purinérgicos, los de tipo Toll (Toll-Like Receptor, TLR) o receptores de quimiocinas, como CX3CR1, CXCR3 o CCR2 (Beggs et al., 2012; Basbaum et al., 2009; Zhou et al., 2010; Ji et al., 2013; Guan et al., 2015). En respuesta a la activación de estos receptores, se activan distintas cascadas de señalización intracelular, como la fosforilación de la proteína cinasa activada por mitógenos (MAPK), dando lugar a la síntesis y liberación de mediadores gliales, entre otros, ATP, citocinas proinflamatorias, quimiocinas, proteasas o prostaglandinas. Estos mediadores actúan sobre receptores presentes en las neuronas espinales y en las propias células gliales, participando en la transmisión nociceptiva a través de la sensibilización central, siendo su activación crucial para el desarrollo de alodinia (Beggs et al., 2012; Lolignier et al., 2015; Svensson y Brodin, 2010; Ji et al., 2013; Yaksh et al., 2015).

1.1.4. Estructuras supraespinales implicadas en la transmisión nociceptiva

La mayor parte de la información que envían las neuronas nociceptivas a áreas cerebrales proviene de las láminas I y V y en menor medida de la II de la médula espinal (Todd, 2010). Los axones de estas neuronas forman parte de múltiples proyecciones, entre las que destacan los **tractos espinotalámico lateral**, que envía información sensorial y discriminativa al tálamo, y el tracto **espinoreticular**, que envía la información al tronco del encéfalo donde se produce la integración de dolores peor localizados. Desde el tálamo y el tronco encefálico la información alcanza las áreas somatosensoriales de la corteza cerebral (Basbaum et al., 2009; Braz et al., 2014)

Numerosos estudios describen la implicación de diferentes estructuras cerebrales en el procesamiento y percepción del dolor mediante el uso de nuevas técnicas de imagen, como la tomografía por emisión de positrones (PET) y la resonancia magnética funcional (fMRI) (Davis y Moayedi, 2013; Baliki y Apkarian, 2015). Estas técnicas han permitido "mapear" la compleja red de estructuras implicadas en la experiencia dolorosa, a la que se denomina "matriz del dolor" (Tracey y Johns, 2010; Baliki y Apkarian, 2015), que probablemente refleja la complejidad, subjetividad y multifactorialidad de la experiencia dolorosa.

A pesar de dicha complejidad, esta incluye, de manera esquemática, dos componentes neuroanatómicos: por una parte, las áreas somatosensoriales primarias y secundarias, tálamo y partes posteriores de la ínsula, responsables de los aspecto discriminativo-sensorial y por otra, el córtex cingulado anterior, córtex prefrontal y partes anteriores de la ínsula, implicados principalmente en los aspectos afectivos y cognitivos, la integración sensorial, memoria y toma de decisiones en el relación con el dolor (Fuchs et al., 2014; Cohen y Mao, 2014; Baliki y Apkarian, 2015).

1.2. LAS QUIMIOCINAS

Descubiertas en la década de los 80, las quimiocinas ("citocinas quimiotácticas") constituyen una familia extensa de proteínas con un peso molecular relativamente bajo (7-14 kD). Hasta la fecha han sido descritas al menos 50 quimiocinas, que se clasifican en cuatro familias en función de la presencia y disposición de residuos de cisteína en su región amino-terminal: las de tipo CC presentan dos residuos de cisteína adyacentes, las CXC un aminoácido que separa las dos cisteínas, las CX3C, cuyas dos primeras cisteínas están separadas por 3 aminoácidos, y las XC con un sólo residuo de cisteína en el extremo amino-terminal (Bachelerie et al., 2014). Aunque la mayoría de quimiocinas tienen un nombre inicial, asignado cuando se descubrieron, su nomenclatura se ha sistematizado añadiendo al código anterior letra L, de ligando, y un número. Por ejemplo, la CCL2 corresponde a la quimiocina inicialmente llamada MCP-1 (monocyte chemoattractant protein-1) y la bautizada como MIP-1α (macrophage inflammatory protein -1- alpha) corresponde a la CCL3 en la denominación sistemática. Pese a que la denominación sistemática, que se empleará a lo largo de la tesis, va ganando terreno a la original, existen aún algunas quimiocinas cuyo nombre inicial sigue siendo ampliamente utilizado, como es el caso de la fractalquina (CX3CL1) (White y Wilson, 2008).

Las quimiocinas ejercen sus efectos sobre receptores que se han clasificado con la misma clave inicial, en función del tipo de quimiocinas que actúa sobre ellos, pero seguidos de la letra R, de receptor. Se reconoce la existencia de 11 tipos de receptores CC (CCR1-CCR11), 7 receptores CXC (CXCR1-CXCR7), 1 receptor CX3C (CX3CR1) y 1 receptor XC (XCR1).La relación entre las quimiocinas y sus receptores es compleja dado que no suele existir selectividad entre una quimiocina y un receptor, sino que varios receptores pueden ser estimulados por distintas quimiocinas y muchas de ellas pueden, a su vez, unirse a diferentes receptores (Charo y Ransohoff, 2006; Bachelerie et al., 2014; Bachelerie et al., 2016). Entre las escasas excepciones a esta regla cabe destacar la unión exclusiva del CX3CL1 (fractalquina) al receptor CX3CR1 como la más conocida y estudiada. Otros ejemplos los constituyen la unión de CCL20 al receptor CCR6 y la de CXCL16 al receptor CXCR6 (D´Haese et al., 2010; Bachelerie et al., 2016).

Los receptores de quimiocinas se expresan en diferentes líneas celulares como mastocitos, células dendríticas y otras células no hematopoyéticas, neuronas, microglía, astroglía, células de Schwann y leucocitos, especialmente en monocitos/macrófagos y neutrófilos o basófilos (Ellis y Bennet, 2013;

Bachelerie et al., 2014). La transducción de la mayoría de estos receptores está acoplada a proteínas G, principalmente G_q (Kuang et al., 1996), aunque existe un grupo de "receptores atípicos" cuyo sistema de señalización es independiente de proteínas G (Cancellieri et al., 2013; Vacchini et al., 2016).

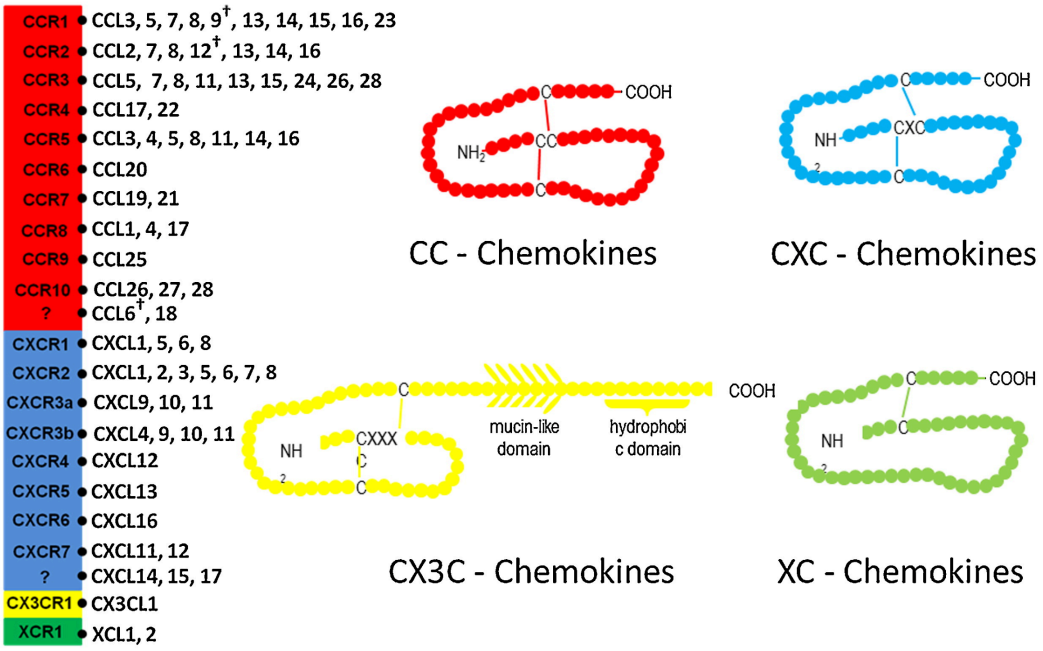

Figura 3. Clasificación de las quimiocinas en función de la posición de sus residuos de cisteína en el extremo N-terminal y representación de la unión de distintas quimiocinas a distintos receptores (Dawes y McMahon, 2013).

Las quimiocinas inflamatorias desempeñan un papel crucial en la respuesta inflamatoria al orquestar la extravasación de leucocitos de la sangre hacia el tejido dañado. Así, las quimiocinas de la serie CC atraen a linfocitos, monocitos y neutrófilos a los lugares en los que se ha producido el daño. Una de las más ampliamente estudiadas, la CCL2 actúa como factor quimiotáctico y activador de monocitos y es igualmente un potente agente quimiotáctico de células dendríticas, basófilos y linfocitos T de memoria (Gerszten et al., 1999; Deshmane et al., 2009; Bachelerie et al., 2014). Otras quimiocinas de la misma familia bastante caracterizadas son las quimiocinas CCL3 y la CCL5 o RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) que desempeñan un papel crucial en la regulación de la migración endotelial de los monocitos, células dendríticas y NK (Maurer y von Stebut, 2004; Griffith et al., 2014).

Las quimiocinas de la familia CXC, en cambio, atraen principalmente polimorfonucleares (PMN). La quimiocina CXCL8 (o interleucina-8, IL-8) activa además monocitos (Gerszten et al., 1999). Asimismo, se ha descrito recientemente que el receptor CXCR3 se encuentra sobreexpresado en linfocitos T CD4+, y

facilita la diferenciación de estas células en linfocitos Th1 (Groom et al., 2012).Por su parte la CXCL16 media la interacción entre células presentadoras de antígenos y linfocitos T (Charo y Ransohoff, 2006) y la XCL1 y la XCL2, que conforman la familia XC se denominan también linfotactinas por ser el único grupo de quimiocinas que solamente atraen linfocitos (Kennedy et al., 1995).

A nivel clínico, se ha descrito la participación de varias quimiocinas en la patogénesis de muchos procesos crónicos, como la artritis reumatoide (Turner et al., 2014; Szekanecz y Koch, 2016), entre las que destacan por su relevancia las quimiocinas de la familia CC, CCL2, CCL3 y CCL5, (Lebre et al., 2011; Szekanecz et al., 2011; Tak et al., 2013; White et al., 2013; Szekanecz y Koch, 2016) y de la familia CXC, CXCL9 y CXCL10 (Lee et al., 2013; Kotrych et al., 2015). Además, también se ha demostrado la participación de las quimiocinas en el desarrollo cerebral, la curación de heridas, en enfermedades cardiovasculares (Charo y Ransohoff, 2006; Turner et al., 2014; Roy et al., 2014; Satish et al., 2015; Sozzani et al., 2015), en procesos neurológicos que se acompañan de reacciones inmunitarias como la esclerosis múltiple (Ubogu et al., 2006) .

Al ser un aspecto central de nuestro trabajo, le dedicaremos una sección aparte a su papel en el mantenimiento y desarrollo de estados dolorosos, un aspecto cuyo conocimiento ha progresado mucho durante los últimos años (Zhang y Oppenheim, 2005; Ubogu et al., 2006; Gao y Ji, 2010, Dawes y McMahon., 2013; Zhou et al., 2015; Knerlich-Lukoschus y Held-Feindt, 2015).

1.2.1 Papel de las quimiocinas en la transmisión nociceptiva

El mayor conocimiento de la participación de las quimiocinas en la modulación de la transmisión nociceptiva ha servido para plantear la posibilidad de actuar sobre ellas como una diana potencialmente útil en el tratamiento del dolor, especialmente crónico. Hasta ahora se ha demostrado la implicación en el dolor de numerosas quimiocinas entre las que destacan algunas que son miembros de la familia CC, como la CCL2, CCL3, CCL5 o CCL7 o de la CXC (CXCL1, CXCL10, CXCL12…) además de la fractalquina (Dawes Y McMahon, 2013; Biber y Bodekke, 2014; Zhou et al., 2015; Knerlich-Lukoschus y Held-Feindt, 2015; Guo y Gao, 2015; Li et al., 2016). Aunque no será una cuestión tratada en nuestro trabajo, otro aspecto destacable es la relación existente entre las quimiocinas y el sistema opioide que se basa en la coexpresión de receptores opioides y diversos tipos de receptores de quimiocinas, y cuya estimulación

conjunta parece eficaz para prevenir la tolerancia e hiperalgesia derivadas de los opioides (Zhang et al., 2004; Mélik Parsadaniantz et al., 2015; Tarakanov y Fuxe, 2015).

La expresión de receptores de quimiocinas se ha demostrado tanto en neuronas de los DRG (Abbadie et al., 2005; Biber y Boddeke, 2014) como de la médula espinal (Qin et al., 2005; Van Steenwinckel et al., 2011) o en otros tipos celulares relacionados con procesos de dolor crónico como las células de Schwan o las células de microglía y de astroglía (Gao y Ji, 2010; Ji et al., 2013; Knerlich-Lukoschus y Held-Feindt, 2015; Tsuda, 2016). Por otra parte, su participación en procesos nociceptivos viene avalada *in vivo* por la reacción hiperalgésica que ocurre tras la administración periférica o central de diversas quimiocinas. En este sentido, el trabajo inicial fue el referido a la CXCL8, anteriormente llamada IL-8 (Cunha et al., 1991) y ha sido seguido por otros donde se muestran los efectos nociceptivos directos de CCL5 tras su administración intraplantar (Oh et al., 2001) así como de CCL2, CCL3, CCL4, CXCL1, CXCL12 o CX3CL1 (Eijkelkamp et al., 2010; Saika et al., 2012; Dawes y McMahon, 2013; Pevida et al., 2014a; 2014b). A nivel periférico, varias quimiocinas desencadenan mecanismos celulares responsables de la excitación o sensibilización neuronal como pueden ser la sensibilización de receptores de la familia TRP, como el TRPV1 y el TRPA1 (Zhang et al., 2005; Jung et al., 2008), la regulación al alza de la excitabilidad de canales de sodio (Nav1.8), de calcio o el aumento de la permeabilidad a ambos cationes (Bhangoo et al., 2007; Belkouch et al., 2011; Dawes y McMahon, 2013). Asimismo, también se ha demostrado que la administración de quimiocinas como la CCL2, CCL3 o CX3CL1 a nivel espinal o CCL5 en la sustancia gris periacueductal provocan síntomas nociceptivos (Milligan et al., 2004; Benamar et al., 2008; Kiguchi et al., 2010a; Baamonde et al., 2011).

La participación de las quimiocinas en los procesos nociceptivos ha sido demostrada en diferentes modelos de dolor crónico, especialmente de **dolor neuropático** (White y Wilson, 2008; Zhang et al., 2012b; Liou et al., 2013; Biber y Bodekke, 2014; Knerlich-Lukoskus y Held-Feind, 2015; Tsuda, 2016) en el que resulta de especial importancia el papel jugado por quimiocinas liberadas desde células inflamatorias, principalmente macrófagos o neutrófilos (Ellis y Bennett, 2013; Liou et al., 2013). De entre todas, la mejor caracterizada tanto a nivel periférico como central es la CCL2 cuyos efectos están mediados por receptores CCR2. Así, un daño nervioso provoca un aumento en la liberación de CCL2 (Zhang et al., 2007; Jung et al., 2009; Jeon et al., 2011) o la sobreexpresión de CCR2 (Abbadie et al., 2003; Zhang et al., 2012b) en neuronas de DRG, de modo que las respuestas nociceptivas provocadas por una lesión neuropática se reducen en ratones carentes de CCR2 (Abbadie et al., 2003). Otras

quimiocinas cuya participación en las respuestas neuropáticas ha sido probada son la CCL3 a través del receptor CCR1 (Kiguchi et al., 2010a) o CCR5 (Kiguchi et al., 2010b; Matsushita et al., 2014), la CCL5 a través del CCR1 (Pevida et al.,2014b), la CCL4 a través de CCR5 (Saika et al., 2012), la fractalquina a través del CX3CR1 (Zhuang et al., 2007; Li et al., 2016) y, más recientemente, la CXCL12 actuando en receptores CXCR4 (Luo et al., 2016).

Puesto que el trabajo recogido en esta tesis se ha realizado en modelos experimentales de dolor inflamatorio y neoplásico, se describirán con mayor detalle los datos relativos a la participación de las quimiocinas en estas situaciones (secciones 1.2.1.1. y 1.3.2). En cada una de esas secciones, se comentarán asimismo algunos aspectos generales relacionados con los principales modelos de dolor inflamatorio y neoplásico utilizados en el laboratorio.

1.2.1.1. Implicación de las quimiocinas en el dolor inflamatorio

La inflamación es un proceso por el cual el organismo responde a un daño tisular o infección y que implica tanto el reclutamiento de células inmunes como la liberación de mediadores para eliminar el estímulo nocivo y promover la regeneración tisular. En los primeros momentos tras la agresión, se produce una rápida activación y proliferación de células inmunes residentes, como neutrófilos, eosinófilos o basófilos además de macrófagos. Estas células inmunes liberan mediadores que conforman la denominada “sopa inflamatoria”, en la que se incluyen citocinas, neurotrofinas, aminas, quimiocinas, purinas o prostaglandinas. Todas estas moléculas pueden actuar como sensibilizadores de nociceptores (Rittner et al., 2005; Ellis y Bennet, 2013; de Miguel et al., 2014) y muchas de ellas mediarán el reclutamiento de células inflamatorias circulantes, como neutrófilos, macrófagos y linfocitos T que pueden contribuir, a su vez, a amplificar la respuesta inflamatoria a través de la liberación de más mediadores (Rittner et al., 2005; Ellis y Bennet, 2013). Además de constituir una importante respuesta ante el daño tisular, la reacción inflamatoria desempeña un papel crucial en otros procesos patológicos como el cáncer, la diabetes, la aterosclerosis o el alzheimer (Charo y Ransohoff, 2006; Turner et al., 2014). Respecto a la nocicepción, los procesos neoplásicos y neuropáticos comparten también la presencia de un importante componente inflamatorio (Marchand et al., 2005; Ellis y Bennet., 2013; McMahon et al., 2015).

Una de las enfermedades inflamatorias crónicas de mayor relevancia en la sociedad es la artritis reumatoide, que se estima que afecta a un 1% de la población mundial (Gibofsky, 2012; Smolen et al.,

2016). Esta patología se caracteriza por presentar inflamación de las articulaciones y dolor en ocasiones intenso, que puede incluso persistir aunque la inflamación se controle (Lee et al., 2011; Wigerblad et al., 2016). Aunque la terapia actual ha supuesto un gran avance en su tratamiento (Smolen et al., 2016), los principales grupos de fármacos utilizados para controlar el dolor asociado son glucocorticoides, antiinflamatorios no esteroideos (AINE), opioides, paracetamol o antidepresivos tricíclicos. Los efectos adversos que presentan estos fármacos junto con la reducida eficacia que presentan en ocasiones (Whittle et al., 2012; Perrot et al., 2013) ponen de manifiesto la necesidad de desarrollar nuevas estrategias analgésicas.

Para la generación de modelos experimentales de dolor inflamatorio, se ha utilizado la inyección en la piel, pata, músculo, articulación y en diversos órganos viscerales de animales de experimentación de diversas sustancias. Entre ellas, algunas producen dolor inflamatorio agudo asociado a un reclutamiento de neutrófilos y otras provocan respuestas más duraderas asociadas a la infiltración de macrófagos, mimetizando así estados inflamatorios agudos y crónicos. Entre las numerosas moléculas utilizadas para producir reacciones inflamatorias como el zimosán, la formalina o el LPS (lipopolisacárido) (revisado en Muley et al., 2016) aunque las sustancias más ampliamente utilizadas para el estudio de las respuestas inflamatorias en animales de experimentación son la carragenina y el coadyuvante de Freund (CFA) (Pircio et al., 1975; Honoré et al., 2000; Menéndez et al., 2004; Baamonde et al., 2007; Gregory et al., 2013; Pan et al., 2014; McCarson, 2015).

En el caso de la carragenina, la respuesta inflamatoria aguda se establece rápidamente, de modo que hay estudios realizados incluso 3 horas tras la inyección (García-Ramallo et al., 2002; Okamoto et al., 2013), aunque en nuestro laboratorio, lo habitual es utilizar los ratones se 6 h tras la administración de carragenina (Baamonde et al., 2004; González-Rodríguez, 2010a; 2010b). En relación con el CFA, también existen numerosos protocolos para establecer el periodo idóneo para realizar estudios tras su administración. Aunque hay numerosos estudios que apoyan que la cronicidad de la reacción inflamatoria está establecida a partir de 3 días tras su administración de CFA (Honoré et al., 2000; Pan et al., 2014; Zhu et al., 2015), en nuestro laboratorio hace años que utilizamos los ratones 7 días después de haber recibido la administración intraplantar de CFA (Menéndez et al., 2004; Baamonde et al., 2004; Baamonde et al., 2007; Menéndez et al., 2006).

Aunque no se dispone de muchos datos relacionados con la posibilidad de producir analgesia bloqueando la acción de quimiocinas en tejidos inflamados, lo cierto es que varias moléculas de esta

familia son capaces, al igual que otros mediadores liberados en los procesos inflamatorios, de sensibilizar los nociceptores periféricos participando en la producción de hiperalgesia.

Respecto a la familia de quimiocinas del grupo CC, la mayoría de los datos conocidos están en relación con la CCL2 y su receptor CCR2. En clínica, se ha relacionado la intensidad del dolor en pacientes con inflamación en la articulación temporomandibular con la expresión de CCL2 (Wang et al., 2009; Ogura et al., 2010; Slade et al., 2011). Experimentalmente, se ha comprobado que la concentración de CCL2 aumenta en tejidos inflamados por carragenina (García-Ramallo et al., 2002; Lucarini et al., 2015) o en los DRG de ratas inflamadas por CFA (Jeon et al., 2008). Aunque los ratones knockout de CCR2 inflamados con CFA muestran respuestas nociceptivas similares a los animales sin esta mutación (Abbadie et al., 2003), se ha descrito que en ratones que sobreexpresan CCL2 tanto la reacción inflamatoria como la hiperalgesia en respuesta al CFA se encuentran aumentadas (Menetski et al., 2007). Asimismo, en un modelo de inflamación visceral, el antagonismo dual de CCR2 y CCR5 ha resultado eficaz para contrarrestar los síntomas nociceptivos derivados de la administración de formalina y carragenina en ratas (Okamoto et al., 2013). Por último, en un modelo de inflamación de la vejiga en ratas, el dolor visceral se relacionó con un aumento de CCL2 y fue inhibido por la administración de un antagonista de CCR2 (Arms et al., 2013).

Aunque se dispone de menos datos, existen también algunas evidencias de la participación de CCL3 y CCL5 en el dolor inflamatorio. Así, la expresión de ambas puede aumentar en respuesta al tratamiento con carragenina y CFA (García-Ramallo et al., 2002; Wang et al., 2006). En estudios comportamentales, se ha observado que tanto el bloqueo de la expresión de CCL3 como su neutralización evitan el desarrollo de dolor en un modelo de prostatitis experimental en ratones. En cuanto al receptor CCR1, que puede ser activado por CCL3 y CCL5, se ha descrito que su bloqueo puede reducir parcialmente el dolor visceral en respuesta al ácido acético y la hiperalgesia mecánica provocada por CFA (Lewis et al., 2014).

Respecto a la familia CXC, existen trabajos que demuestran la participación de las quimiocinas CXCL8 y CXCL1 en la hiperalgesia inflamatoria provocada por carragenina (Cunha et al., 1991; Cunha et al., 2003; Cunha et al., 2005), aunque estos resultados no fueron confirmados al estudiar la inflamación crónica producida por CFA (Brack et al., 2004a; 2004b). Asimismo, hay trabajos previos que describen la eficacia para contrarrestar síntomas hipernociceptivos derivados de procesos inflamatorios de antagonistas

CXCR2, sobre los que actúan la quimiocina CXCL18, o de antagonistas duales CXCR1/CXCR2 (Manjavachi et al., 2010; Lopes et al., 2016).

Por último, respecto a la familia CX3C, se ha descrito una reducción de los síntomas dolorosos en ratones deficientes de CX3CR1 a los que se les había administrado zimosán (Staniland et al., 2010), lo que sugiere la implicación de la fractalquina, hecho que ha sido confirmado en ratas tratadas con carragenina (Souza et al., 2013).

1.3. EL DOLOR NEOPLÁSICO

Se estima que en torno a un 75-90% de personas con cáncer avanzado experimentan síntomas dolorosos (Falk y Dickenson, 2014). Ello puede estar relacionado no sólo con la propia progresión del tumor (destrucción del tejido, daño de aferencias nerviosas, distensión de una zona cerrada rica en nociceptores, componente inflamatorio del tumor...) sino también con el fracaso del tratamiento analgésico y, entre un 20 y un 40% de las veces, con el dolor neuropático causado por el tratamiento con fármacos antineoplásicos.

Entre todas estas situaciones, las metástasis óseas son la causa más frecuente de dolor canceroso moderado o severo (Coleman et al., 2014) y el cáncer de próstata, mama, pulmón y el mieloma los tipos de cáncer que más frecuentemente metastatizan en hueso (Coleman, 2006; Li et al., 2014; Coleman et al., 2014). El dolor generado por los tumores óseos se describe a menudo como continuado y constante, aumenta su intensidad al avanzar el desarrollo de la enfermedad y puede ser relativamente resistente al tratamiento farmacológico (Mercadante, 2013). Durante la progresión y el crecimiento del tumor es frecuente la aparición de dolor irruptivo, definido como una exacerbación del dolor inducida bien por el movimiento o de manera espontánea en episodios cortos e intermitentes de dolor extremo incluso en presencia de tratamiento analgésico (Zeppetella et al., 2011; Davies et al., 2014). En la actualidad, tan sólo la mitad de las personas tratadas con las terapias convencionales experimentan alivio de dicho dolor (Meuser et al., 2001; Davies et al., 2014).

Tratando de dar una orientación global al tratamiento del dolor neoplásicos, en 1990 la Organización Mundial de la Salud, desarrolló una escalera terapéutica basada en la administración gradual de analgésicos en función de la evolución del grado de dolor que sufre el paciente (WHO, 1990; 1996).Los

fármacos incluidos en la escalera analgésica son los AINE, solos o en combinación con opiáceos débiles y, llegado el caso, los opiáceos potentes. Cada uno de estos pasos puede ir acompañado además por el uso de coadyuvantes, como bisfosfonatos, glucocorticoides o psicofármacos (Mercadante y Fulfaro, 2007). Las terapias basadas en esta escalera generan un alivio significativo del dolor oncológico óseo en un buen número de pacientes aunque se considera que aproximadamente entre el 27% (Kane et al., 2015) y el 45% (Meuser et al., 2001; Sloski et al., 2015) permanecen refractarios al tratamiento o padecen efectos adversos en un grado no tolerable. Debe tenerse en cuenta el hecho de que la creciente cronificación de los procesos neoplásicos prolonga los tratamientos y acentúa la aparición de efectos adversos, como la lesión gastrointestinal en el caso de los AINE o la depresión respiratoria, el estreñimiento, o la tolerancia y dependencia en el caso de los opiáceos. Estas limitaciones justifican la búsqueda de alternativas terapéuticas para el control del dolor neoplásico, en la que el desarrollo de modelos experimentales en animales de experimentación juega un papel fundamental.

1.3.1. Modelos experimentales de dolor neoplásico

El primer modelo de dolor neoplásico experimental se estableció en 1999 a través de la inoculación de células NCTC 2472 de fibrosarcoma en la cavidad medular de la parte distal del fémur del ratón (Schwei et al., 1999). Este modelo abrió la posibilidad de realizar estudios sobre los mecanismos del dolor neoplásico en ratones analizando los daños ocasionados en el tejido óseo, el comportamiento nociceptivo y los diferentes cambios neuroquímicos que ocurren a nivel del tumor o en la médula espinal. Durante los años siguientes se publicaron modificaciones de este modelo, en las células se inocularon en distintas localizaciones, como el calcáneo (Wacnik et al., 2001), la tibia (Menéndez et al., 2003) o el húmero (Wacnik et al., 2003; Zhao et al., 2004). Asimismo, se diseñaron nuevos modelos basados en la inoculación de células tumorales de distinto origen en diferentes huesos y tanto en ratones como en ratas. Dada la elevada prevalencia del dolor neoplásico de origen óseo, se utilizaron frecuentemente líneas celulares con capacidad de metastatizar en hueso, como algunas derivadas de cáncer de mama, próstata o pulmón, que poseen gran afinidad por las vértebras, pelvis, costillas y huesos largos (Reale et al., 2001; Coleman, 2006).

En general, la inoculación de las células tumorales se ha hecho en huesos de las extremidades con el objeto de poder valorar alteraciones en la locomoción o la reactividad ante la aplicación de estímulos nociceptivos. Aunque en la actualidad son numerosas las líneas celulares que se han inoculado en

distintos huesos (revisión en Slosky et al., 2015), hasta la fecha, los modelos más ampliamente utilizados en ratones para el estudio de estrategias terapéuticas para contrarrestar el dolor neoplásico óseo son el modelo de osteosarcoma inducido por inoculación de células NCTC 2472 (Sevcik et al., 2005a; Sevcik et al., 2005b; Menéndez et al., 2006; Baamonde et al., 2007; González-Rodríguez et al., 2009; Ghilardi et al., 2010; Curto-Reyes et al., 2010; Pevida et al., 2012; 2014a; 2014b) y melanoma B16-F10 (Curto-Reyes et al., 2010; González-Rodríguez et al., 2012; Pevida et al., 2012; 2014b) y en ratones atímicos, los modelos derivados de la inoculación en el fémur de células de cáncer de próstata AC1 (Halvorson et al., 2005) o por la inoculación intraplantar de células escamosas de carcinoma oral humano (Schmidt et al., 2007). Por otra parte, en ratas existen numerosos trabajos relativos al uso de un modelo de dolor neoplásico óseo por inoculación en la cavidad medular de la tibia y fémur, mayoritariamente, de células de cáncer de mama Walker 256 (Roudier et al., 2006; Hu et al., 2012a; 2012b; Bu et al., 2014; Jin et al., 2015; Ren et al., 2015; Wang et al., 2016) y de próstata AT-3 (Zhang y Lao, 2012).

Otro tipo de modelos de dolor neoplásico pretendían generar tumores localizados en órganos viscerales. El ejemplo más representativo es el de cáncer de páncreas en ratones transgénicos, en los que se evalúan parámetros como la presentación de vocalización y adquisición de una postura encorvada (Lindsay et al., 2005).

Aunque las alteraciones en la reactividad a estímulos dolorosos, la presencia de dolor espontáneo o el menor uso del miembro afectado es bastante general en estos modelos de dolor canceroso, las modificaciones neuroquímicas subyacentes no presentan un patrón único (Honoré et al., 2000). El dolor neoplásico óseo es un síndrome complejo que implica la interacción entre las propias células tumorales, células inflamatorias, nervios periféricos y las células óseas. Así, el dolor neoplásico óseo puede presentar alguna característica de neuropático, al producirse infiltración y compresión de fibras nerviosas. Los primeros estudios inmunohistoquímicos sobre la inervación mostraron una destrucción de fibras aferentes peptidérgicas y no peptidérgicas que inervan el hueso por parte de las células tumorales (Schwei et al., 1999; Peters et al., 2005). Estudios más recientes apuntan que además, debido a la liberación de NGF por las células tumorales, se forman neuromas que coexisten con una reorganización patológica de fibras aferentes sensitivas y simpáticas que inervan el periostio y representan un mecanismo del componente neuropático del dolor asociado a cáncer óseo (Jiménez-Andrade et al., 2011; Falk y Dickenson, 2014; Mantyh, 2014).

En la médula espinal se ha descrito un incremento de expresión de c-Fos, así como del péptido dinorfina, un rasgo compartido con otros modelos de dolor inflamatorio y neuropático (Honoré et al., 2000). Asimismo, se observó una regulación al alza de diversos mediadores hipernociceptivos, como CCL2 (Pevida et al., 2014a), CXCL1 (Xu et al., 2014), IL-1β (Baamonde et al., 2007), y receptores, como CCR2 (Vit et al., 2006; Hu et al., 2013; Ren et al., 2015) o TLR4 (Li et al., 2013) y una disminución de los receptores opioides mu (Yamamoto et al., 2008). En lo que respecta a la respuesta de las células de la glía en las zonas medulares que reciben las aferencias de nociceptivas tumorales, mientras que, como se mencionó anteriormente, la reacción de la microglía resulta menos evidente, dependiendo de diversos factores como el tipo de células inoculadas (Honoré et al., 2000; Ji et al., 2013; Pevida et al., 2014a; Ducourneau et al., 2014). Los datos relativos a la activación de la astroglía parecen más uniformes, ya que ha sido descrita en modelos basados en la inoculación de células de sarcoma, cáncer de colon, mama o próstata en la cavidad medular del fémur o tibia (Schwei et al., 1999; Honoré et al., 2000; Hald et al., 2009; Doré-Savard et al., 2010; Pevida et al., 2014a; Xu et al., 2014).

1.3.2. Implicación de las quimiocinas en el dolor neoplásico experimental

En el contexto de la búsqueda de nuevas dianas para inhibir el dolor tumoral, varios trabajos realizados durante los últimos han evaluado la implicación de diferentes quimiocinas tanto en el desarrollo y progresión del tumor (Izhak et al., 2012; Dairaghi et al., 2012; Guilloton et al., 2012; Aldinucci y Colombatti, 2014) como en el desarrollo y mantenimiento de los síntomas nociceptivos asociados (revisado en Guo y Gao, 2015 y Zhou et al., 2015).

Khasabova et al. (2007) describieron la liberación de CCL2 en un modelo de fibrosarcoma inducido por inoculación de células NCTC 2472 en el calcáneo de ratones. Este trabajo vincula una serie de cambios neuroquímicos, como la regulación al alza de los receptores TRPV1 y de los canales de Ca^{+2} dependientes de voltaje, al aumento de niveles de CCL2. Asimismo, Vit et al. (2006) mostraron un ligero aumento de receptores CCR2 a nivel espinal en este mismo modelo. Trabajos posteriores mostraron, mediante pruebas de comportamiento, la implicación de diversas quimiocinas en el desarrollo y establecimiento del dolor neoplásico experimental. Hasta la fecha y durante la realización de esta tesis, se ha demostrado la participación de diversas quimiocinas en este contexto de dolor, recogidas en la Tabla 1.

Tabla 1 Modificación de las respuestas nociceptivas obtenidas en modelos de dolor neoplásico experimental mediante la inhibición del efecto de diversas quimiocinas.

Quimiocina	Receptor	Tratamiento	Linea celular	Nivel	Parámetro estudiado			Referencia
					Hiperalgesia térmica	Alodinia mecánica	Hiperalgesia mecánica	
CCL2	Anticuerpo anti-CCL2		Walker 256 (mama)	Espinal		↓		Hu et al., 2012a
	CCR2	Antagonista CCR2	NCTC 2472 (osteosarcoma)	Periferia	↓	↔	↓	Pevida et al., 2012
		Anticuerpo anti-CCL2		Espinal	↓			Pevida et al., 2014a
	CCR2	Antagonista CCR2	Walker 256 (mama)	Espinal	↓	↓		Ren et al., 2015
CCL5	**CCR1**	Antagonista CCR1 Anticuerpo anti-CCL5	NCTC 2472 (osteosarcoma)	Periferia	↓	↔	↓	Pevida et al., 2014b
	Anticuerpo anti-CCL5		Walker 256 (mama)	Espinal			↓	Hang et al., 2013
CXCL1	Anticuerpo anti-CXCL1		Walker 256 (mama)	Espinal	↓	↓		Wang et al., 2016
	CXCR2	Anticuerpo anti-CXCL1 Antagonista CXCR2	RM-1 (próstata)	Espinal	↓	↓		Xu et al., 2014
CXCL10	**CXCR3**	Anticuerpo anti-CXCL10 Antagonista CXCR3	Walker 256 (mama)	Espinal		↓		Bu et al., 2014
		Antagonista CXCR3				↓		Guan et al., 2015
CXCL12	**CXCR4**	Antagonista CXCR4 Anticuerpo anti-CXCL12	Walker 256 (mama)	Espinal	↓	↓		Shen et al., 2014; Hu et al., 2015
	Anticuerpo anti-CXCL12					↓		Hang et al., 2016
	CX3CR1	Anticuerpo anti-CX3CR1	Walker 256 (mama)	Espinal	↓	↓		Yin et al., 2010; Hu et al., 2012b

Casillas en blanco, parámetro no estudiado. ↓ Disminución. ↔ No modificación.

Respecto a las quimiocinas de la serie CC, se ha descrito su implicación en el establecimiento de la hiperalgesia derivada de la inoculación de distintas células tumorales, especialmente células de fibrosarcoma NCTC 2472 y de cáncer de mama Walker 256 en ratas. A nivel periférico, se ha descrito la liberación de CCL2 y CCL5 tanto *in vitro*, en el medio de cultivo (Schiller et al., 2009; Pevida et al., 2014b) como *in vivo* en la pata inoculada con células de fibrosarcoma NCTC 2472, y se ha demostrado la implicación de la quimiocina CCL2 y su receptor, CCR2 (Pevida et al., 2012) y de la quimiocina CCL5 a través del receptor CCR1 (Pevida et al., 2014b) en el modelo de dolor neoplásico derivado de la inoculación intratibial de células de fibrosarcoma NCTC 2472. Asimismo, a nivel espinal se ha mostrado

un aumento de CCL2 (Hu et al., 2012a; Pevida et al., 2014a; Ren et al., 2015; Jin et al., 2015), que a través del receptor CCR2 estimula la astroglía (Pevida et al., 2014b) y la microglía (Hu et al., 2013; Pevida et al., 2014b; Jin et al., 2015). Otro trabajo ha relacionado el efecto analgésico derivado de la inhibición de CCR2 con una desregulación en la expresión espinal de la subunidad NR2B del receptor de NMDA y de la nNOS (Ren et al., 2015)

En relación con las quimiocinas de la familia CXC, otros trabajos, desarrollados en un modelo de dolor neoplásico derivado de la inoculación de células de cáncer de mama Walker 256 en ratas, muestran la contribución CXCL12/CXCR4 al mantenimiento y desarrollo del dolor neoplásico óseo a través de la sensibilización de neuronas y la activación de astrocitos y microglía (Shen et al., 2014; Liu et al., 2014; Hu et al., 2015). Además, se indica que la vía MAPK podría estar asociada a la neuroinflamación en situaciones de cáncer mediada por CXCL12/CXCR4 (Hu et al., 2015).

En cuanto al sistema CXCL10/CXCR3, Bu et al. (2014) y Guan et al. (2015) han mostrado cómo el bloqueo de este eje, bien mediante la administración intratecal del anticuerpo anti-CXCL10 o bien mediante el antagonismo del receptor, inhibe la activación de la microglía, sugiriendo que la función de esta quimiocina en el desarrollo del dolor neoplásico está mediada por la activación de esta célula glial. Además también describen que la activación del receptor CXCR3 en la médula podría igualmente estar mediado a través de las activación de la vía de la proteina cinasa B (Akt) y ERK. Por otra parte, se ha descrito que la inhibición de la quimiocina CXCL10 y/o su receptor CXCR3 podrían potenciar la analgesia opioide en animales con dolor neoplásico experimental, habiéndose propuesto esta estrategia como terapia adyuvante para paliar el dolor neoplásico (Ye et al., 2014).

2.Justificación y objetivos

Las quimiocinas son péptidos de bajo peso molecular (7-14kD) cuya función más conocida se relaciona con la respuesta inmune y particularmente con la quimiotaxis de granulocitos, macrófagos o linfocitos (Bachelerie et al., 2014; Griffith et al., 2014). Junto a este papel, el reconocimiento de su participación en la amplificación de las señales nociceptivas que ocurre en diferentes estados patológicos es un hecho cada vez mejor documentado (Biber y Boddeke, 2014; Guo y Gao, 2015; Knerlich-Lukoschus y Held-Feindt, 2015).

Actualmente, se reconoce la participación en los procesos nociceptivos de unas 30 quimiocinas (Dawes y McMahon, 2013). Así, se dispone de bastante información sobre la función hiperalgésica de quimiocinas como CCL2, CCL3, CXCL1 o CX3CL1, siendo probablemente la CCL2 (Monocyte Chemoattractant Protein-1, MCP-1) la quimiocina cuya función nociceptiva está mejor documentada. En general, estos péptidos amplifican respuestas nociceptivas a través del estímulo de receptores expresados tanto en los nociceptores como a nivel de la médula espinal o en estructuras superiores como la sustancia gris periacueductal (Benamar et al., 2008; Kiguchi et al., 2012; Dawes y McMahon, 2013). De acuerdo con ello, su presencia en la proximidad de los nociceptores (Qin et al., 2005; Dawes y McMahon, 2013) o a nivel central (Van Steenwinckel et al., 2011) puede producir hiperalgesia o alodinia en animales de experimentación. Puesto que en varios procesos patológicos que cursan con dolor se observa un aumento en la concentración de determinadas quimiocinas, se ha propuesto que la administración de antagonistas de sus receptores o de anticuerpos que las neutralicen puede ser un abordaje eficaz para aliviar dicho dolor.

Una buena parte de la información disponible en relación con la participación de las quimiocinas en procesos dolorosos se ha obtenido inicialmente en modelos de dolor neuropático provocados por lesión nerviosa (Kiguchi et al., 2012), daño espinal (Knerlich-Lukoschus y Held-Feindt, 2015) o tratamiento con antitumorales neurotóxicos (Pevida et al., 2013; Li et al., 2016). En contrapartida, su implicación en la instauración del dolor que acompaña a otros procesos, como la inflamación y el cáncer, ha sido estudiada en menor medida.

En relación con la inflamación y la CCL2, existen datos clínicos procedentes de pacientes con inflamación temporomandibular que correlacionan el aumento en la

expresión de dicha quimiocina y la intensidad del dolor (Ogura et al., 2010; Slate et al., 2011). A nivel experimental, los resultados publicados son menos concluyentes. Así, aunque se ha demostrado que los ratones carentes del receptor CCR2, sobre el que actúa la CCL2, desarrollan hiperalgesia y alodinia inflamatoria con normalidad, también se ha descrito que la sobreexpresión de CCL2 provoca el aumento de la hiperalgesia inflamatoria (Menetski et al., 2007) o que el dolor visceral secundario a la inflamación vesical puede estar relacionado con un aumento de la expresión de CCL2 (Arms et al., 2013). Dentro de la serie de quimiocinas CC, también se han relacionado con el dolor inflamatorio los receptores CCR1, sobre los que pueden actuar la CCL3 y la CCL5 y, de acuerdo con ello, se ha descrito que la administración de un antagonista de CCR1 reduce parcialmente la hiperalgesia mecánica de ratones inflamados (Lewis et al., 2014). El escaso conocimiento de la participación de las quimiocinas en el dolor inflamatorio nos impulsó a evaluar la implicación de los receptores CCR1 y CCR2 y las quimiocinas capaces de activarlos en dos modelos de laboratorio habitualmente utilizados para el estudio de la inflamación aguda y crónica en ratones.

En relación con el dolor neoplásico, hay un buen número de trabajos que estudian la función de diferentes quimiocinas (revisado en Zhou et al., 2015). Aunque también en este caso hay datos correspondientes a otras moléculas de la serie CC como CCL5 (Hang et al.,2013; Pevida et al., 2014b), la CCL2 es la molécula sobre la que se dispone de más información (Hu et al., 2012; Pevida et al., 2012; 2014a; Jin et al., 2015; Ren et al., 2015). En buena medida, ello se debe a que existen estudios en pacientes con cáncer en los que se observa un aumento de la expresión de CCL2 en algunos tipos de tumores sólidos (Guilloton et al., 2012; Lim et al., 2016) como el de próstata (Zhang et al., 2010a; 2010b; Izumi et al., 2015; Tsaur et al., 2015), un tipo de cáncer muy prevalente y que cursa frecuentemente con dolor cuando aparecen metástasis óseas (Coleman et al., 2014). El interés de estudiar la implicación del efecto del eje CCL2/CCR2 en tumores prostáticos se acentúa teniendo en cuenta que existen resultados que demuestran que la CCL2 puede actuar estimulando directamente su crecimiento (Zhang et al., 2010a; 2010b; Ito et al., 2015). Sin embargo, hasta el momento presente, el estudio experimental de la participación de la CCL2 en el dolor tumoral (Pevida et al., 2012; Hu et al., 2012; Pevida et al., 2014a; Ren et al., 2015; Jin et al., 2015) no se ha realizado nunca en un modelo derivado de la presencia de células

de cáncer de próstata en hueso. Esta carencia nos animó a desarrollar un modelo de dolor tumoral de origen prostático con el que poder estudiar la implicación de quimiocinas, y en particular la CCL2, en las respuestas a estímulos nociceptivos.

Por lo tanto, nuestra hipótesis plantea qu**e *la inhibición de la acción de determinadas quimiocinas de la serie CC puede ser eficaz para contrarrestar las respuestas dolorosas en ratones con inflamación aguda y crónica así como con un tumor óseo derivado de la presencia de células tumorales de próstata***.

Para ello se han establecido los siguientes objetivos:

1) Estudiar la implicación de los receptores CCR2 y de su principal ligando endógeno, la quimiocina CCL2, en las respuestas hipernociceptivas medidas en ratones con inflamacion aguda o crónica.

2) Explorar la participación de receptores CCR1 y de sus ligandos endógenos CCL3 y CCL5 en las respuestas hipernociceptivas medidas en ratones con inflamación aguda o crónica.

3) Inocular en la tibia de ratones inmunocompetentes células RM1 derivadas de cáncer de próstata y estudiar en ellos el desarrollo del tumor así como las respuestas nociceptivas derivadas y determinar la posible participación de las quimiocinas CCL2, CCL3 y CCL5.

3. Artículos

3.1. ARTÍCULO 1

Llorián-Salvador M, Pevida M, González-Rodríguez S, Lastra A, Fernández-García MT, Hidalgo A, Menéndez L, Baamonde A. **Analgesic effects evoked by a CCR2 antagonist or an anti-CCL2 antibody in inflamed mice**. Fundam Clin Pharmacol. 2016;30:235-247.

OBJETIVO

Estudiar la implicación de los receptores CCR2 y de su principal ligando endógeno, la quimiocina CCL2, en las respuestas hipernociceptivas medidas en ratones con inflamación aguda producida tras la administración de carragenina o crónica provocada por coadyuvante de Freund (CFA).

MÉTODOS

- Ratones CD-1 inflamados por inyección intraplantar de carragenina 6 h antes o de CFA 1 semana antes.
- ELISA para la cuantificación de los niveles de CCL2 en las patas.
- Tinción hematoxilina-eosina para el análisis histológico del tejido inflamado
- Ensayos inmunohistoquímicos para la localización de CCL2 en tejidos inflamados.
- Placa caliente unilateral para medir la hiperalgesia térmica.
- Prueba de von Frey para medir la alodinia mecánica

RESULTADOS Y CONCLUSIONES

- La administración sistémica aguda del antagonista del receptor CCR2, RS504393, revirtió la hiperalgesia térmica, pero no la alodinia mecánica, que aparece en ratones con inflamación aguda y crónica. La administración aguda de RS504393 no produjo efecto antiinflamatorio.

- La administración intraplantar de pequeñas dosis de RS504393 también antagonizó la hiperalgesia inflamatoria, sugiriendo que el efecto de este fármaco ocurre a través de receptores expresados en el tejido inflamado.

- La expresión de CCL2 está aumentada en los tejidos inflamados y se localiza especialmente en macrófagos. La administración intraplantar de un anticuerpo anti-CCL2 evita la hiperalgesia térmica.

En conclusión, el aumento local de la concentración de CCL2 en respuesta a una inflamación aguda o crónica contribuye al desarrollo de la hiperalgesia inflamatoria pero no de la alodinia mecánica. De acuerdo con ello, la hiperalgesia puede ser inhibida tanto a través del antagonismo del receptor CCR2 como de la neutralización de la CCL2, sin que esta acción antihiperalgésica dependa de un efecto antiinflamatorio paralelo.

doi: 10.1111/fcp.12182

ORIGINAL ARTICLE

Analgesic effects evoked by a CCR2 antagonist or an anti-CCL2 antibody in inflamed mice

María Llorián-Salvador[a], Marta Pevida[a], Sara González-Rodríguez[a], Ana Lastra[a], María-Teresa Fernández-García[b], Agustín Hidalgo[a], Ana Baamonde[a], Luis Menéndez[a]*

[a]*Facultad de Medicina, Laboratorio de Farmacología, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, C/Julián Clavería 6, 33006 Oviedo, Spain*

[b]*Unidad de Histopatología Molecular en Modelos Animales de Cáncer, IUOPA, Universidad de Oviedo, C/Julián Clavería 6, 33006 Oviedo, Spain*

Keywords
carrageenan,
CCL2,
CCR2,
chemokines,
complete Freund's adjuvant,
inflammation,
mouse

Received 24 July 2015;
revised 22 December 2015;
accepted 25 January 2016

*Correspondence and reprints:
luismen@uniovi.es

ABSTRACT

Chemokine CCL2, also known as monocyte chemoattractant protein-1 (MCP-1), is a molecule that in addition to its well-established role in chemotaxis can also act as nociceptor sensitizer. The upregulation of this chemokine in inflamed tissues could suggest its involvement in inflammatory hypernociception. Thus, we have measured CCL2 levels in mice with acute or chronic inflammation due to the intraplantar (i.pl.) injection of carrageenan or complete Freund's adjuvant (CFA), respectively, and we have studied whether inflammatory hyperalgesia or allodynia could be attenuated by blocking CCR2 receptors or neutralizing CCL2 with an anti-CCL2 antibody. A remarkable increase in CCL2 concentration was detected by ELISA in paw homogenates coming from carrageenan- or CFA-inflamed mice, being its expression mainly localized in macrophages, as shown by immunohistochemical assays. The s.c. (0.3–3 mg/kg) or i.pl. (0.3–3 μg) administration of the CCR2 antagonist, RS 504393, dose dependently inhibited thermal hyperalgesia measured in acutely or chronically inflamed mice, whereas s.c. administration of this drug did not reduce inflammatory mechanical allodynia. Furthermore, the inhibition of inflammatory hyperalgesia after the administration of an anti-CCL2 antibody (0.1–1 μg; i.pl.) suggests that CCL2 could be the endogenous chemokine responsible for CCR2-mediated hyperalgesic effects. Besides, the acute administration of the highest antihyperalgesic dose of RS 504393 assayed did not reduce paw tumefaction or modify the presence of inflammatory cells. These results indicate that the blockade of the CCL2/CCR2 system can counteract inflammatory hyperalgesia, being this antinociceptive effect unrelated to a decrease in the inflammatory reaction.

INTRODUCTION

The chemokine CCL2, also referred to as monocyte chemoattractant protein-1 (MCP-1), is a key mediator in inflammatory processes [1] able to induce the migration and infiltration of CCR2-expressing monocytes/macrophages which, in turn, become a major source of this chemokine [2]. Apart from this proinflammatory effect, CCL2 can evoke nociceptor sensitization [3,4] and, accordingly, the administration of CCL2 into the rat paw induces mechanical [4,5] and thermal [4] hyperalgesia. Although the relevance of CCR2 in

nociceptive processing is well established [6], the demonstration of the presence of these receptors in peripheral nerves remains elusive probably due to a low level of expression, the difficulty to detect them in small structures, or a limited distal transport [7]. Nevertheless, CCR2 mRNA expression has been demonstrated in dorsal root ganglia (DRG) cells of rodents [8], and CCR2 protein has been also detected in rat DRG by both immunohistochemical [7,8] and Western blot [9,10] assays. Although it has been suggested that CCR2 expression could be predominant in small-diameter DRG cells probably corresponding to nociceptors [7], other authors describe that their presence is similar in medium and large DRG neurons [11].

The previously reported correlation between CCL2 expression and pain intensity in patients with oral or temporomandibular joint inflammation [12,13] as well as the enhanced presence of this chemokine in joints of patients with rheumatoid arthritis [14] makes attractive to study the possibility of inducing antinociceptive effects in inflammatory settings by blocking CCR2. Initial reports have suggested that these receptors did not play a relevant role in inflammatory pain because knockout mice lacking the CCR2 gene develop inflammatory hyperalgesia and allodynia, whereas neuropathic hypernociception is almost totally suppressed [6]. In contrast, it has been shown more recently that a mixed CCR2/CCR5 antagonist can evoke analgesia in the formalin test in mice and in carrageenan-treated rats [15] and that referred visceral pain related to enhanced CCL2 expression measured in rats with cyclophosphamide-induced bladder inflammation can be inhibited by the administration of a CCR2 antagonist [7]. The greater thermal inflammatory hyperalgesia observed in genetically modified mice that overexpress CCL2 [16] further supports that this chemokine could play a hypernociceptive role in inflammation.

The present experiments were designed to explore the involvement of CCL2 and CCR2 in the hypernociceptive responses measured in mice receiving the intraplantar injection of carrageenan or complete Freund's adjuvant (CFA), two standard inducers of acute or chronic inflammation, respectively. An augmented presence of CCL2 mRNA [17] or CCL2 protein [18] has been detected in inflamed tissues some few hours after the administration of carrageenan, and also, the increased expression of CCL2 mRNA in inflamed tissue [19] or of CCL2 protein in DRG [20] was demonstrated some days after the injection of CFA. In the present study, we have initially checked by ELISA and immunohistochemical methods whether the local presence of CCL2 is increased 6 h after the administration of carrageenan or 7 days after the injection of CFA. Next, we have performed behavioral studies to measure whether the administration of a CCR2 antagonist or an anti-CCL2 antibody can evoke antinociceptive effects in inflamed mice. Finally, we have studied whether the analgesic effect produced by the administration of the CCR2 antagonist could be related to the inhibition of carrageenan- or CFA-evoked inflammation.

MATERIAL AND METHODS

Animals

Swiss male mice (28–32 g) from the Animalario of the Universidad de Oviedo (Reg. 33044 13A) exposed to a light–dark cycle of 12 h and with free access to water and food were used. Experiments were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Spain) and carried out in accordance with the EU Directive 2010/63/EU for the Protection of Laboratory Animals. Mice were employed only once.

Drugs

Acute and chronic inflammation were induced by intraplantar (i.pl.) administration into the right hind paw of 25 μL of lambda carrageenan (type IV Sigma; 2% dissolved in saline) or complete Freund's adjuvant (Sigma), respectively. Control mice received an i.pl. injection of the same volume of saline.

RS 504393 (Tocris), dissolved in 5% DMSO at the highest concentration used and further in saline, or the corresponding solvent was administered subcutaneously (s.c.) under the fur of the neck in a volume of 10 mL/kg or i.pl. in 25 μL. Goat anti-CCL2 antibody (R&D AF-479-NA) or goat serum (Sigma) was diluted in saline and i.pl. administered in 25 μL. The anti-CCL2 antibody shows less than 0.01% cross-reactivity with other recombinant mouse chemokines, as described by the manufacturer.

Enzyme-linked immunosorbent assay (ELISA)

CCL2 was measured in tissue homogenates prepared from the plantar side of the hind paw of inflamed and control mice, as previously described [21]. Tissues were harvested, frozen in liquid nitrogen, and stored at −80 °C. After thawing, paws were homogenized with a drill (Minicraft MB130, Rocky Hill, USA) in a volume of 3 μL/mg of buffer consisting in 0.1 M Tris, 0.15 M

NaCl, 0.5% CTAB (Fluka), and a protease inhibitor (1 tablet/50 mL buffer, Roche Diagnostics). Next, homogenates were centrifuged (15,000 *g*, 15 min, 4 °C), and protein concentration of supernatants was measured by BCA protein assay (Pierce) by using a spectrophotometer (Nanodrop 2000C, ThermoScientific). Measurements were performed with a CCL2/MCP-1 sandwich enzyme-linked immunosorbent assay kit (R&D, DuoSet® mouse, Minneapolis, MN, USA). In brief, 20 μg of homogenates were incubated for 2 h with the coating antibody and, after washing, a 2-h period with a second biotinylated anti-mouse CCL2 was followed by a 20-min incubation period with streptavidin–peroxidase. Color was developed by adding tetramethylbenzidine–H_2O_2 (1 : 1), and the reaction was terminated with 2N H_2SO_4. The color was quantified spectrophotometrically at 450 nm subtracting the readings obtained at 570 nm. Values obtained came from at least 4–6 independent samples performed in duplicate.

Immunohistochemical assays

The plantar side of inflamed or noninflamed paws was fixed with 4% formaldehyde for 12–16 h, and 4-μm cross-sections of paraffin-embedded formalin-fixed blocks were stained with hematoxylin and eosin. Immunohistochemical assays were also performed in paraffin-embedded 4-μm sections coming from paws fixed during 4–6 h with 4% formaldehyde. Sections were deparaffinized and incubated at room temperature with an anti-CCL2 antibody (30 min; 3.6 μg/ml; polyclonal goat anti-mouse, R&D), a polyclonal bridge rabbit anti-goat immunoglobulins/HRP (30 min; 1 : 200; Dako), and a secondary antibody (30 min; Dako Envision+ System-HRP-labeled polymer anti-rabbit). As a positive control of CCL2 staining, slices coming from mesenteric mouse lymph nodes were used. As negative controls, experiments were performed in paw sections in the absence of primary antibody. As a control of the specificity of the anti-CCL2 antibody staining, a saturating concentration (5 : 1) of a fragment of the mouse CCL2 peptide corresponding to the epitope recognized by the antibody (amino acids Gln24-Arg96, Biolegend) was incubated during 1 h at 37°C with the primary antibody. Next, sections were incubated with this antibody–peptide mixture for 30 min, and the immunohistochemical procedure was continued as usually.

For double-immunolabeling assays addressed against CCL2 and CD68, plantar paw skin was fixed in 4% formaldehyde, cryoprotected by immersion for 12–24 h in 15% sucrose and 24 h in 30% sucrose dissolved in PBS 0.01 M at 4 °C; 30-μm-thick sections were obtained using a freezing microtome (Microm HM430) and serially collected on gelatin-coated slides (Super-Frost® Plus, Menzel–Glaser, Braunschweig, Germany). The sections were initially incubated in cold acetone (Prolabo) for 10 min, rinsed during 30 min in PBS (0.01 M), and further incubated at 4 °C overnight in a humid chamber with a mixture of the antibodies against CCL2 (rabbit polyclonal; Torrey-Pines, 1 : 200) and CD68 (clone KP1, mouse monoclonal, Dako, 1 : 15) in 0.01 M PBS. Next, the sections were rinsed during 30 min in PBS (0.01 M) and incubated for 90 min with green Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, 1:250 in 0.01 M PBS) to reveal CCL2 staining, and after 30 min in 0.01 M PBS, they were further incubated for 90 min with Texas Red-conjugated goat anti-mouse IgG (Molecular Probes; 1:100 in 0.01 M PBS). Finally, the sections were washed for 30 min in PBS and mounted. Staining was detected by using a Leica TCS SP2 confocal microscope (Leica Microsystems, Heidelberg GmbH) with a Leica DM IRE2 automatic fluorescence inverted microscope. A 488-nm krypton/argon laser line was used to excite Alexa Fluor 488, and a helium/neon laser line of 543 nm to excite Alexa Fluor 546 or Texas Red. Optical section was acquired with an objective CS.PL APO 63x/1.40 oil and a zoom of 1.46.

Nociceptive assays

Unilateral hot plate test

As previously described [22], mice were gently restrained and the plantar side of the tested paw placed on a hot plate (IITC Life Science) set at 48.6 °C. Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at 2-min intervals, and the mean of two measures was considered. A cutoff of 20 s was established.

Von Frey test

Twenty minutes after habituation, von Frey filaments 2.44, 2.83, 3.22, 3.61, 4.08, 4.56 were applied to the hind paw plantar side of mice [21]. Starting with the 3.61 filament, a positive response (lifting, shaking, or licking of the paw) was followed by the application of the immediate thinner filament or the immediate thicker one if the response was negative. The 50% response threshold was calculated using the following formula: 50% g threshold = $(10^{Xf+\kappa\delta})/10000$, where Xf is the value of the last von Frey filament applied; κ is a correction factor based on the pattern of responses

(from the Dixon's calibration table); and δ is the mean distance in log units between stimuli (here, 0.4).

Measurement of paw tumefaction

Before and at different time points after the injection of the inflammatory agents, the maximal dorsoplantar diameter of the right hind paw was measured by using a caliper in order to quantify the development of macroscopic tumefaction.

Statistical analysis

Mean values and their corresponding standard errors were calculated. The values of CCL2 levels obtained in homogenates coming from inflamed tissue by ELISA were compared with those from saline-treated by the Student's *t*-test, and comparisons over time were made by the Dunnett's *t*-test. Thermal withdrawal latencies were compared by a Student's *t*-test when two groups were considered, while comparisons among different groups were made by ANOVA followed by the Newman–Keuls test or the Dunnett's *t*-test when compared with the solvent group (either in dose dependency or time course studies). Threshold values obtained by the von Frey test in ipsilateral and contralateral paws were compared by the Mann–Whitney *U*-test, and comparisons with the solvent-treated group were made with an initial Kruskal–Wallis analysis followed by the Dunn's test. Paw sections obtained in mice treated with the CCR2 antagonist or solvent were compared by the Student's *t*-test. The criterion for statistical significance was $P < 0.05$.

RESULTS

Increased presence of CCL2 in hind paws inflamed with carrageenan or CFA

CCL2 levels were measured by ELISA in homogenates coming from paws treated with carrageenan 6 h before or with CFA 1 week before. Whereas the concentrations measured in control mice injected with saline either 6 h or 1 week before were, respectively, 8.34 ± 1.31 and 14.23 ± 3.12 pg/mg protein, a dramatic enhancement in CCL2 levels occurred in paws inflamed with carrageenan or CFA (*Figure 1a*). To determine the duration of the increase in CCL2 levels detected in both models, further measurements were performed at different times after the administration of the inflammatory agent. As shown in *Figure 1b*, the elevation in CCL2 concentration measured 6 h after carrageenan administration remains unaltered 1 week after and importantly decreases at week 2. In contrast, about 75% of enhanced CCL2 levels measured 1 week after CFA remain at week 3 and an almost 50% of the initial increase is still present 1 month after CFA administration (*Figure 1c*).

In order to more precisely ascertain the localization of CCL2 in inflamed paws, immunohistochemical studies were designed. As a positive control, CCL2 staining was observed in macrophages and not in lymphocytes in a mouse mesenteric lymph node (*Figure 2a*). In paws inflamed either with carrageenan (*Figure 2d*) or with CFA (*Figure 2e*), CCL2 staining was mainly detected in the cytoplasm of macrophages, identified by

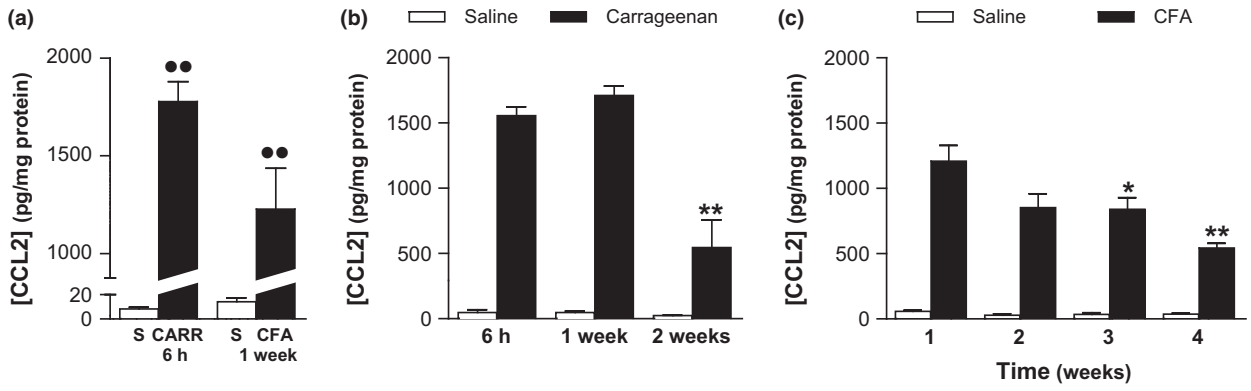

Figure 1 CCL2 concentrations detected by ELISA in homogenates coming from hind paws of mice inflamed with carrageenan (CARR) or complete Freund's adjuvant (CFA). (a) Levels of CCL2 measured 6 h after i.pl. administration of carrageenan and 1 week after the i.pl administration of CFA or saline. (b) CCL2 levels measured 6 h, 1 week, and 2 weeks after the i.pl. administration of carrageenan or saline. (c) CCL2 levels measured 1, 2, 3, and 4 weeks after the i.pl. administration of CFA or saline. Means and corresponding SEM are represented (n = 4–6). ●●$P < 0.01$ compared to solvent-treated groups, Student's *t*-test; *$P < 0.05$, **$P < 0.01$ compared with mice treated 6 h before with carrageenan or 1 week before with CFA, Dunnett's *t*-test.

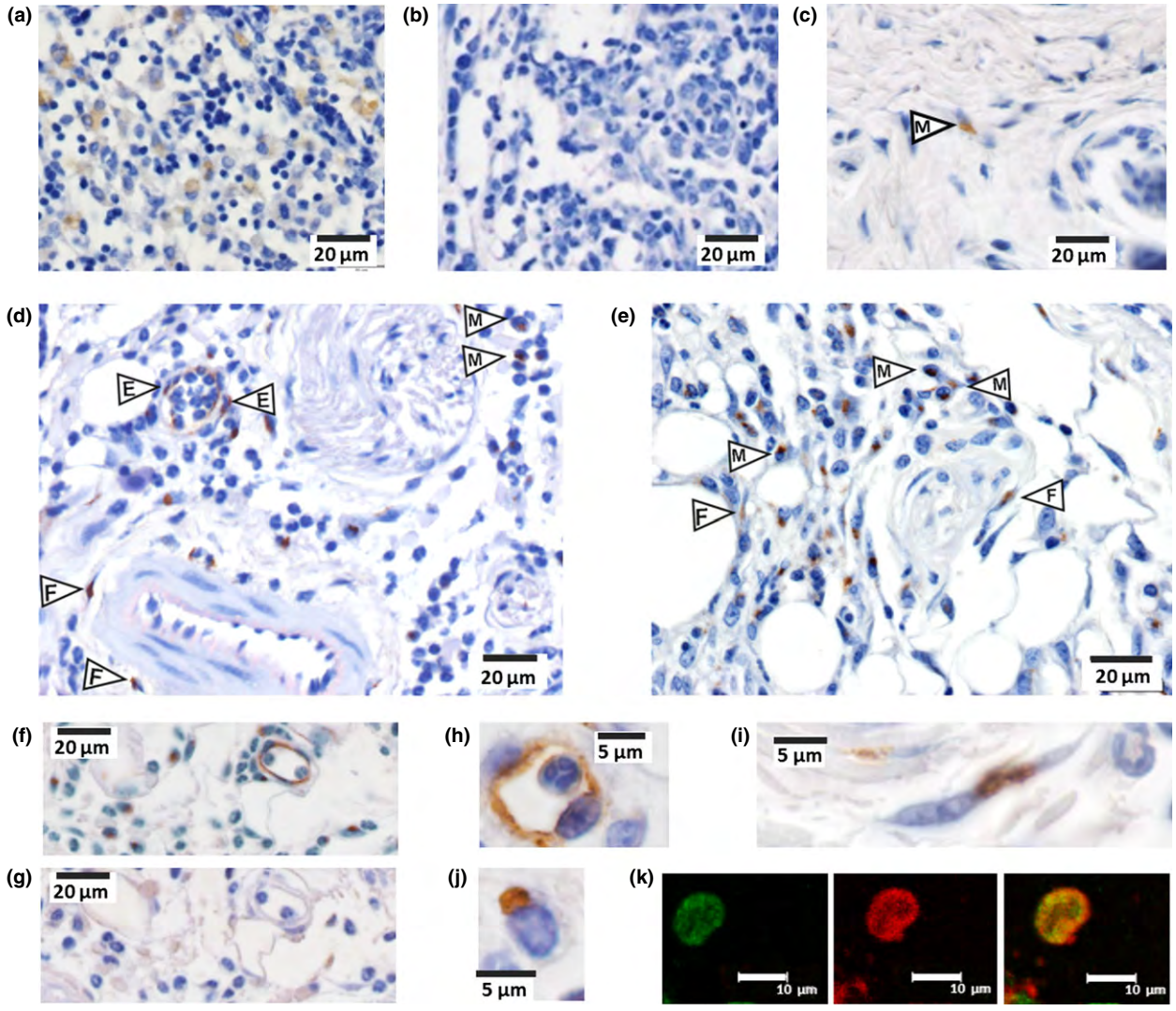

Figure 2 Immunohistochemical localization of CCL2 in inflamed paws. (a) Positive control of CCL2 staining performed in a mouse mesenteric lymph node in which macrophage positive for CCL2 can be observed. (b) Lack of CCL2 staining in a carrageenan-inflamed paw section in the absence of primary antibody (negative control). (c) Example of CCL2 staining in an isolated macrophage (M) present in the normal dermis of a solvent-treated paw. (d, e) Immunohistochemical CCL2 staining of the plantar side of paws inflamed with CARR (d) or CFA (e). Arrow heads with M, F, and E point stained macrophages, fibroblasts, and endothelial cells, respectively. (f, g) CCL2 staining in macrophages and endothelial cells obtained in a carrageenan-inflamed paw (f) and inhibition of the staining when a serial section was incubated with the fragment of the mouse CCL2 peptide amino acids Gln24-Arg96 (g). In (h–j), high-magnification representative images amplified from *Figure 1d* taken with a 100× objective with oil immersion and including CCL2-positive endothelial cells from a small capillary that contains in its light an unmarked polymorphonuclear cell (h), a fibroblast (i), and a macrophage (j). (k) Colocalization of CCL2 with CD68 in a macrophage present in a hind paw 6 h after carrageenan administration. Images obtained from a single stack with the anti-CCL2 antibody stained in green (left), the anti-CD68 antibody stained in red (center), and the double labeling in yellow (right).

their medium size and characteristic morphology with a unique nucleus (*Figure 2j*) and also by the double staining with the anti-CCL2 antibody and the anti-CD68 antibody observed in carrageenan-inflamed mice (*Figure 2k*). CCL2 staining was also observed in the cytoplasm of resident cells such as fibroblasts with their typical spindle shape and oval nucleus (*Figure 2i*). Furthermore, CCL2 expression was also detected in capillary endothelial cells (*Figure 2h*) of carrageenan- (*Figure 2d*), but not of CFA-inflamed paws.

In contrast, CCL2 staining was almost absent in noninflamed paws and was only observed in some isolated macrophages of normal dermis (*Figure 2c*). In assays performed in the absence of primary antibody, no staining was observed in inflamed paws (negative control) (*Figure 2b*). Finally, CCL2 staining in inflamed paws was inhibited when the antibody was preincubated with a saturating concentration (5 : 1) of the mouse CCL2 peptide fragment corresponding to Gln24-Arg96 that matches the sequence of CCL2 targeted by the anti-CCL2 antibody used (*Figure 2f, g*).

Antihyperalgesic, but not antiallodynic, effect evoked by the CCR2 antagonist RS 504393 in carrageenan- or CFA-inflamed mice

The s.c. administration of the CCR2 antagonist RS 504393 (0.3–3 mg/kg, 30 min before) dose dependently inhibited thermal hyperalgesia measured in mice inflamed with either carrageenan (*Figure 3a*) or CFA (*Figure 3b*). Paw withdrawal latencies were of about 14 s in noninflamed paws and of 7–9 s in inflamed ones. The dose of 0.3 mg/kg of RS 504393 did not modify either carrageenan- or CFA-evoked hyperalgesia, 1 mg/kg evoked an intermediate effect, and 3 mg/kg produced withdrawal latencies indistinguishable from those measured in noninflamed paws. As shown in *Figure 3c*, the antihyperalgesic effect evoked by 3 mg/kg of RS 504393 was already significant 15 min after its administration, maximal at 30 min, partially persisted at 45 min, and completely disappeared 60 min after its injection.

Because a previous report described that another CCR2 antagonist that is structurally analogous to RS 504393 can evoke more marked effects when repeat-

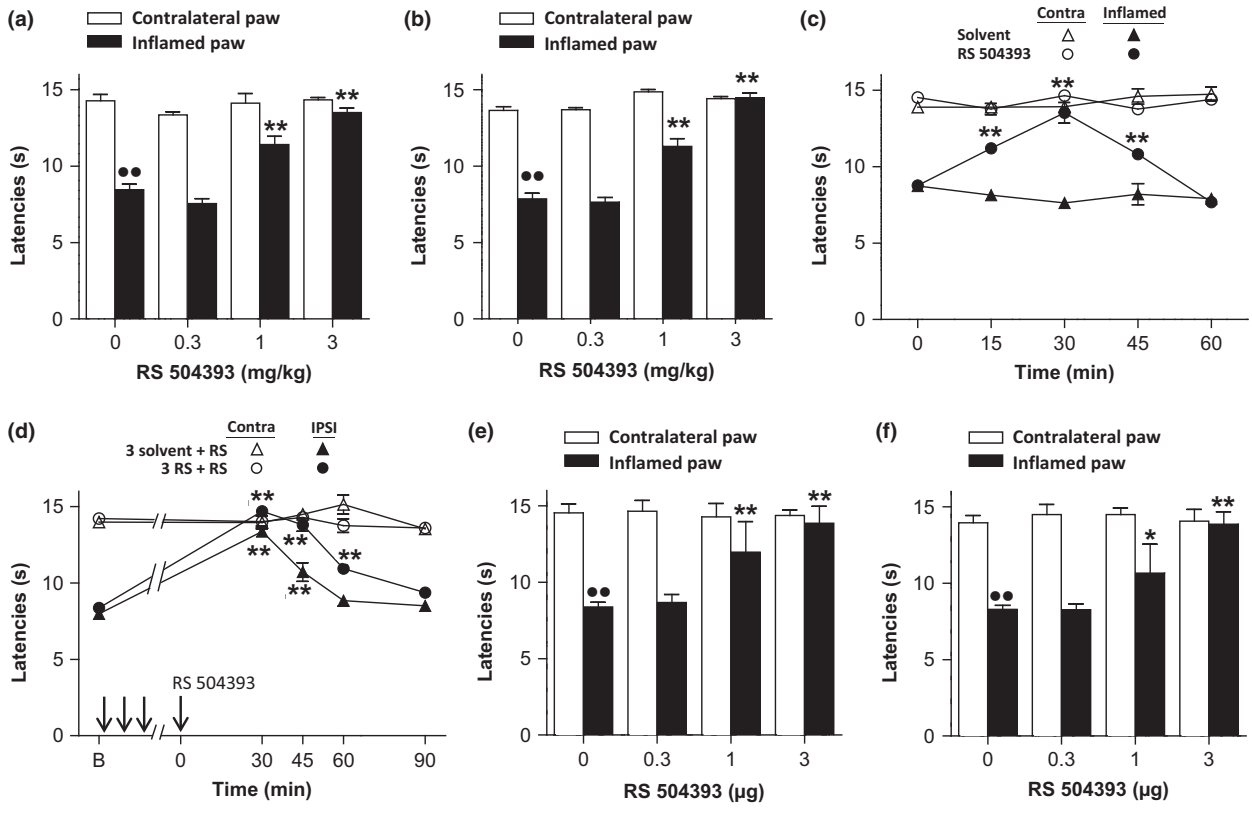

Figure 3 Inhibition of thermal hyperalgesia induced by the s.c. administration of RS 504393 (0.3–3 mg/kg, 30 min before) in mice inflamed with CARR 6 h before (a) or CFA 1 week before (b) and time course of the antihyperalgesic effect evoked by 3 mg/kg of RS 504393 in mice inflamed with CFA (c). (d) Time course of the antihyperalgesic effect evoked by the repeated s.c. administration of 3 mg/kg of RS 504393 four times every 6 h in mice inflamed with CFA; (b), baseline withdrawal latencies before treatment; arrows represent RS 504393 or solvent administration. (e, f) Inhibition of thermal hyperalgesia by the i.pl. administration of RS 504393 (0.3–3 µg, 30 min before) in mice inflamed with CARR 6 h before (e) or CFA 1 week before (f). Data are expressed as means ± SEM (n = 5–8). ●●$P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Student's t-test; *$P < 0.05$ **$P < 0.01$ compared with solvent-treated group, Dunnett's t-test.

edly administered 4 times every 6 h [23], a new experiment was performed in order to test whether this possibility also occurs in relation to the antihyperalgesic effects of RS 504393. Mice treated with CFA 1 week before received the administration of either solvent or RS 504393 (3 mg/kg; s.c.) 18, 12, and 6 h before testing, and then, the effect of 3 mg/kg of RS 504393 was assessed by the unilateral hot plate in both groups. As represented in *Figure 3d*, the antihyperalgesic effect evoked by systemic RS 504393 is more maintained if mice received previously this same drug, being maximal 30 and 45 min after the last administration and only completely disappearing 90 min after.

Furthermore, considering that the increase in CCL2 concentration was measured locally at inflamed territories, we were interested in elucidating whether inflammatory hyperalgesia could also be inhibited through the local blockade of CCR2 receptors at this level. Both the hyperalgesia measured 6 h after the administration of carrageenan (*Figure 3e*) and that measured 1 week after CFA administration (*Figure 3f*) were dose dependently inhibited 30 min after the i.pl. administration of 0.3–3 μg of RS 504393.

In contrast with the results obtained in the unilateral hot plate test, the administration of a dose of the CCR2 antagonist even three times higher (10 mg/kg) did not modify mechanical allodynia measured in mice inflamed with carrageenan (*Figure 4a*) or CFA (*Figure 4b*). In any case, we aimed to confirm that CCL2 is a molecule able to evoke mechanical allodynia by testing the effect of the exogenous i.pl. administration of different doses of this chemokine (60–600 ng). The 50% threshold values measured by the von Frey test were reduced after the i.pl. administration of CCL2, this allodynic effect reaching a statistical significance with the dose of 600 ng (*Figure 4c*).

Antihyperalgesic effect evoked by the administration of an anti-CCL2 antibody to carrageenan- or CFA-inflamed mice

In order to elucidate whether the antihyperalgesic effect evoked by RS 504393 could be related to the presence of CCL2 during the inflammatory process, we have studied the effect of the administration of a selective anti-CCL2 antibody. We initially checked the ability of this antibody to counteract the hyperalgesic reaction induced by the local administration of CCL2. The i.pl. administration of CCL2 (2-20 ng, 30 min before) induced dose-dependent thermal hyperalgesia (*Figure 5a*) that was completely prevented by the coadministration of 1 μg of the anti-CCL2 antibody (*Figure 5b*). Supporting the involvement of CCL2 in inflammatory hyperalgesia, the i.pl. injection of this anti-CCL2 antibody (0.1–1 μg, 30 min before) dose dependently inhibited thermal hyperalgesia produced by carrageenan (*Figure 5c*) or CFA (*Figure 5d*). The analgesic effect evoked by the i.pl administration of 1 μg of the anti-CCL2 antibody in CFA-inflamed mice was already significant 15 min after its administration, peaked at 30 min, and completely disappeared 60 min after (*Figure 5e*).

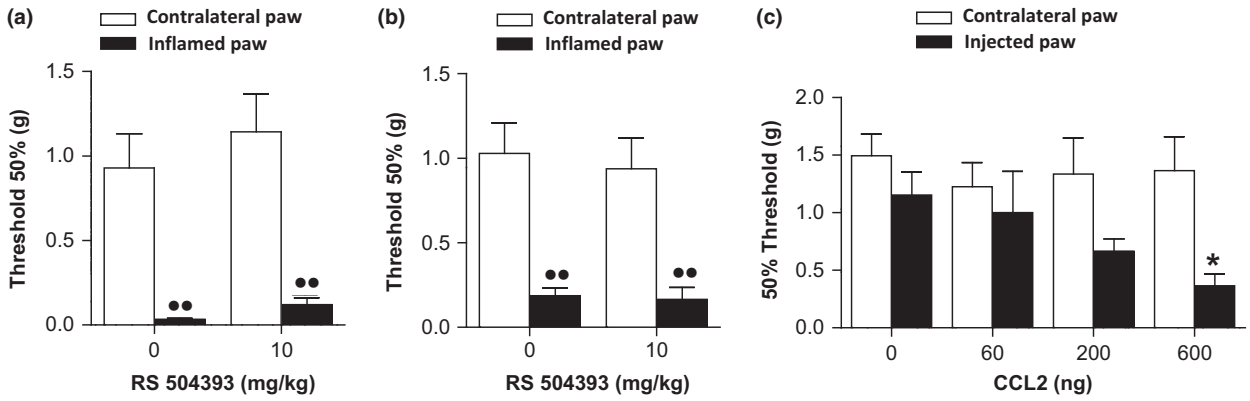

Figure 4 In (a) and (b), lack of effect of RS 504393 (s.c.; 10 mg/kg, 30 min before) on mechanical allodynia measured in mice inflamed with carrageenan (a) and CFA (b). (c) Allodynic effect evoked after the i.pl. administration of CCL2 (60–600 ng/25 μL, 30 min before) to naïve mice. Data are expressed as means ± SEM (n = 6–12). ●●$P < 0.01$ compared with its corresponding, contralateral, left paw, Mann–Whitney U-test; *$P < 0.05$ compared with the ipsilateral paw of solvent-treated group, Dunn's test.

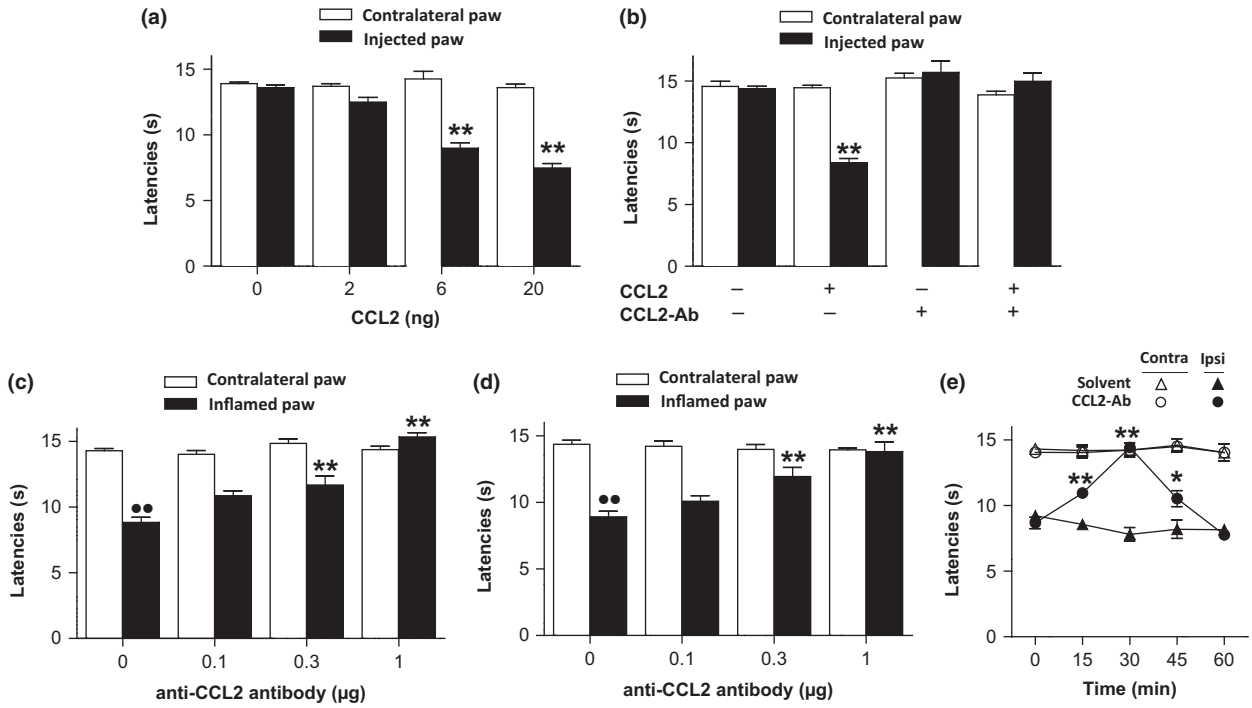

Figure 5 Hyperalgesic effect evoked after the i.pl. administration of CCL2 (2–20 ng/25 μL, 30 min before) to naïve mice (a) and prevention of the hyperalgesia evoked by 20 ng of CCL2 by the coadministration of 1 μg of anti-CCL2 antibody (b). Inhibition of thermal hyperalgesia evoked by CARR (c) or CFA (d) after the i.pl. administration of anti-CCL2 antibody (0.1–1 μg/25 μL, 30 min before) into the inflamed paw of mice and time course of the antihyperalgesic effect evoked by the i.pl. administration of 1 μg of the anti-CCL2 antibody to mice inflamed with CFA (e). Means and corresponding SEM are shown (n = 6–8). ●●$P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Student's t-test; **$P < 0.01$ compared with solvent-treated group, Dunnett's t-test (a, c, e) or Newman–Keuls test (b).

Lack of acute anti-inflammatory effect after the administration of the CCR2 antagonist RS 504393 to mice treated with carrageenan or CFA

Because the antinociceptive effects evoked by the CCR2 antagonist RS 504393 in inflammatory states could be related to a putative acute anti-inflammatory effect, we have measured paw tumefaction and performed an histological analysis of carrageenan- or CFA-inflamed paws of mice treated with the maximal antihyperalgesic dose of RS 504393 or solvent either once or twice.

The single administration of 3 mg/kg of RS 504393 at a time identical to that used when testing its nociceptive properties (30 min before testing) did not modify the increase in the paw size measured 6 h after carrageenan administration (*Figure 6a*) or the inflammatory reaction observed in hematoxylin–eosin-stained slices (*Figure 6b, c*). Accordingly, the typical pattern of acute inflammation, based on the presence of abundant polymorphonuclear neutrophils and some macrophages, was unaltered in mice receiving the administration of RS 504393 30 min before (*Figure 6d, e*). In addition, the administration of 3 mg/kg of RS 504393 30 min before testing did not reduce the degree of chronic inflammation evoked by the administration of CFA 1 week before (*Figure 6f, h*) or the presence of macrophages, lymphocytes, fibroblasts, or polymorphonuclear neutrophils (*Figure 6i, j*) in the inflamed paw.

In order to explore whether a more maintained exposition to RS 504393 could produce anti-inflammatory effects, we have also assayed a protocol in which the CCR2 antagonist was administered twice. In acutely inflamed mice, RS 504393 was administered 30 minutes before and 2.5 h after carrageenan and paw sections were measured before and 1.5, 2.5, 4.5, and 6.5 h after the first administration of drug or solvent (*Figure 7a*). As before, paw tumefaction was similar in mice treated with the CCR2 antagonist or solvent. In accordance, the administration of RS

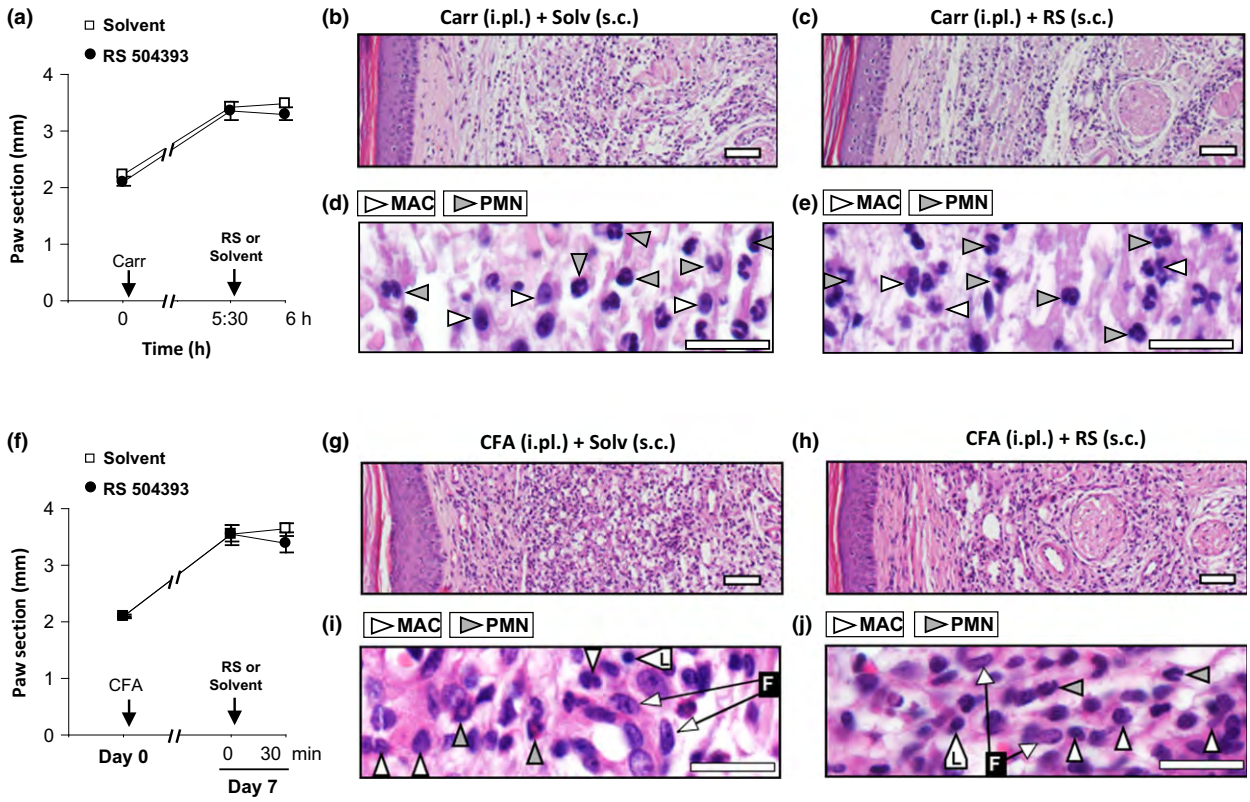

Figure 6 Lack of anti-inflammatory effect following the administration of 3 mg/kg of RS 504393 (s.c.) or solvent 30 min before testing to mice treated with carrageenan (CARR) or CFA. Measures of the right hind paw sections were taken before the injection of the inflammatory agent, just before administering RS 504393 or solvent and 30 min after in mice treated with CARR (a) or CFA (f). Means and corresponding standard errors are represented (n = 5–7). Representative examples of hematoxylin–eosin-stained sections corresponding to the paws of mice after the last paw section measurement are shown for CARR-inflamed mice treated with solvent (b, d) or RS 504393 (c, e) and CFA-inflamed mice treated with solvent (g, i) or RS 504393 (h, j). Arrows indicate the presence of macrophages (MAC), polymorphonuclear neutrophils (PMN), lymphocytes (L), and fibroblasts (F). Scale bars represent 50 μm (b, c, g, and h) or 20 μm (d, e, i, and j).

504393 did not modify the degree of inflammatory reaction observed in hematoxylin–eosin-stained sections coming from carrageenan-inflamed paws collected at the end of the experiment (*Figure 7b, c*). In chronically inflamed mice by the injection of CFA 1 week before, a similar pattern of administration of the CCR2 antagonist (at time 0 and 3 h) did not modify paw tumefaction measured 1.5, 2.5, 4.5, or 6.5 h after the first administration of RS 504393 (*Figure 7d*) or the histological features observed in inflamed paws (*Figure 7e, f*).

DISCUSSION

We show here that the blockade of CCR2 or the neutralization of CCL2 inhibits thermal hyperalgesia, but not mechanical allodynia, in mice with acute or chronic peripheral inflammation. This antihyperalgesic effect does not seem to rely on the inhibition of the inflammatory reaction, because the acute administration of the more effective dose of the CCR2 antagonist did not modify the degree of inflammation.

Initially, we checked whether the levels of CCL2 are increased in the inflamed paws of mice injected with carrageenan or CFA. Previous reports have described increased levels of CCL2 from 4 h after the s.c. injection of carrageenan into the back of mice [18] or 24 h after the i.pl. administration of carrageenan in rats [17]. However, to our knowledge, no previous quantification of CCL2 levels has been carried out in CFA-inflamed tissues. In our ELISA experiments, a remarkable local increase in CCL2 levels was measured in

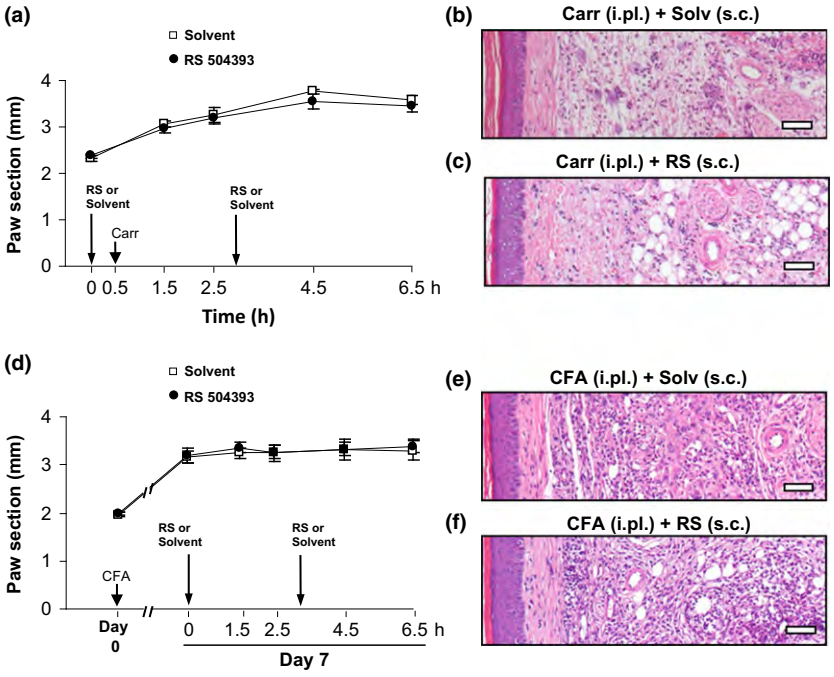

Figure 7 Lack of anti-inflammatory effect following two administrations of 3 mg/kg of RS 504393 (s.c.) or solvent at time 0 and 3 h after to mice treated with carrageenan (CARR) or CFA. Measures of the right hind paw sections were taken before the injection of the inflammatory agent, just before the first administration of RS 504393 and 1.5, 2.5, 4.5, or 6.5 h after in mice treated with CARR (a) or CFA (d). Means and corresponding standard errors are represented (n = 5–6). Representative examples of hematoxylin–eosin-stained sections corresponding to the paws of mice after the last paw section measurement for CARR-inflamed mice treated with solvent (b) or RS 504393 (c) and CFA-inflamed mice treated with solvent (e) or RS 504393 (f). Scale bars represent 50 μm.

paw homogenates 6 h or 1 week after the i.pl. administration of carrageenan or CFA, respectively. The maintenance of increased CCL2 concentrations in mice treated with CFA fits well with the chronic nature of CFA-evoked inflammation and is in accordance with its use in behavioral studies related to chronic inflammatory pain [24].

Besides, an increased expression of CCL2, mainly localized in macrophages, was observed in both carrageenan- and CFA-inflamed mice by immunohistochemical experiments. The expression of this chemokine was also detected in resident cells of acutely or chronically inflamed paws, such as fibroblasts. Interestingly, CCL2 staining was present in endothelial cells of small arterioles in mice acutely, but not chronically, inflamed, being its expression at this level probably linked to its chemotactic role in the initial steps of inflammation.

The systemic administration of RS 504393, a CCR2 antagonist [25], dose dependently inhibits thermal hyperalgesia in mice with either acute or chronic peripheral inflammation. The antihyperalgesic effect evoked by the highest dose assayed of RS 504393 (3 mg/kg) was rather brief reaching its maximum 30 min after administration and completely disappearing in 1 h. However, the repeated administration of this drug every 6 h induced a more maintained antihyperalgesic effect that could rely on its accumulation as previously described with an RS 504393 analog administered with the same schedule [23].

The antihyperalgesic effect produced in response to the i.pl. administration of low doses of the CCR2 antagonist both in acute and in chronic inflamed mice supports the involvement of local CCR2 expressed at the injured tissue. This implication of peripheral CCR2 in inflammatory nociceptive processing seems compatible with the upregulation of these receptors described in DRG of rodents with cystitis [7], osteoarthritis [26], or paw inflammation induced by CFA injection [6]. In addition, the inhibitory effect evoked by RS 504393 demonstrates that endogenous molecules able to bind this receptor contribute to the establishment of inflammatory hypernociception. The fact that the local administration of a dose of anti-CCL2 antibody that prevents the hyperalgesic response evoked by exogenous CCL2 completely antagonized carrageenan- and CFA-induced hyperalgesia strongly supports the participation of CCL2 in CCR2-mediated inflammatory hyperalgesia. Although other chemokines such as CCL7 (MCP-3), CCL8, and CCL11 bind to CCR2 in mice [27], CCL2 is the main endogenous CCR2 agonist involved in pain processing [3,7,16,20,21,28–31]. Thus, the increased CCL2 levels present in inflamed tissues together with the antihyperalgesic effect produced by an anti-CCL2 antibody strongly suggest that CCL2 is acting as an endogenous CCR2 agonist in both

carrageenan- and CFA-treated mice. Previous data support the possibility that the hyperalgesic effect evoked by CCR2 activation at periphery could be mediated through TRPV1 sensitization [30], a type of receptors clearly involved in nociceptive heat transduction [32]. Besides, the lack of antiallodynic effect following the administration of RS 504393 suggests that CCR2 activation is not involved in the production of mechanical allodynia in peripheral inflammatory processes. Supporting this fact, it has been reported that inflammatory thermal hyperalgesia, but not mechanical allodynia, is amplified in mice that overexpress CCL2 [16] and, as shown here and elsewhere [6], only very high doses of CCL2 can induce mechanical allodynia when i.pl. administered. In mice with enhanced local levels of CCL2 due to the development of bone tumors in response to the inoculation of fibrosarcoma NCTC 2472 [21] or prostate RM-1 [31] tumoral cells, the administration of a CCR2 antagonist also inhibits thermal hyperalgesia without modifying mechanical allodynia. In contrast with these data, other reports have described that the administration of a CCR2 antagonist [7,28] or an anti-CCL2 antibody [29] inhibits mechanical allodynia in rats with bladder inflammation [7], neuropathic injury [8,28], or bone cancer [29]. The potential involvement of different mechanisms in the establishment of allodynia [33] could explain that the pharmacological modulation of this symptom can vary depending on the pathological process.

It has been reported that paw tumefaction evoked by CFA is augmented in mice overexpressing CCL2 [16] and that a maintained blockade of the CCL2/CCR2 system can evoke anti-inflammatory effects. Thus, a reduction in CFA-evoked inflammation was observed in rats treated during several days with a molecule able to inhibit CCL2-induced chemotaxis of monocytes/macrophages [34] and the inflammation evoked by 2,4-dinitrofluorobenzene in mice was partially prevented after the blockade of CCR2 during 2 days [35]. Therefore, it could be possible that a putative anti-inflammatory action induced by the acute administration of RS 504393 could explain the antihyperalgesic effect evoked by this CCR2 antagonist in our experiments. In order to check this possibility, we have measured the degree of tumefaction and examined microscopically the inflammatory reaction that carrageenan or CFA produces in mice treated with the maximal antihyperalgesic dose of RS 504393 administered either 30 min before testing, as performed in hyperalgesic assays, or injected twice and measured at different time points. The volume of edema, the intensity of the inflammatory reaction, and the presence of inflammatory cells remained unmodified in mice treated with the CCR2 antagonist, thus indicating that RS 504393 acutely administered did not produce immediate anti-inflammatory effects. These results agree with previous reports indicating that CCR2 deletion does not reduce formalin-evoked inflammation [6] or corneal inflammation evoked by cauterization [36]. In fact, it has been argued that the blockade of a single chemokine receptor might not be a good anti-inflammatory strategy due to the redundancy of inflammatory signals triggered by different chemokines [37]. In any case, although we cannot exclude the possibility that a maintained treatment with adequate doses of RS 504393 could exert an anti-inflammatory action as previously suggested [34,35], our results indicate that the reduction in inflammatory hyperalgesia evoked by the acute administration of RS 504393 is related to a specific analgesic effect and does not occur secondarily to a putative reduction in carrageenan- or CFA-mediated paw inflammation.

Globally, the present data confirm that the levels of CCL2 increase in inflamed sites and show that both CCL2 neutralization and CCR2 blockade can reduce inflammatory hyperalgesia, but not mechanical allodynia, in mice. The effects of CCL2/CCR2 axis blockade have been previously assessed in experimental settings of neuropathic [8,28,38] or tumoral [21,29,31] pain. To our knowledge, only one previous report has explored the analgesic effect evoked by a CCR2 antagonist in an inflammatory setting [7]. This study described that CCL2 concentrations measured in inflamed bladder of rats are transiently increased some hours after the acute, but not chronic, administration of cyclophosphamide and that the administration of a CCR2 antagonist inhibits the referred pain reactivity at this initial inflammatory stage [7].

CONCLUSION

Our results indicate that CCL2 levels are increased after acute or chronic inflammation of somatic tissues and that CCR2 blockade or CCL2 neutralization counteracts thermal hyperalgesia, but not mechanical allodynia of inflammatory origin. In addition, the absence of an anti-inflammatory effect following the acute administration of antihyperalgesic doses of RS 504393 indicates that the inhibition of hyperalgesia after CCR2 inhibition is due to an intrinsic antinociceptive effect

not secondary to the inhibition of the inflammatory reaction.

ACKNOWLEDGEMENTS

Grants were provided by Ministerio de Economía y Competitividad (MINECO, SAF2012-36271) and Fundación para la Investigación Científica y Técnica de Asturias and FEDER (European Union) (FC-15-GRU-PIN14-125). S.G-R. is recipient of a postdoctoral grant from Programa Clarín (Asturias)-Marie Curie-Cofund. IUOPA is supported by Fundación Bancaria Caja de Ahorros de Asturias (Asturias, Spain). The authors appreciate the excellent technical assistance given by Marta Alonso Guervós and Ángel Martínez Nistal (Servicios Científico-Técnicos, Universidad de Oviedo) for fluorescence microscopy and imaging processing.

REFERENCES

1 Melgarejo E., Medina M.A., Sánchez-Jiménez F., Urdiales J.L. Monocyte chemoattractant protein-1: a key mediator in inflammatory processes. Int. J. Biochem. Cell Biol. (2009) **41** 998–1001.

2 Deshmane S.L., Kremlev S., Amini S., Sawaya B.E. Monocyte chemoattractant protein-1 (MCP-1): an overview. J. Interferon Cytokine Res. (2009) **29** 313–326.

3 Belkouch M., Dansereau M.A., Réaux-Le Goazigo A. et al. The chemokine CCL2 increases Nav1.8 sodium channel activity in primary sensory neurons through a Gß?-dependent mechanism. J. Neurosci. (2011) **31** 18381–18390.

4 Qin X., Wan Y., Wang X. CCL2 and CXCL1 trigger calcitonin gene-related peptide release by exciting primary nociceptive neurons. J. Neurosci. Res. (2005) **82** 51–62.

5 Bogen O., Dina O.A., Gear R.W., Levine J.D. Dependence of monocyte chemoattractant protein 1 induced hyperalgesia on the isolectin B4-binding protein versican. Neuroscience (2009) **159** 780–786.

6 Abbadie C., Lindia J.A., Cumiskey A.M. et al. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. Proc. Natl Acad. Sci. USA (2003) **100** 7947–7952.

7 Arms L., Girard B.M., Malley S.E., Vizzard M.A. Expression and function of CCL2/CCR2 in rat micturition reflexes and somatic sensitivity with urinary bladder inflammation. Am. J. Physiol. Renal. Physiol. (2013) **305** F111–F122.

8 Zhu X., Cao S., Zhu M.D., Liu J.Q., Chen J.J., Gao Y.J. Contribution of chemokine CCL2/CCR2 signaling in the dorsal root ganglion and spinal cord to the maintenance of neuropathic pain in a rat model of lumbar disc herniation. J. Pain (2014) **15** 516–526.

9 Wang C.H., Zou L.J., Zhang Y.L., Jiao Y.F., Sun J.H. The excitatory effects of the chemokine CCL2 on DRG somata are greater after an injury of the ganglion than after an injury of the spinal or peripheral nerve. Neurosci. Lett. (2010) **475** 48–52.

10 Yang Z., Luo W., Wang J., Tan Y., Fu R., Fang B. Chemokine ligand 2 in the trigeminal ganglion regulates pain induced by experimental tooth movement. Angle Orthod. (2014) **84** 730–736.

11 Zhang H., Boyette-Davis J.A., Kosturakis A.K. et al. Induction of monocyte chemoattractant protein-1 (MCP-1) and its receptor CCR2 in primary sensory neurons contributes to paclitaxel-induced peripheral neuropathy. J. Pain (2013) **14** 1031–1044.

12 Wang X.M., Hamza M., Wu T.X., Dionne R.A. Upregulation of IL-6, IL-8 and CCL2 gene expression after acute inflammation: correlation to clinical pain. Pain (2009) **142** 275–283.

13 Ogura N., Satoh K., Akutsu M. et al. MCP-1 production in temporomandibular joint inflammation. J. Dent. Res. (2010) **89** 1117–1122.

14 Taylor P.C., Peters A.M., Paleolog E. et al. Reduction of chemokine levels and leukocyte traffic to joints by tumor necrosis factor alpha blockade in patients with rheumatoid arthritis. Arthritis Rheum. (2000) **43** 38–47.

15 Okamoto M., Suzuki T., Watanabe N. Modulation of inflammatory pain in response to a CCR2/CCR5 antagonist in rodent model. J Pharmacol Pharmacother. (2013) **4** 208–210.

16 Menetski J., Mistry S., Lu M. et al. Mice overexpressing chemokine ligand 2 (CCL2) in astrocytes display enhanced nociceptive responses. Neuroscience (2007) **149** 706–714.

17 Yang H.Y., Mitchell K., Keller J.M., Iadarola M.J. Peripheral inflammation increases Scya2 expression in sensory ganglia and cytokine and endothelial related gene expression in inflamed tissue. J. Neurochem. (2007) **103** 1628–1643.

18 García-Ramallo E., Marques T., Prats N., Beleta J., Kunkel S.L., Godessart N. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. J. Immunol. (2002) **169** 6467–6473.

19 Wang Y., Wei D., Lai Z., Le Y. Triptolide inhibits CC chemokines expressed in rat adjuvant-induced arthritis. Int. Immunopharmacol. (2006) **6** 1825–1832.

20 Jeon S.M., Lee K.M., Park E.S., Jeon Y.H., Cho H.J. Monocyte chemoattractant protein-1 immunoreactivity in sensory ganglia and hindpaw after adjuvant injection. NeuroReport (2008) **19** 183–186.

21 Pevida M., González-Rodríguez S., Lastra A., Hidalgo A., Menéndez L., Baamonde A. CCL2 released at tumoral level contributes to the hyperalgesia evoked by intratibial inoculation of NCTC 2472 but not B16-F10 cells in mice. Naunyn Schmiedebergs Arch. Pharmacol. (2012) **385** 1053–1061.

22 González-Rodríguez S., Llames S., Hidalgo A., Baamonde A., Menéndez L. Potentiation of acute morphine-induced analgesia measured by a thermal test in bone cancer-bearing mice. Fundam. Clin. Pharmacol. (2012) **26** 363–372.

23 Mitchell L.A., Hansen R.J., Beaupre A.J., Gustafson D.L., Dow S.W. Optimized dosing of a CCR2 antagonist for amplification of vaccine immunity. Int. Immunopharmacol. (2013) **15** 357–363.

24 Miranda H.F., Romero M.A., Puig M.M. Antinociceptive and anti-exudative synergism between dexketoprofen and tramadol in a model of inflammatory pain in mice. Fundam. Clin. Pharmacol. (2012) **26** 373–382.
25 Mirzadegan T., Diehl F., Ebi B. et al. Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. J. Biol. Chem. (2000) **275** 25562–25571.
26 Miller R.E., Tran P.B., Das R. et al. CCR2 chemokine receptor signaling mediates pain in experimental osteoarthritis. Proc. Natl Acad. Sci. USA (2012) **109** 20602–20607.
27 Charo I.F., Hills R., Horuk R., Matsushima K., Murphy P.M., Oppenheim J.J. Chemokine receptors: CCR2. IUPHAR/BPS Guide to PHARMACOLOGY, http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=59. Last modified on 17/02/2015. Accessed on 27/03/2015
28 Van Steenwinckel J., Reaux-Le Goazigo A., Pommier B. et al. CCL2 released from neuronal synaptic vesicles in the spinal cord is a major mediator of local inflammation and pain after peripheral nerve injury. J. Neurosci. (2011) **31** 5865–5875.
29 Hu J.H., Zheng X.Y., Yang J.P., Wang L.N., Ji F.H. Involvement of spinal monocyte chemoattractant protein-1 (MCP-1) in cancer induced bone pain in rats. Neurosci. Lett. (2012) **17** 60–63.
30 Pflücke D., Hackel D., Mousa S.A. et al. The molecular link between C-C-chemokine ligand 2-induced leukocyte recruitment and hyperalgesia. J. Pain (2013) **14** 897–910.
31 Llorián-Salvador M., Pevida M., Fernández-García M.T. et al. Hypernociceptive responses following the intratibial inoculation of RM1 prostate cancer cells in mice. Prostate (2015) **75** 70–83.
32 Caterina M.J., Schumacher M.A., Tominaga M., Rosen T.A., Levine J.D., Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature (1997) **389** 816–824.
33 Lolignier S., Eijkelkamp N., Wood J.N. Mechanical allodynia. Pflugers Arch. (2015) **467** 133–139.
34 Laborde E., Macsata R.W., Meng F. et al. Discovery, optimization, and pharmacological characterization of novel heteroaroylphenylureas antagonists of C-C chemokine ligand 2 function. J. Med. Chem. (2011) **54** 1667–1681.
35 Brodmerkel C.M., Huber R., Covington M. et al. Discovery and pharmacological characterization of a novel rodent-active CCR2 antagonist, INCB3344. J. Immunol. (2005) **175** 5370–5378.
36 Oshima T., Sonoda K.H., Tsutsumi-Miyahara C. et al. Analysis of corneal inflammation induced by cauterisation in CCR2 and MCP-1 knockout mice. Br. J. Ophthalmol. (2006) **90** 218–222.
37 Pease J.E., Horuk R. Chemokine receptor antagonists: part 1. Expert Opin. Ther. Pat. (2009) **19** 39–58.
38 Serrano A., Paré M., McIntosh F., Elmes S.J., Martino G. Blocking spinal CCR2 with AZ889 reversed hyperalgesia in a model of neuropathic pain. Mol. Pain. (2010) **6** 90.

3.2. ARTÍCULO 2

Llorián-Salvador M, González-Rodríguez S, Lastra A, Fernández-García MT, Hidalgo A, Menéndez L, Baamonde A. **Involvement of CC chemokine receptor 1 and CCL3 in acute and chronic inflammatory pain in mice.** Basic Clin Pharmacol Toxicol. 2015 Dec 12. [En prensa]

OBJETIVO

Estudiar la participación de receptores CCR1 y de sus ligandos endógenos CCL3 y CCL5 en las respuestas hipernociceptivas medidas en ratones con inflamación aguda producida tras la administración de carragenina o crónica provocada por el coadyuvante de Freund (CFA).

MÉTODOS

- Ratones CD-1 inflamados por inyección intraplantar de carragenina 6 h antes o de CFA 1 semana antes.
- ELISA para la cuantificación de los niveles de CCL3 y CCL5 en las patas y en la médula espinal.
- PCR cuantitativa (q-PCR) para medir la expresión de mRNA de CCL3 en tejidos inflamados.
- Tinción hematoxilina-eosina para el análisis histológico del tejido inflamado.
- Ensayos inmunohistoquímicos para la localización de CCL3.
- Placa caliente unilateral para medir la hiperalgesia térmica.
- Prueba de von Frey para medir la alodinia mecánica.

RESULTADOS Y CONCLUSIONES

- La administración aguda del antagonista de receptores CCR1, J113863, inhibió la hiperalgesia térmica medida tanto en ratones con inflamación aguda como crónica pero sólo la alodinia mecánica debida a inflamación crónica.

- El grado de inflamación producida por carragenina o CFA no se modificó tras la administración aguda de dosis analgésicas de J113863 descartando que este efecto pudiera deberse a una posible acción antiinflamatoria.

- La expresión del mRNA de CCL3 así como de la propia proteína CCL3, principalmente localizada en macrófagos y neutrófilos, aumentó en las patas de ratones inflamadas con carragenina o CFA. Sin embargo, la inflamación no produjo cambios locales de los niveles de CCL5 ni modificó la expresión de CCL3 o de CCL5 en la médula espinal.

- La inhibición de la hiperalgesia inflamatoria producida por la administración de un anticuerpo anti-CCL3 parece indicar que el estímulo de receptores CCR1 en tejidos inflamados es debido a un aumento de la expresión de dicha quimiocina.

En resumen, los datos recogidos en esta publicación indican que la hiperalgesia y la alodinia inflamatorias pueden contrarrestarse mediante el bloqueo de receptores CCR1 independientemente de un posible efecto antiinflamatorio. Tanto en la inflamación provocada por carragenina como por CFA, la inhibición del dolor producida tras la administración de un antagonista CCR1 parece deberse al bloqueo de la acción ejercida por la CCL3.

BCPT Basic & Clinical Pharmacology & Toxicology

Basic & Clinical Pharmacology & Toxicology Doi: 10.1111/bcpt.12543

Involvement of CC Chemokine Receptor 1 and CCL3 in Acute and Chronic Inflammatory Pain in Mice

María Llorián-Salvador[1], Sara González-Rodríguez[1], Ana Lastra[1], María Teresa Fernández-García[2], Agustín Hidalgo[1], Luis Menéndez[1] and Ana Baamonde[1]

[1]Laboratory of Pharmacology, School of Medicine, University Institute of Oncology of Asturias (IUOPA), University of Oviedo, Asturias, Spain and [2]Molecular Histopathology in Animal Models of Cancer Unit, IUOPA, University of Oviedo, Asturias, Spain

(Received 3 September 2015; Accepted 6 December 2015)

Abstract: Chemokines are chemotactic cytokines whose involvement in nociceptive processing is being increasingly recognized. Based on the previous description of the involvement of CC chemokine receptor type 1 (CCR1) in pathological pain, we have assessed the participation of CCR1 and its endogenous ligands CCL3 and CCL5 in hyperalgesia and allodynia in mice after acute inflammation with carrageenan and chronic inflammation with complete Freund's adjuvant (CFA). The subcutaneous administration of the CCR1 antagonist J113863 (3–30 mg/kg; 30 min. before) dose dependently inhibited carrageenan- and CFA-evoked thermal hyperalgesia and mechanical allodynia produced by CFA, but not by carrageenan. The maximal dose of J113863 did not modify the increase in paw thickness induced by carrageenan or CFA. An almost ten times augmentation of CCL3 levels was detected by ELISA assays in both carrageenan and CFA paws, but not in spinal cords of inflamed mice, whereas CCL5 concentrations remained unaltered. Accordingly, a marked increase of CCL3 mRNA expression was observed in inflamed paws, with CCL3 protein detected in neutrophils and macrophages by immunohistochemical experiments. The intraplantar administration of an anti-CCL3 antibody (0.3–3 μg) blocked thermal hyperalgesia in carrageenan- and CFA-inflamed mice as well as CFA-evoked mechanical allodynia. Our data suggest that the increased concentrations of CCL3 present in inflamed tissues can be involved in acute and chronic inflammatory hyperalgesia as well as in chronic mechanical allodynia, and that these hypernociceptive symptoms can be counteracted by its neutralization with an antibody or by the blockade of CCR1 receptors.

Chemokines are chemotactic cytokines involved in inflammatory processes, mainly by favouring immune cell recruitment. Several chemokines activate nociceptive pathways at both peripheral and central level, and this explains their participation in different types of pathological pain [1–4]. CC chemokine receptor type 1 (CCR1) is expressed in neurons of dorsal root ganglia (DRG) [5,6] as well as in central structures related to pain [7,8]. The stimulation of CCR1 expressed in nociceptors causes Ca^{2+} influx and enhances capsaicin responsiveness [6], and the local administration of CCR1 agonists elicits nociceptive behaviours in mice [9,10]. CCR1 is up-regulated in sensory neurons [11] and the spinal cord [7,12] after nerve injury and increased CCR1 mRNA has also been measured in rats 24 hr after receiving carrageenan [13], supporting its involvement in nociceptive processing. Functionally, it has been reported that some types of neuropathic pain can be alleviated by blocking CCR1 expression with siRNA [11], and that the administration of a CCR1 antagonist can prevent some types of murine bone cancer pain [10] and partially reduce writhing responses in mice receiving intraperitoneal acetic acid or mechanical hyperalgesia in inflamed mice [14].

Among the different chemokines able to activate CCR1 [15], CCL3 (MIP-1α, macrophage inflammatory protein-1α) and CCL5 (RANTES, reduced upon activation normal T-cell expressed and secreted) are particularly related to nociception. CCL3 mRNA is up-regulated in the spinal cord of inflamed rats [16] and the spinal expression of CCL3 increases in mice with prostatitis [17] or nerve injury [11] being its neutralization with a specific antibody able to evoke analgesic effects [12]. CCL5 has been also previously related to hypernociceptive reactions associated with neuropathic injury [18] and tumour development [10,19,20].

In this study, we examined the possible role played by CCR1 in inflammatory pain bearing in mind the relevance that the blockade of CCR1 could have in clinical inflammatory settings [21]. We measured the analgesic responses obtained after the administration of the CCR1 antagonist J113863, formerly named 2q [22,23], in two standard models of acute and chronic local inflammation in rodents based on the intraplantar (i.pl.) injection of carrageenan or complete Freund's adjuvant (CFA). The possibility that the antinociceptive effect produced by the CCR1 antagonist could be secondary to a putative acute anti-inflammatory effect was explored, and finally, we have also tried to elucidate whether an increased expression of CCL3 or CCL5 could occur in these inflammatory settings.

Materials and Methods

Animals. Swiss male mice from the Animalario of the Universidad de Oviedo (Reg. 33044 13A) were used. Experiments were approved by the Comité Ético de Experimentación Animal de la Universidad de Oviedo (Spain) and performed according to the ethical standards in Directive 86/609/EEC, 'European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes'.

Author for correspondence: Ana Baamonde, Laboratory of Pharmacology. School of Medicine. University of Oviedo. C/ Julián Clavería 6. 33006 Oviedo. Asturias. Spain. (fax +34 985103551, e-mail arbaiza@uniovi.es).

Unilateral hot plate test. As previously described [10,24], mice were gently restrained and the plantar side of the paw placed on a hot plate (IITC Life Sciences, Woodland Hills, CA, USA) set at 48.6°C. Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at 2-min. intervals, and the mean of two measures was considered. Cut-off was 20 sec.

von Frey test. Filaments 2.44, 2.83, 3.22, 3.61, 4.08, 4.56 (Stoelting) were applied to the paw plantar side [10]. Starting with the 3.61 filament, a positive response was followed by the application of the immediate thinner filament or the immediate thicker one if the response was negative. The 50% response threshold was calculated using the following formula: 50% g threshold = $(10^{Xf+\kappa\delta})/10{,}000$; Xf, is the value of the last von Frey filament applied; κ, correction factor based on pattern of responses (Dixon's calibration table); δ, mean distance in log units between stimuli (here, 0.4).

Measurement of paw thickness. The maximal dorsoplantar section of the paw was measured with a calliper to obtain a macroscopic measure of paw swelling [25].

Drugs. Lambda carrageenan (type IV Sigma, Steinheim, Germany; 2% in saline) or complete Freund's adjuvant (CFA; Sigma) was intraplantarly (i.pl.) injected (30 µl). Control mice were treated with saline. J113863 (Tocris, UK) and dexamethasone (Sigma) were solved in 10% DMSO in distilled water and injected subcutaneously (s.c.) under the fur of the neck or intraperitoneally (i.p.), respectively, in a volume of 10 ml/kg. Goat anti-CCL3 antibody (R&D AF-450-NA, Minneapolis, USA) or goat serum (Sigma) diluted in saline at the same IgG concentration was i.pl. administered in 25 µl. Recombinant murine CCL3 (PrepoTech, Rocky Hill, USA) was dissolved in distilled water.

Enzyme-linked immunosorbent assay (ELISA). As previously described [10], the paw plantar side and L4-L6 spinal cord segments were placed in buffer (0.1 M Tris, 0.15 M NaCl, 0.5% CTAB (Fluka, Saint Louis, USA) containing a protease inhibitor (1 tablet/10.5 ml buffer, Complete Mini Roche Diagnostics, Mannheim, Germany). Next, 3 µl (paw) or 6 µl (spinal cord) of buffer per mg tissue was added and tissues were homogenated with a drill (Minicraft MB130, Rocky Hill, USA), centrifuged (15,000 × *g*, 15 min, 4°C) and their supernatant protein concentration measured.

ELISA (CCL3/MIP-1α and CCL5/RANTES R&D DuoSet mouse) assays were performed following the instructions of the manufacturer by adding 40 or 10 µg protein (for CCL3 and CCL5 measurements, respectively) of homogenates coming from paws or 30 or 50 µg protein (for CCL3 and CCL5 measurements, respectively) of spinal cord homogenates. Values obtained came from at least six independent samples performed in duplicate.

Immunohistochemical assays. Paws were fixed with 4% formaldehyde for 4 hr, and 4 µm sections of paraffin-embedded formalin-fixed blocks were stained with haematoxylin and eosin. Initially, antigen retrieval was performed in deparaffinized sections incubated in PTLink with Envision FLEX Target Retrieval Solution Low pH (Dako, Carpinteria, USA) for 20 min at 95°C, further blocked during 20 min. with Protein Block Serum-Free (Dako) and next incubated with an anti-CCL3 antibody (polyclonal goat anti-mouse, R&D, 30 µg/ml for 30 min. at RT). A 30-min. incubation with a polyclonal rabbit anti-goat immunoglobulins/HRP (1:200, Dako) was followed by 30 min. at room temperature with secondary antibody Envision Rabbit (Dako). The conjugate's peroxidase label was visualized by diaminobenzidine DAB+ (Dako), and slices were counterstained with Mayer's haematoxylin.

Real-time quantitative polymerase chain reaction (qPCR). mRNA was extracted from paw plants using EZNA® Total RNA Kit I Omega bio-tek (Norcross, Georgia, USA), and RNA concentration was determined using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer. The first-strand cDNA was synthesized from 1 µg of total RNA priming by oligo(dT) using SuperScript reverse transcription kit (Life technologies, ThermoFisherScientific, Carlsbad, CA, USA). Next, real-time quantitative PCR was performed on a 7900HT Fast Real-time PCR System (Applied Biosystems, ThermoFisher Scientific, Spain) using specific primers for the mouse gene of CCL3 accession number NM_011337.2 (forward 5′ CCA GCC AGG TGT CAT TTT CCT 3′ and reverse 5′ TCC AAG ACT CTC AGG CAT TCA GT 3′) and for the β-actin gene accession number NM_007393.4 (forward 5′ GCA GCT CCT TCG TTG CCG GT 3′ and reverse 5′ TAC AGC CCG GGG AGC ATC GT 3′) [26]. Duplicates of six independent samples of control or inflamed paws were used in each case, and data were analysed by the $2^{-\Delta\Delta Ct}$ method [27].

Statistical analysis. Thermal withdrawal latencies were compared by Student's *t*-test when the values obtained in ipsilateral and contralateral paws were compared in each group, whilst comparisons among different groups or with the solvent group were made by ANOVA followed by the Newman–Keuls or Dunnett's *t*-tests, respectively. Threshold values obtained by the von Frey test in ipsilateral and contralateral paws were compared by the Mann–Whitney's *U*-test, and comparisons with the solvent-treated group were made with an initial Kruskal–Wallis analysis followed by Dunn's test. A two-way (treatment x time) ANOVA for matched values was used to compare paw thickness measured in mice at different times, and a further Newman–Keuls *t*-test was used to compare values at particular times between drug- and solvent-treated mice. Values related to the levels of CCL3 and CCL5 and CCL3 mRNA expression were compared by Student's *t*-test. Significances were established at $p < 0.05$.

Results

Antinociceptive effects induced by the CCR1 antagonist J113863 in mice with acute or chronic peripheral inflammation.

The s.c. administration of J113863 (3–30 mg/kg; 30 min. before) dose dependently inhibited thermal hyperalgesia measured in mice inflamed with either carrageenan (fig. 1A) or CFA (fig. 1B). In both cases, 3 mg/kg did not modify hyperalgesia, 10 mg/kg evoked an intermediate effect ($p < 0.01$ *versus* vehicle) in carrageenan- or CFA-treated mice and 30 mg/kg completely suppressed hyperalgesia ($p < 0.001$ *versus* vehicle).

Furthermore, the administration of 30 mg/kg of J113863 significantly reduced mechanical allodynia in mice inflamed with CFA ($p < 0.01$ *versus* vehicle) (fig. 2B). In contrast, the same dose did not modify carrageenan-evoked allodynia (fig. 2A). Interestingly, the administration of 30 mg/kg of J113863 did not reduce mechanical allodynia measured 6 hr after the injection of CFA (fig. 2C).

The acute administration of the maximal antinociceptive dose of J113863 does not reduce carrageenan- or CFA-induced inflammation in mice.

To assess the possible involvement of an acute anti-inflammatory effect in the antihyperalgesic action evoked by J113863, we designed experiments to measure whether the acute admin-

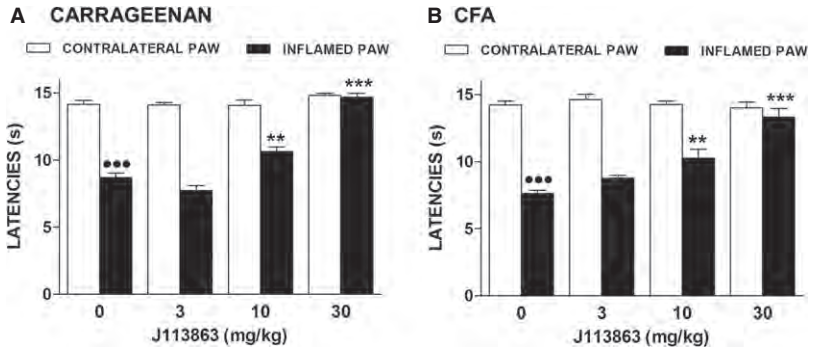

Fig. 1. The administration of J113863 inhibits thermal hyperalgesia in mice with acute or chronic inflammation. Inhibition of thermal hyperalgesia measured by the unilateral hot plate test after the s.c. administration of J113863 (3–30 mg/kg, 30 min. before) in mice inflamed with carrageenan 6 hr before (A) or CFA 1 week before (B). Data are expressed as means ± S.E.M. (n = 5–6). ●●●$p < 0.001$ compared with its corresponding, contralateral, left paw of solvent-treated group, Student's *t*-test; **$p < 0.01$, ***$p < 0.001$ compared with the ipsilateral paw of solvent-treated group, Dunnett's *t*-test.

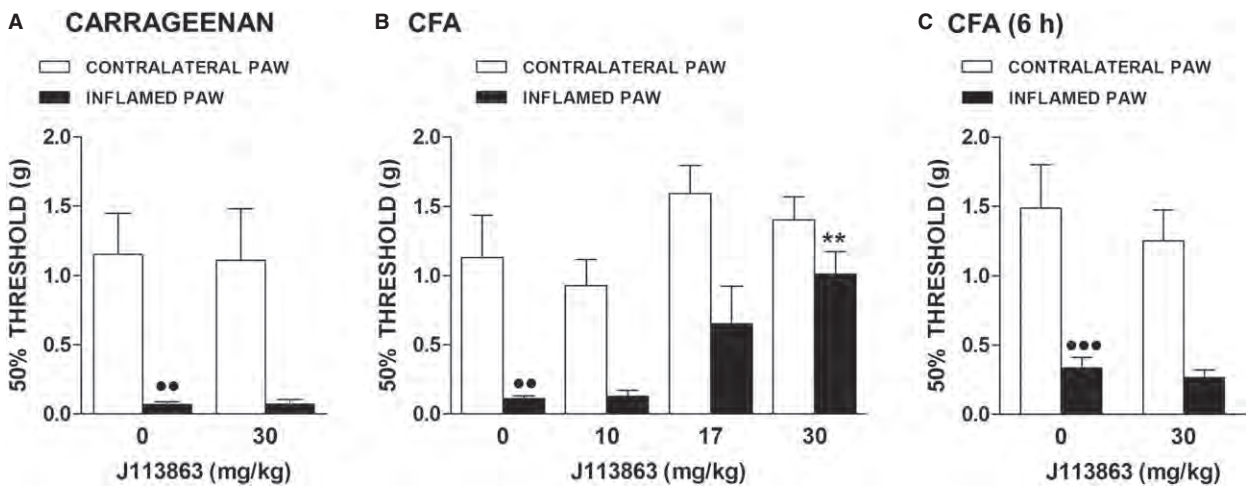

Fig. 2. The acute administration of J113863 inhibits mechanical allodynia in mice with chronic, but not acute, inflammation. (A) Lack of effect of J113863 (30 mg/kg s.c., 30 min. before) on mechanical allodynia measured by the von Frey test in mice inflamed with carrageenan 6 hr before. (B) Inhibition of mechanical allodynia evoked by J113863 (10–30 mg/kg s.c., 30 min. before) in mice inflamed with CFA 1 week before. (C) Lack of effect of J113863 (30 mg/kg s.c., 30 min. before) on mechanical allodynia evoked by the administration of CFA 6 hr before. Data are expressed as means ± S.E.M. (n = 5–10). ●●$p < 0.01$; ●●●$p < 0.001$ compared with its corresponding, contralateral, left paw of solvent-treated group, Mann–Whitney's *U*-test; **$p < 0.01$, compared with the ipsilateral paw of solvent-treated group, Dunn's test.

istration of the CCR1 antagonist could avoid the increase in dorsoplantar diameter of hind paws treated with carrageenan or CFA. As shown in fig. 3A, a time-dependent inhibition of hind paw swelling is evoked in mice receiving carrageenan after the administration of dexamethasone (10 mg/kg, i.p.) being the measure of dorsoplantar diameter significantly reduced 4.5 hr and 6.5 hr after dexamethasone administration ($p < 0.01$ and $p < 0.001$ *versus* solvent-treated mice) (fig. 3A). In contrast, neither a single injection of J113863 (30 mg/kg) 30 min. before carrageenan (fig. 3B) nor two administrations of this dose 30 min. before and 3 hr after carrageenan modified the increase in paw tumefaction (fig. 3C).

Similarly, a control experiment with dexamethasone was performed in mice that received 1 week before CFA intraplantarly. As shown in fig. 3D, the administration CFA evoked a significant increase in paw diameter that was significantly reduced 4.5 hr and 6.5 hr after the i.p. administration of 10 mg/kg of this glucocorticoid ($p < 0.05$ and $p < 0.01$ *versus* solvent-treated mice). In contrast, as occurred in carrageenan-treated mice, a single administration of 30 mg/kg of J113863 did not modify CFA-evoked paw swelling measured 30 min. after (fig. 3E) and the repeated injection of this CCR1 antagonist with a 3.5-hr interval did not reduce paw diameter at any time studied (fig. 3F).

Measurement of CCL3 and CCL5 levels in inflamed paws and spinal cord of acutely or chronically inflamed mice.

CCL3 concentrations measured by ELISA in solvent-treated paws were about 3 pg/mg protein, and an almost ten times enhancement was obtained in paws inflamed with either carrageenan or CFA ($p < 0.01$ and $p < 0.0001$, for car-

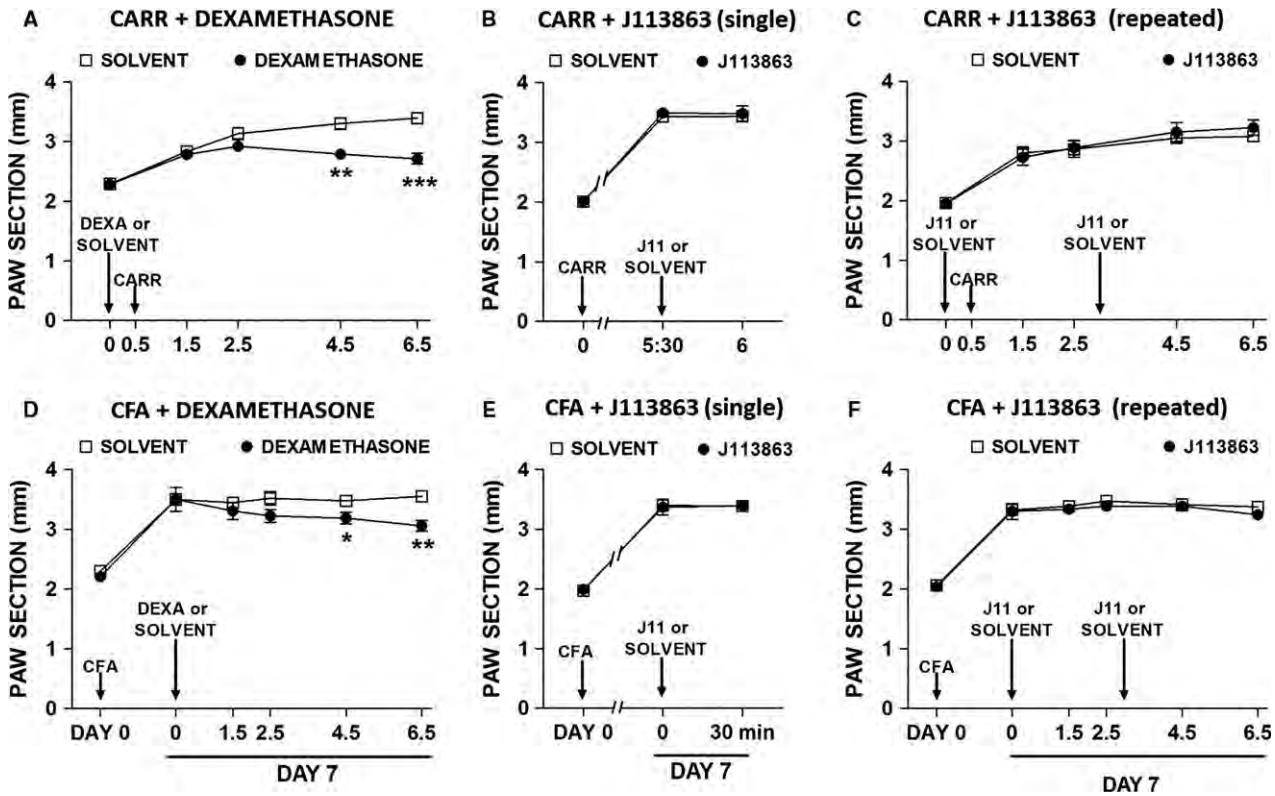

Fig. 3. The acute administration of J113863 does not modify carrageenan- or CFA-evoked increase of paw thickness. In (A) and (D), decreased paw section values obtained in carrageenan (CARR, A)- or CFA(D)-inflamed mice treated with a single injection of dexamethasone (10 mg/kg) or solvent. Right hind paw sections were measured just before administering dexamethasone or solvent prior to the injection of carrageenan or 1 week after the injection of CFA and 1.5, 2.5, 4.5 and 6.5 hr after. Means and corresponding standard errors are represented (n = 5). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ comparisons between solvent- or dexamethasone-treated mice at different times, Newman–Keuls test. In (B) and (E), lack of changes in paw section following the single s.c. administration of 30 mg/kg of J113863 (J11) or solvent 30 min. before testing to mice i.pl. injected with carrageenan (CARR, B) or CFA (E). Right hind paw sections were measured prior to the injection of the inflammatory agent, just before administering J113863 or solvent and 30 min. after. Means and corresponding standard errors are represented (n = 5). In (C) and (F), lack of changes in paw tumefaction following two s.c. administrations of 30 mg/kg of J113863 (J11) or solvent at time 0 and 3 hr after to mice treated with carrageenan (CARR, C) or CFA (F). Right hind paw sections were measured prior to the injection of the inflammatory agent, just before the first administration of J113863 and 1.5, 2.5, 4.5 or 6.5 hr after in mice treated with CARR or CFA. Means and corresponding standard errors are represented (n = 4).

rageenan and CFA, respectively, *versus* solvent-treated groups) (fig. 4A). In contrast, the concentration of CCL3 measured in spinal cord homogenates was not altered by acute or chronic inflammation (fig. 4C). Inflammation produced by either carrageenan or CFA did not modify the concentration of CCL5 in paws (fig. 4B) or spinal cords (fig. 4D).

Increase in CCL3 mRNA and immunohistochemical localization of CCL3 in acute- and chronic-inflamed paws.

A significant increase in CCL3 mRNA expression was detected by qPCR when comparing the relative fold change expression obtained in paws inflamed with carrageenan (fig. 5A) or CFA (fig. 5B) with their corresponding solvent-treated controls ($p < 0.01$ and $p < 0.001$, respectively).

Immunohistochemical experiments were designed to detect CCL3 expression in inflamed paws. Initial experiments demonstrated CCL3 staining in corneal epithelium that was used as a control tissue (fig. 6A) and its complete disappearance when tissues were incubated in the absence of anti-CCL3 antibody (fig. 6B). In carrageenan (fig. 6C)- and CFA (fig. 6D)- inflamed paws, CCL3 immunostaining was detected in neutrophils (fig. 6E) and macrophages (fig. 6F), with expression in macrophages being more frequent in chronically than in acutely inflamed mice. CCL3 immunostaining was almost completely absent in control paws (not shown).

Antinociceptive effect induced by the administration of an anti-CCL3 antibody in mice with acute or chronic peripheral inflammation.

We performed an initial experiment to determine the ability of an anti-CCL3 antibody to prevent CCL3-evoked hyperalgesia. The i.pl. injection of 250 ng of CCL3 evokes significant thermal hyperalgesia ($p < 0.0001$ *versus* contralateral non-injected paw) that was completely antagonized by the co-administration of 3 μg of the anti-CCL3 antibody ($p < 0.0001$; fig. 7A). Interestingly, the administration of the same dose of this antibody completely reverted thermal hyperalgesic responses in carrageenan (fig. 7B)- and CFA-inflamed (fig. 7C) mice

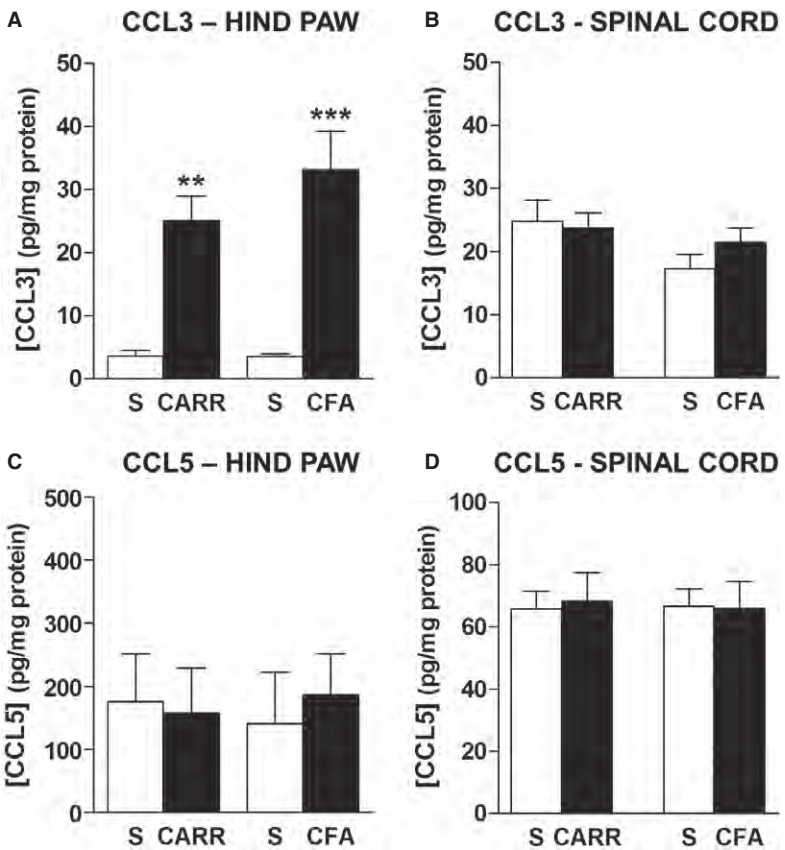

Fig. 4. Increased CCL3 concentrations are present in inflamed paws. CCL3 and CCL5 concentrations measured by ELISA in hind paws (A, C) or spinal cord (B, D) of mice treated with an i.pl. injection of carrageenan (CARR) or saline (S) 6 hr before or with CFA or solvent (S) 1 week before. (A) CCL3 concentrations measured in the right hind paws of mice treated with saline, carrageenan or CFA (n = 5–7). (B) CCL3 concentrations measured in the lumbar spinal cord of mice treated with saline, carrageenan or CFA (n = 6–7). (C) CCL5 concentrations measured in the right hind paws of mice treated with saline, carrageenan or CFA (n = 6). (D) CCL5 concentrations measured in the lumbar spinal cord of mice treated with saline, carrageenan or CFA (n = 5). Means and their S.E.M. are represented. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with its corresponding saline-treated group, Student's *t*-test.

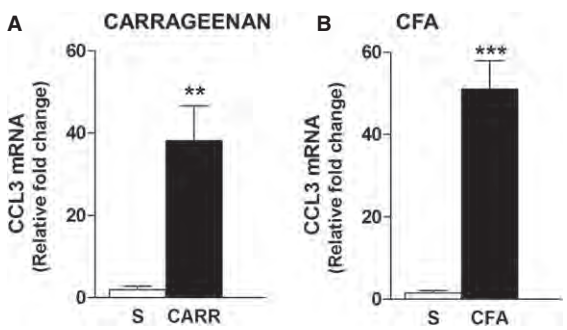

Fig. 5. mRNA CCL3 expression is increased in inflamed paws. Expression of CCL3 mRNA determined by qPCR in the plantar side of hind paws treated either with an i.pl. injection of carrageenan (CARR) or saline (S) 6 hr before or with CFA or saline (S) 1 week before. Relative expression levels of CCL3 mRNA in paws treated with saline or carrageen (A) or saline or CFA (B). The expression of β-actin mRNA was used as internal control. Means and their S.E.M. are represented (n = 6). $^{**}p < 0.01$, $^{***}p < 0.001$, compared with its corresponding saline-treated group, Student's *t*-test.

($p < 0.0001$ *versus* solvent in both cases). With respect to CFA-evoked mechanical allodynia, the co-administration of 3 or 5 μg of the antibody did not significantly modify threshold values, and the administration of 10 μg of anti-CCL3 antibody restored the threshold values to normal ($p < 0.01$, compared to vehicle) (fig. 7D).

Discussion

Our data demonstrate an antinociceptive effect following the acute administration of the CCR1 antagonist J113863 in mice with acute or chronic inflammation induced by the i.pl. administration of carrageenan 6 hr or CFA 1 week before, respectively. The antinociceptive action evoked by J113863 is not due to an anti-inflammatory effect and seems related to the blockade of the hypernociceptive action exerted by the chemokine CCL3, whose levels are augmented in inflamed paws. No changes in CCL5 concentration were detected in response to inflammation.

The administration of a CCR1 antagonist has been previously reported to reduce acetic acid-induced mouse visceral pain and mechanical hyperalgesia measured 24 hr after CFA injection in rats [14]. Our results demonstrating the complete and dose-dependent inhibition of inflammatory hyperalgesia and allodynia produced by J113863 extend and reinforce the notion that the blockade of CCR1 can constitute an interesting

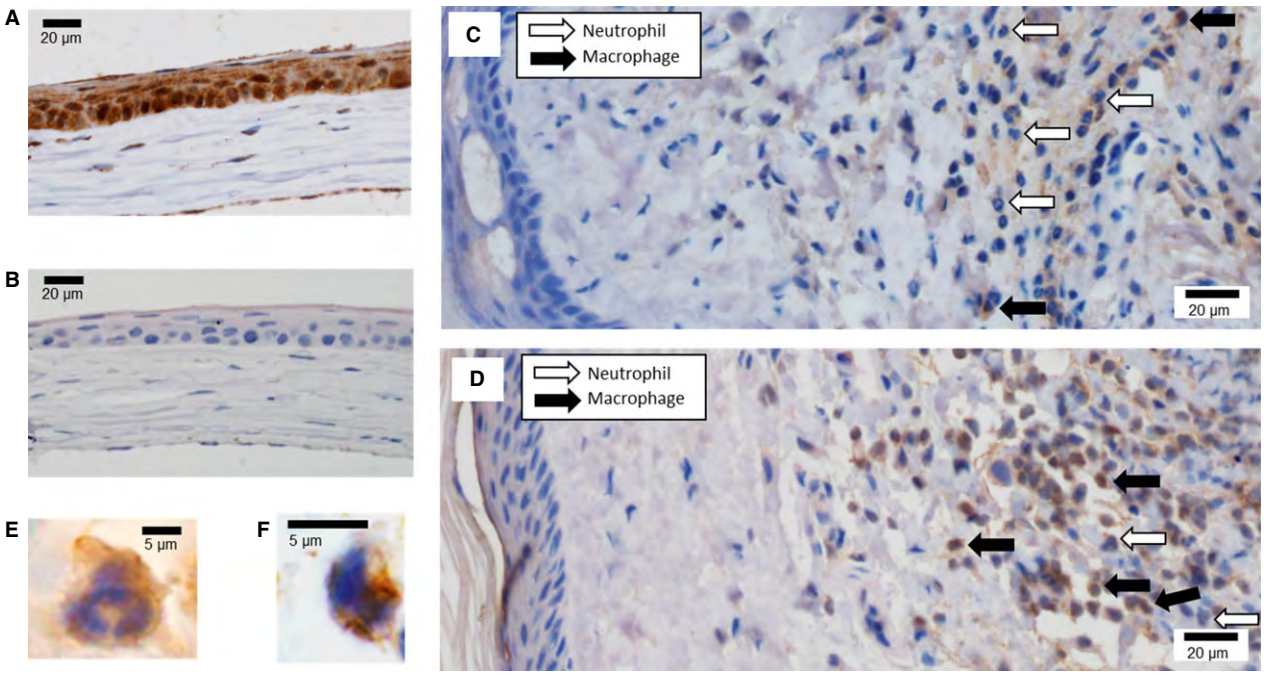

Fig. 6. Expression of CCL3 in neutrophils and macrophages in carrageenan- and CFA-inflamed paws. CCL3 staining in hind paws of mice inflamed with carrageenan 6 hr before or CFA 1 week before. (A) Positive control of CCL3 staining performed in mouse corneal epithelium. (B) Negative control of CCL3 staining in corneal epithelium in the absence of primary antibody. (C–D) Immunohistochemical CCL3 staining of the plantar side of paws inflamed with carrageenan (C) or CFA (D). White and black arrows point stained CCL3-expressing neutrophils and macrophages, respectively. (E) Example of CCL3 staining in a neutrophil present in a CFA-treated mouse. (F) Example of CCL3 staining in an isolated macrophage present in a carrageenan-treated mouse.

target to achieve analgesia in inflammatory settings. Although this CCR1 antagonist inhibited hyperalgesia evoked by either acute or chronic inflammation, only mechanical allodynia due to CFA but not to carrageenan was reduced after its administration. We have no mechanistic explanation for the exclusive effect of J113863 on allodynia induced by CFA, but the duration of the inflammatory process rather than the inflammatory agent used seems to be critical for differences in response since the early acute allodynia detected 6 hr after CFA administration was also CCR1-insensitive, as occurred 6 hr after carrageenan. The poor ability of a quaternary ammonium compound such as J113863 [28] to cross the blood–brain barrier makes unlikely the involvement of spinal mechanisms in its anti-allodynic effect in chronic inflammation. The different response to J113863 in carrageenan- or CFA-treated mice seems unrelated to changes in the expression of CCL3 or CCL5 in inflamed tissues because it was similar after carrageenan or CFA treatment.

Once demonstrated that the acute administration of J113863 can evoke analgesic effects in inflamed mice, we have tried to determine whether they could derive from a putative anti-inflammatory action. This possibility seemed feasible because the acute treatment with J113863 prior to the inflammatory stimulus can prevent cell infiltration or inhibit the production of particular chemokines and TNF-α [23] and consistent anti-inflammatory responses can be achieved after its chronic administration [28]. We initially checked that a single administration of dexamethasone (10 mg/kg) effectively reduced paw swelling in inflamed mice. In contrast, paw thickness evoked by carrageenan or CFA was not reduced after one or two administrations of the maximal antihyperalgesic dose of J113863, thus suggesting that the analgesic effect evoked by J113863 occurs independently of an anti-inflammatory effect.

The analgesic effects exhibited by J113863 could indicate an increased expression in inflamed tissues of a chemokine able to activate CCR1. CCL3 and CCL5 were considered as the more probable candidates to play such a role due to their well-recognized hypernociceptive effects [6,9,29] and to the previous description that both CCL3 [7,11,30] and CCL5 [10,19,31] can be up-regulated under different pathological conditions. In contrast, we have not considered the possible involvement of CCL7 (monocyte chemotactic protein 3, MCP-3) because, although it can also bind to CCR1, its pro-nociceptive effects seem related to CCR2 activation [32]. Our results indicate that neither CCL3 nor CCL5 spinal levels are increased in acutely or chronically inflamed mice. However, an increase of CCL3 mRNA expression was measured by qPCR in paws of carrageenan- and CFA-inflamed paws, as previously shown in mice treated with carrageenan [29] and CCL3 protein levels quantified by ELISA were dramatically increased in paws coming from mice receiving carrageenan or CFA. These results agree with the previously described increase of CCL3 levels measured 4–6 hr after the administration of carrageenan in a murine air pouch model [23]. The finding that CCL3 staining appears in macrophages and neutrophils of inflamed paws is in accordance with previous

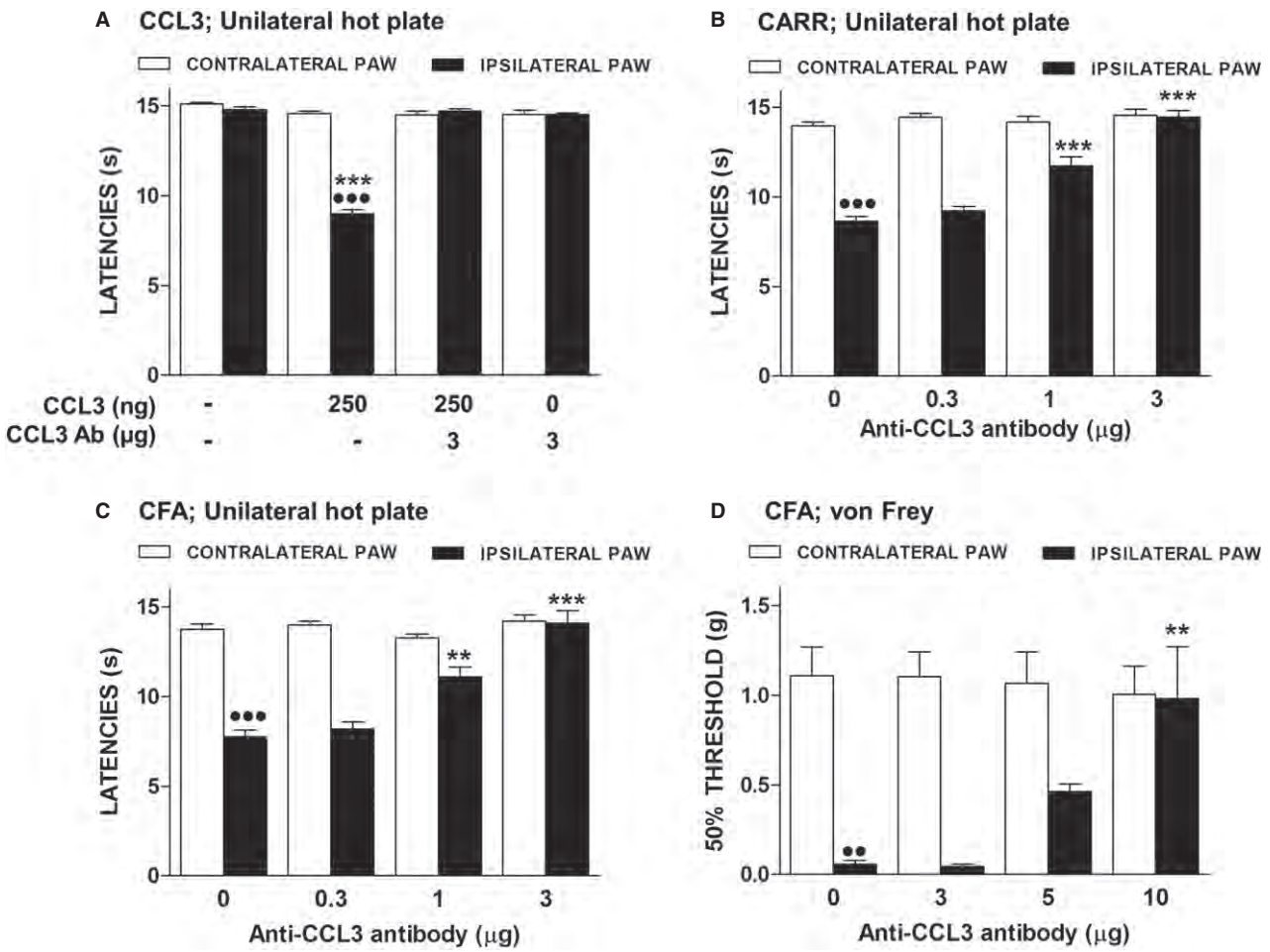

Fig. 7. Inflammatory thermal and mechanical allodynia are reduced by the local administration of an anti-CCL3 antibody. (A) Thermal hyperalgesia measured by the unilateral hot plate test 75 min. after the i.pl. administration of 250 ng of CCL3 and its reversion by the co-administration of 3 μg of an anti-CCL3 antibody. (B) Inhibition of thermal hyperalgesia after the i.pl. administration of an anti-CCL3 antibody (0.3–3 μg, 30 min. before) in mice inflamed with carrageenan (CARR) 6 hr before. (C) Inhibition of thermal hyperalgesia after the i.pl. administration of an anti-CCL3 antibody (0.3–3 μg, 30 min. before) in mice inflamed with CFA 1 week before. (D) Anti-allodynic effect evoked by the i.pl. administration of an anti-CCL3 antibody (3–10 μg, 30 min. before) on mechanical allodynia evoked by the administration of CFA 1 week before. Data are expressed as means ± S.E.M. (n = 4–9). In (A–C) ●●●$p < 0.001$ compared with its corresponding, contralateral, left paw of solvent group, Student's *t*-test; ***$p < 0.001$ compared with solvent-treated group, Dunnett's *t*-test. In (D) ●●$p < 0.001$ compared with its corresponding, contralateral, left paw of solvent group, Mann–Whitney's *U*-test; **$p < 0.01$, compared with solvent-treated group, Dunn's test.

studies showing that CCL3 expression can be triggered by inflammatory mediators such as TNF-α in macrophages [33] or IL-1 in neutrophils [34]. However, in contrast to the up-regulation described in rat joints inflamed with monosodium iodoacetate [3], no change in CCL5 levels was found in carrageenan- or CFA-treated paws, suggesting that different inflammatory settings can produce particular modifications in the level of chemokines.

Finally, as the involvement of CCR1 in inflammatory hyperalgesia could be due to the local increase of CCL3 in inflamed paws, we assessed whether the neutralization of this chemokine could effectively lead to antihyperalgesic effects. The i.pl. injection of an anti-CCL3 antibody completely counteracted both carrageenan- and CFA-evoked thermal hyperalgesia as well as mechanical allodynia evoked by CFA, an effect similar to that seen with the CCR1 antagonist. The fact that the dose of anti-CCL3 antibody necessary to antagonize CFA-induced mechanical allodynia is higher than the dose that inhibits thermal hyperalgesia suggests that a greater concentration of CCL3 is required to produce allodynia. In any case, these results are compatible with the possibility that the analgesic effects evoked by J113863 could be due to the blockade of nociceptive responses triggered by CCL3.

In conclusion, our results indicate that CCL3 levels are augmented in acutely or chronically inflamed paws of mice, probably due to its release from macrophages and neutrophils and that hypernociceptive symptoms present in these settings can be counteracted by its neutralization with an anti-CCL3 antibody. The enhanced presence of CCL3 can also explain the immediate analgesic effects, independent from an anti-inflammatory action, evoked by the administration of the CCR1 antagonist J113863 in carrageenan- and CFA-inflamed mice. These

results add new insights to the previously reported information related to the involvement of CCR1 in neuropathic [7,11,12,35] or neoplastic [10] pain. The possibility of inhibiting inflammatory pain following the acute administration of a CCR1 antagonist seems particularly interesting considering that CCR1 antagonists are already being tested in clinical trials, and that a CCR1 antagonist has been shown to improve rheumatoid arthritis without inducing remarkable adverse reactions [21].

Acknowledgements

Grants were provided by Ministerio de Economía y Competitividad (MINECO, SAF2012-36271) and Fundación para la Investigación Científica y Técnica de Asturias and FEDER (European Union) (FC-15-GRUPIN14-125). S.G-R. is recipient of a postdoctoral grant from Programa Clarín (Asturias)-Marie Curie-Cofund. IUOPA is supported by Fundación Bancaria Caja de Ahorros de Asturias (Asturias, Spain).

References

1 Abbadie C, Bhangoo S, De Koninck Y, Malcangio M, Melik-Parsadaniantz S, White FA. Chemokines and pain mechanisms. Brain Res Rev 2009;**60**:125–34.
2 White FA, Wilson NM. Chemokines as pain mediators and modulators. Curr Opin Anaesthesiol 2008;**21**:580–5.
3 Dawes JM, McMahon SB. Chemokines as peripheral pain mediators. Neurosci Lett 2013;**557** Pt A:1–8.
4 de Miguel M, Kraychete DC, Meyer Nascimento RJ. Chronic pain: cytokines, lymphocytes and chemokines. Inflamm Allergy Drug Targets 2014;**13**:339–49.
5 Zhang N, Rogers TJ, Caterina M, Oppenheim JJ. Proinflammatory chemokines, such as C-C chemokine ligand 3, desensitize mu-opioid receptors on dorsal root ganglia neurons. J Immunol 2004;**173**:594–9.
6 Zhang N, Inan S, Cowan A, Sun R, Wang JM, Rogers TJ *et al.* A proinflammatory chemokine, CCL3, sensitizes the heat- and capsaicin-gated ion channel TRPV1. Proc Natl Acad Sci USA 2005;**102**:4536–41. Erratum in: Proc Natl Acad Sci USA. 2005;102:7050.
7 Knerlich-Lukoschus F, von der Ropp-Brenner B, Lucius R, Mehdorn HM, Held-Feindt J. Spatiotemporal CCR1, CCL3(MIP-1α), CXCR4, CXCL12(SDF-1α) expression patterns in a rat spinal cord injury model of posttraumatic neuropathic pain. J Neurosurg Spine 2011;**14**:583–97.
8 Rajan AJ, Asensio VC, Campbell IL, Brosnan CF. Experimental autoimmune encephalomyelitis on the SJL mouse: effect of gamma delta T cell depletion on chemokine and chemokine receptor expression in the central nervous system. J Immunol 2000;**164**:2120–30.
9 Oh SB, Tran PB, Gillard SE, Hurley RW, Hammond DL, Miller RJ. Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. J Neurosci 2001;**21**:5027–35.
10 Pevida M, Lastra A, Meana A, Hidalgo A, Baamonde A, Menéndez L. The chemokine CCL5 induces CCR1-mediated hyperalgesia in mice inoculated with NCTC 2472 tumoral cells. Neuroscience 2014;**259**:113–25.
11 Kiguchi N, Maeda T, Kobayashi Y, Fukazawa Y, Kishioka S. Macrophage inflammatory protein-1alpha mediates the development of neuropathic pain following peripheral nerve injury through interleukin-1beta up-regulation. Pain 2010;**149**:305–15.
12 Kiguchi N, Kobayashi Y, Maeda T, Saika F, Kishioka S. CC-chemokine MIP-1α in the spinal cord contributes to nerve injury-induced neuropathic pain. Neurosci Lett 2010;**484**:17–21.
13 Yang HY, Mitchell K, Keller JM, Iadarola MJ. Peripheral inflammation increases Scya2 expression in sensory ganglia and cytokine and endothelial related gene expression in inflamed tissue. J Neurochem 2007;**103**:1628–43.
14 Lewis ND, Muthukumarana A, Fogal SE, Corradini L, Stefanopoulos DE, Adusumalli P *et al.* CCR1 plays a critical role in modulating pain through hematopoietic and non-hematopoietic cells. PLoS One 2014;**9**:e105883.
15 Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ *et al.* International Union of Basic and Clinical Pharmacology. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacol Rev 2014;**66**:1–79.
16 Chen JJ, Dai L, Zhao LX, Zhu X, Cao S, Gao YJ. Intrathecal curcumin attenuates pain hypersensitivity and decreases spinal neuroinflammation in rat model of monoarthritis. Sci Rep 2015;**5**:10278.
17 Wong L, Done JD, Schaeffer AJ, Thumbikat P. Experimental autoimmune prostatitis induces microglial activation in the spinal cord. Prostate 2015;**75**:50–9.
18 Yin Q, Fan Q, Zhao Y, Cheng MY, Liu H, Li J *et al.* Spinal NF-κB and chemokine ligand 5 expression during spinal glial cell activation in a neuropathic pain model. PLoS One 2015;**10**:e0115120.
19 Hang LH, Shao DH, Chen Z, Chen YF, Shu WW, Zhao ZG. Involvement of spinal CC chemokine ligand 5 in the development of bone cancer pain in rats. Basic Clin Pharmacol Toxicol 2013;**113**:325–8.
20 Hang LH, Li SN, Shao DH, Chen Z, Chen YF, Shu WW. Evidence for involvement of spinal RANTES in the antinociceptive effects of triptolide, a diterpene triepoxide, in a rat model of bone cancer pain. Basic Clin Pharmacol Toxicol 2014;**115**:477–80.
21 Tak PP, Balanescu A, Tseluyko V, Bojin S, Drescher E, Dairaghi D *et al.* Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. Ann Rheum Dis 2013;**72**:337–44.
22 Naya A, Sagara Y, Ohwaki K, Saeki T, Ichikawa D, Iwasawa Y *et al.* Design, synthesis, and discovery of a novel CCR1 antagonist. J Med Chem 2001;**44**:1429–35.
23 García-Ramallo E, Marques T, Prats N, Beleta J, Kunkel SL, Godessart N. Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. J Immunol 2002;**169**:6467–73.
24 Menéndez L, Lastra A, Hidalgo A, Baamonde A. Unilateral hot plate test: a simple and sensitive method for detecting central and peripheral hyperalgesia in mice. J Neurosci Methods 2002;**113**:91–7.
25 Baamonde A, Lastra A, Fresno MF, Llames S, Meana A, Hidalgo A *et al.* Implantation of tumoral XC cells induces chronic, endothelin-dependent, thermal hyperalgesia in mice. Cell Mol Neurobiol 2004;**24**:269–81.
26 Lange-Sperandio B, Trautmann A, Eickelberg O, Jayachandran A, Oberle S, Schmidutz F *et al.* Leukocytes induce epithelial to mesenchymal transition after unilateral ureteral obstruction in neonatal mice. Am J Pathol 2007;**171**:861–71.
27 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 2001;**25**:402–8.
28 Amat M, Benjamim CF, Williams LM, Prats N, Terricabras E, Beleta J *et al.* Pharmacological blockade of CCR1 ameliorates murine arthritis and alters cytokine networks in vivo. Br J Pharmacol 2006;**149**:666–75.
29 Eijkelkamp N, Heijnen CJ, Willemen HL, Deumens R, Joosten EA, Kleibeuker W *et al.* GRK2: a novel cell-specific regulator of

severity and duration of inflammatory pain. J Neurosci 2010;**30**:2138–49.

30 Kim D, You B, Lim H, Lee SJ. Toll-like receptor 2 contributes to chemokine gene expression and macrophage infiltration in the dorsal root ganglia after peripheral nerve injury. Mol Pain 2011;**7**:74.

31 Liou JT, Yuan HB, Mao CC, Lai YS, Day YJ. Absence of C-C motif chemokine ligand 5 in mice leads to decreased local macrophage recruitment and behavioural hypersensitivity in a murine neuropathic pain model. Pain 2012;**153**:1283–91.

32 Imai S, Ikegami D, Yamashita A, Shimizu T, Narita M, Niikura K *et al.* Epigenetic transcriptional activation of monocyte chemotactic protein 3 contributes to long-lasting neuropathic pain. Brain 2013;**136**:828–43.

33 Leichtle A, Hernandez M, Ebmeyer J, Yamasaki K, Lai Y, Radek K *et al.* CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF-/- mice. J Immunol 2010;**184**:3087–97.

34 Paulsson JM, Moshfegh A, Dadfar E, Held C, Jacobson SH, Lundahl J. In-vivo extravasation induces the expression of interleukin 1 receptor type 1 in human neutrophils. Clin Exp Immunol 2012;**168**:105–12.

35 Kiguchi N, Kobayashi Y, Saika F, Kishioka S. Epigenetic upregulation of CCL2 and CCL3 via histone modifications in infiltrating macrophages after peripheral nerve injury. Cytokine 2013;**64**:666–72.

3.3. ARTÍCULO 3

Llorián-Salvador M, Pevida M, Fernández-García MT, Lastra A, Obaya A, Cal S, Hidalgo A, Menéndez L, Baamonde A. **Hypernociceptive responses following the intratibial inoculation of RM1 prostate cancer cells in mice**. Prostate. 2015 75:70-83.

OBJETIVO

Estudiar las respuestas nociceptivas derivadas de la inoculación en la tibia de células RM1 derivadas de cáncer de próstata en ratones inmunocompetentes y determinar la posible participación de las quimiocinas CCL2, CCL3 y CCL5 en la hipernocicepción tumoral.

MÉTODOS

- Ratones C57BL/6 inoculados con células RM1 en la cavidad medular de la tibia.
- Transfección con luciferasa de las células RM1 para la obtención de imágenes de bioluminiscencia que permitan seguir la evolución del crecimiento del tumor.
- Tinción hematoxilina-eosina para el análisis histológico de los tumores.
- Ensayos inmunohistoquímicos para estudiar la posible activación de astroglía y microglía en la médula espinal durante el desarrollo del tumor.
- Placa caliente unilateral para medir la hiperalgesia térmica.
- Test de presión sobre la pata para medir la hiperalgesia mecánica.
- Prueba de von Frey para medir la alodinia mecánica.
- ELISA para la cuantificación de los niveles de CCL2, CCL3 y CCL5 en el medio de cultivo, tumores y médula espinal de ratones inoculados con células RM1.

RESULTADOS Y CONCLUSIONES

- La inoculación de 1000 células RM1 en la cavidad medular de la tibia provocó el desarrollo de tumores óseos con un patrón mixto osteoblástico/osteoclástico y la activación de astroglía, pero no de microglía, en la médula espinal.

- El crecimiento tumoral produjo hiperalgesia y alodinia a partir del cuarto día tras la inoculación. Ambos parámetros alcanzaron su máximo entre 12 y 16 días después de la inoculación de las células tumorales.

- La administración aguda de morfina o de ácido zoledrónico inhibió la hiperalgesia térmica y la alodinia mecánica de forma dependiente de la dosis.

- Las células RM1 en cultivo pueden liberar CCL2 y CCL5, pero no CCL3. En los tumores se observó un aumento de la concentración de CCL2 pero no de las otras quimiocinas. La administración de un antagonista de receptores CCR2, el RS504393, inhibió la hiperalgesia térmica, pero no la alodinia mecánica, en ratones con tumor.

En conjunto, nuestros resultados describen el desarrollo de hiperalgesia y alodinia en ratones inmunocompetentes inoculados con células RM1 procedentes de cáncer de próstata. Sus características histopatológicas así como su respuesta a morfina o ácido zoledrónico le confieren utilidad como modelo experimental para el estudio del dolor neoplásico de origen prostático. La implicación de la CCL2 en sus respuestas hipernociceptivas sugiere el interés de los antagonistas de receptores CCR2 como posibles analgésicos en este contexto.

Hypernociceptive Responses Following the Intratibial Inoculation of RM1 Prostate Cancer Cells in Mice

María Llorián-Salvador,[1] Marta Pevida,[1] María Teresa Fernández-García,[2] Ana Lastra,[1] Álvaro Obaya,[3] Santiago Cal,[4] Agustín Hidalgo,[1] Luis Menéndez,[1] and Ana Baamonde[1]*

[1]*Laboratorio de Farmacología, Facultad de Medicina, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, Asturias, Spain*
[2]*Unidad Histopatología Molecular en Modelos Animales de Cáncer, IUOPA, Asturias, Spain*
[3]*Área de Fisiología, Facultad de Medicina, IUOPA, Asturias, Spain*
[4]*Área de Bioquímica y Biología Molecular, Facultad de Medicina, IUOPA, Asturias, Spain*

BACKGROUND. Pain due to bone metastases of prostatic origin is a relevant clinical issue. We study here the nociceptive responses obtained in mice receiving the intratibial inoculation of RM1 prostate cancer cells.
METHODS. 10^2–10^5 RM1 cells were inoculated to C57BL/6 mice and tumor development was analysed histologically and with luciferase-expressing RM1 cells. Spinal astroglial (GFAP) or microglial (Iba-1) expression was assessed with immunohistochemical methods and hypernociception was measured by the unilateral hot plate, the paw pressure and the von Frey tests. The analgesic effect of morphine, zoledronic acid or the CCR2 antagonist RS504393 was measured. Levels of the chemokines CCL2, CCL3, and CCL5 were determined by ELISA.
RESULTS. The inoculation of 10^3 RM1 cells induced tumoral growth in bone with a mixed osteoclastic/osteoblastic pattern and evoked astroglial, but not microglial, activation in the spinal cord. Hyperalgesia and allodynia were already established four days after inoculation and dose-dependently inhibited by the s.c. administration of morphine (1–5 mg/kg) or zoledronic acid (1–3 mg/kg). CCL2 and CCL5, but not CCL3, were released by RM1 cells in culture whereas only an increased presence of CCL2 was found in bone tumor homogenates. The administration of the CCR2 antagonist RS504393 (0.3–3 mg/kg) inhibited RM1 induced thermal hyperalgesia without modifying mechanical allodynia.
CONCLUSION. The intratibial inoculation of RM1 cells in immunocompetent mice induces hypernociceptive responses and can be useful to perform studies of bone cancer induced pain related to androgen-independent prostate cancer. The antinociceptive role derived from the blockade of the CCR2 chemokine receptors is further envisaged. *Prostate 75: 70–83, 2015.*

KEY WORDS: **bone cancer-induced pain; prostate bone metastasis; mouse; morphine; zoledronic acid; chemokine CCL2**

INTRODUCTION

Bone metastases can appear during the development of common tumors, such as those affecting the prostate, breast, kidney or lungs and, since the invasion of bones by tumoral cells is frequently associated to pain symptoms [1], the incidence of bone cancer pain has a great impact in clinical settings. The development during the past decade of several experimental models of bone cancer-induced pain based on

Grant sponsor: MEC; Grant number: SAF2012-36271.
*Correspondence to: Ana Baamonde, Laboratorio de Farmacología, Facultad de Medicina, Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, c/ Julián Clavería 6, 33006 Oviedo, Asturias, Spain.
Received 24 June 2014; Accepted 15 August 2014
DOI 10.1002/pros.22893
Published online 27 September 2014 in Wiley Online Library (wileyonlinelibrary.com).

the inoculation of different types of tumoral cells into bones of mice or rats, has allowed the study of the properties of cancer pain and the assay of the efficacy of strategies to counteract it [2]. The multiple variables involved in the development of bone cancer-induced pain, such as the type of bone injury, the presence of a neuropathic or inflammatory reaction, the acidity produced in the tumor environment or the ability of a particular cell line to release different nociceptive mediators [3], justify the interest in studying the nociceptive responses originated by different types of experimental tumors, especially if they resemble a clinical situation.

Prostate cancer is one the most common forms of non-skin cancer in men in developed countries [4]. Although in its initial steps, androgen deprivation can maintain prostate cancer tumor growth under control for a consistent length of time, the process usually becomes insensitive to antiandrogenic therapy [5]. At this androgen-independent stage, more than 90% of patients show bone metastases [6] whose painful symptoms may be resistant to current analgesic therapies [1,5]. Several experimental approaches have been developed in rats following the inoculation of androgen-independent prostate tumoral cells, such as AT-3.1 or AT3B-1 [7–9] or MATLyLu cells [10] to the appropriate syngeneic strain [11]. In athymic mice, a first model was described based in the intrafemoral inoculation of prostatic ACE-1 cells [12]. Aiming to use immunocompetent mice to perform pain studies related to bone metastases of prostatic origin, we have selected the injection of RM1 cells, a cancer cell line derived from a mouse prostate reconstitution model [13]. This cell line can evoke tumoral growth after its intraosseus inoculation [14] and has been proposed as the reference one to experimentally induce androgen-independent prostate cancer [15]. Just recently, when our experiments were in their final phase, a publication has described painful behaviors in response to the intrafemoral inoculation of these RM1 cells [16].

We present here a methodological study of the nociceptive responses measured in C57BL/6 mice intratibially inoculated with RM1 cells. Initially, we have checked the ability of intratibial RM1 cells to evoke tumor growth and studied the histological characteristics of tumors produced as well as their progression with an in vivo image luminescence technique. Next, we have explored if tumor development evokes spinal glial activation and behavioral hypernociception measured by three different behavioral tests. The ability to inhibit these hypernociceptive symptoms after the administration of two reference drugs, such as morphine or zoledronic acid was also assessed and, finally, we have explored the involvement in the hypernociception evoked by this tumor of some chemokines, such as CCL2, CCL3, and CCL5, whose role in bone cancer-induced pain has been previously explored after the inoculation of different types of tumoral cells [17–20]. The results here described, together with those recently reported [16], support the usefulness of this model of bone cancer-induced pain and further suggest that chemokine CCR2 receptors could be useful targets to counteract painful symptoms associated to bone metastases derived from prostate cancer.

MATERIALS AND METHODS

Animals

Experiments were performed in 10–12 week old C57BL/6 male mice bred in the Animalario of the Universidad de Oviedo (Reg. 33044 13A), housed six per cage with a bedding of sawdust and maintained on a 12 hr dark–light cycle with free access to food and water. All the experimental procedures were approved by the Comité tico de Experimentación Animal de la Universidad de Oviedo (Asturias, Spain). Each animal was used only once.

Cell Culture and Inoculation

RM1 cells (kindly donated by Dr. Timothy Thompson, MD Anderson Cancer Center–University of Texas) were cultured in DMEM (low glucose) + glutamin (DMEM + GlutaMAX, Gibco) enriched with 10% fetal calf serum (Gibco), HEPES (1 M) (Cellgro), penicillin (10.000 U/ml)-streptomycin (10 mg/ml) (Biochrom AG). When cells were preconfluent, they were treated with trypsin/EDTA (0.05/0.02%) and detached. The trypsin/EDTA solution was neutralized with growth medium (1:1) and, after centrifugation at 400 *g* for 10 min, the remaining pellet was suspended in PBS.

For cell inoculation, anesthesia was induced by spontaneous inhalation of 3% isoflurane (Isoflo®, Esteve) and maintained by administering 1.5% isoflurane in oxygen through a breathing mask. Different suspensions of 10^2–10^5 cells in 5 μl of PBS were injected into the right tibial medullar cavity and after applying acrylic glue (Hystoacril®, Braun) on the tibial plateau incised area, surgery was finished with a stitch of the skin. Control mice received the inoculation of RM1 cells previously killed by quickly freezing them three times without cryoprotection.

Histology and Control of Tumor Growth

For histological analysis, the inoculated limbs were transferred to a decalcifying solution (4% formaldehyde, 14% formic acid) for 36 hr. Next, these were

thoroughly washed under tap water and conserved in 10% formaldehyde. Finally, 4 μm cross sections of paraffin-embedded formalin-fixed blocks were stained with hematoxylin and eosin. Following antigen retrieval in PTLink with Envision FLEX Target Retrieval Solution High pH, 20 min at 95°C (Dako), immunohistochemical assays with anti-E-cadherin (monoclonal mouse anti-human, Dako, 1:25 for 20 min at room temperature) and anti-vimentin (D21H3, monoclonal rabbit, Cell Signaling, 1:100 overnight at 4°C) antibodies were performed in order to ascertain the epithelial or mesenchymal character of tumoral cells.

To control tumor growth in an IVIS® Lumina in vivo non invasive bioluminescence imaging system (Caliper Life Sciences), RM1 cells were stably transfected with the firefly luciferase (Luc). Luciferase was expressed under ubiquitin-C promoter cloned in p-GL3basic vector (Promega) and cells were transfected using lipofectamine reagent (Life Technologies) as recommended by the manufacturer. Transfected cells were grown in the presence of 0.8 mg/ml geneticin G418 sulphate and individual clones stably expressing luciferase (Luc-RM1) were selected by luminescence intensity in a Fujifilm LAS3000 mini. Three mice were inoculated with 10^3 Luc-RM1 cells and luminescence was measured at days 4, 8, 12, 16, or 20 after inoculation. To measure luminescence, mice were anesthetized with s.c. ketamine hydrochloride (90 mg/kg, Imalgene®) and xylazine (10 mg/kg, Rompum®), received an i.p. injection of 150 mg/kg of D-luciferin (Melford) and 10 min after, they were placed in a light-obstructing chamber in order to take pictures with a 10 min exposition time, that were processed with the Living Image® software.

Drugs and Drug Administration

Morphine hydrochloride (Ministerio de Sanidad, Spain) and zoledronic acid (Sigma) were dissolved in saline. The CCR2 receptor antagonist, RS504393 (Tocris), was solved in 5% DMSO at the highest concentration used. All drugs were s.c. administered under the fur of the neck in a final volume of 10 ml/kg. In all cases control animals received an injection of the corresponding solvents.

Nociceptive Assays

Unilateral hot plate test. As previously described [20], mice were gently restrained and the plantar side of the tested paw placed on a hot plate (IITC Life Science) set at 48.6°C. Measurements of withdrawal latencies from the heated surface of each hind paw were made separately at two minute-intervals and the mean of two measures was considered. A cut-off of 20 sec was established.

Paw pressure test. Mechanical withdrawal latencies were measured by a previously described adaptation of the Randall-Selitto method [20], in which a constant pressure stimulus is used. Mice were gently restrained and a pressure of 450 g was applied to their hind paws with an Ugo Basile 7200 apparatus, until a struggle reaction appears. Measurements of withdrawal latencies of each hind paw were made alternately at two minute-intervals and the mean of two measures made in each hind paw was considered. A 60 sec cut-off was established in order to prevent tissue damage.

Von Frey test. Mechanical allodynia was assessed by applying von Frey filaments (Stoelting) to the plantar side of the paws as previously reported [20]. Mice were placed on a wire mesh platform and 20 min were allowed for habituation. The von Frey filaments 2.44, 2.83, 3.22, 3.61, 4.08, and 4.56 were used and, starting with the 3.61 filament, six measurements were taken in each animal randomly starting by the left or right paw. Based on the "up and down" method, the observation of a positive response (lifting, shaking or licking of the paw) was followed by the application of the immediate thinner filament or the immediate thicker one if the response was negative. The 50% response threshold was calculated using the following formula: 50% g threshold $= (10^{Xf+\kappa\delta})/10{,}000$; where Xf is the value of the last von Frey filament applied; κ is a correction factor based on pattern of responses (from the Dixon's calibration table); δ is the mean distance in log units between stimuli (here, 0.4).

Enzyme-Linked Immunosorbent Assay (ELISA)

CCL2, CCL3, and CCL5 levels were measured in samples of RM1 cell culture medium taken either before incubation or when cells reached confluence, usually two days after their culture in 75 cm^2 flasks. Experiments were also performed in tissue homogenates prepared from hind limbs inoculated with live or killed tumoral cells or the contralateral ones. Following the procedure previously described [18,20], media and tissues were immediately frozen in liquid nitrogen and stored at −80°C until use. The ankle to knee denuded hind limbs, including bone and soft tissue were weighted and homogenized in a volume of 3 μl/mg in a buffer consisting in 0.1 M Tris, 0.15 M NaCl, 0.5% CTAB (Fluka) and a protease inhibitor (1 tablet/50 ml buffer, Roche Diagnostics) with a Polytron PT-MR3100 (Kinematica). Next, homogenates were centrifuged at 15,000 *g* for 15 min at 4°C. Protein concentration of supernatants was measured by a

BCA protein assay (Pierce) by using a spectrophotometer (Nanodrop 2000C, Thermoscientific).

Measurements were performed with the commercially available sandwich enzyme-linked immunosorbent assays R&D, DuoSet® Mouse, CCL2/MCP-1, CCL3/MIP-1α, and CCL5/RANTES. Plates (R&D) were coated overnight at room temperature with an antibody specific for mouse CCL2, CCL3, or CCL5. One hundred microliter of culture media or 100 μg of sample homogenates were added into wells. After washing, a 2 hr incubation period was performed with a second biotinylated anti-mouse CCL2, anti-mouse CCL3, or anti-mouse CCL5 antibody and followed by a 20 min incubation period with streptavidin-peroxidase (HRP). After washing to remove all unbound enzyme, colour was developed by adding tetramethylbenzidine: H_2O_2, (1:1) and the reaction was terminated with a stop solution (2N H_2SO_4). The intensity of the coloured product was quantified spectrophotometrically at 450 nm substracting the readings obtained at 570 nm in order to correct optical background of plates. Values obtained from culture media and limbs came from at least six independent samples performed in duplicate.

Immunohistochemical Assays

As previously described [21] spinal cords for experiments with GFAP and Iba-1 antibodies were taken from mice anesthetized with isoflurane (Isoflo®, Esteve) and decapitated. After sectioning them at thoracic and sacral levels, the lumbar cord enlargement was immersed for 24 hr in 4% formaldehyde diluted in PBS 0.1 M at 4°C. In all cases, spinal cords were cryoprotected by 24 hr immersion in 30% sucrose at 4°C and 30 μm thick sections were obtained using a freezing microtome (Mikrom HM430).

Sections fixed in slices to measure GFAP or Iba-1 immunoreaction, were initially incubated in cold acetone (Prolabo) for 10 min, rinsed during 30 min in PBS (0.01 M) and further incubated at 4°C overnight in a humid chamber with primary antibodies. The primary antibodies used were a polyclonal rabbit anti-glial fibrillary acid protein (GFAP) antibody (Dako; 1:500 in 0.01 M PBS) or a polyclonal rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba-1) antibody (Wako; 1:250 in 0.01 M PBS). Previously to the incubation with the Iba-1 primary antibody, sections were immersed for 30 min in 0.01 M PBS containing 0.1% Triton X-100. After overnight incubation, sections were rinsed for 30 min in 0.01 M PBS, incubated 1.5 hr at room temperature in a dark humid chamber with green Alexa fluor® 488-conjugated goat anti-rabbit IgG (Life Technologies; 1:250 in 0.01 M PBS), washed 30 min in 0.01M PBS (containing 0.1% Triton X-100 for slices with Iba-1 previously) and, finally, mounted and cover slipped using Fluoromount-G® (Southern Biotech). As a procedural control, primary antibodies were omitted and replaced by PBS and under these conditions no staining occurred.

Sections were imaged using a BX61 Olympus microscope with a 4×/0.16NA objective and a quantitative analysis of the GFAP or Iba-1 immunofluorescence was performed by measuring the percentage of green immunoreactive surface with a computer-assisted imaging software analysis system (Image J version 1.43 u, NIH) [21]. For each experiment, the five slices with the greater staining were selected. In each case 5–8 independent experiments were performed.

Statistical Analysis

Mean values and their corresponding standard errors were calculated for the weights of mice, immunohistochemical image analysis, behavioral assays and ELISA measurements. To compare the development of the weight of mice inoculated with RM1 cells, the initial one-way analysis of variance (ANOVA) analysis was followed by the Newman-Keuls post hoc test to detect differences between groups at different times. The immunoreactive surface obtained when labeling with GFAP or Iba-1 were compared by a Student's *t*-test. Thermal withdrawal latencies were compared by a Student's *t* test when two groups were considered, whilst comparisons of time course or with the solvent group were done by an initial ANOVA followed by the Dunnett's *t*-test. Threshold values obtained by the von Frey test in ipsilateral and contralateral paws were compared by the U Mann–Whitney's test and for time-course or comparisons with the solvent-treated group, an initial Kruskal-Wallis analysis followed by the Dunn's test was used. The values of chemokine levels obtained in tissue homogenates were compared by an initial ANOVA followed by the Newman–Keuls test. The criterion for statistical significance was $P < 0.05$.

RESULTS

Tumor Development After the Intratibial Inoculation of 10^2–10^5 RM1 Cells

In order to select an appropriate number of RM1 cells able to produce bone tumors after their intratibial administration, the percentage of mice that develops tumor in the tibia 20 days after the inoculation of 10^2–10^4 RM1 cells was calculated by examining hematoxylin-eosin stained sections of hind limbs. Whereas only 4/6 mice (66.6%) inoculated with 10^2 RM1 cells developed bone tumor, all mice receiving the intra-

tibial administration of 10^3 or 10^4 cells showed tumoral bone injury at day 20 (Fig. 1A). The weight of these same mice was recorded every four days after inoculation and a significant loss in body weight was only observed 20 days after the inoculation of 10^4 RM1 cells (Fig. 1B). RM1-evoked mortality was assessed in a separate group of mice inoculated with 10^3–10^5 (N = 9–13 per group). One hundred percent mortality was observed 20 days after the intratibial inoculation of 10^5 RM1 cells and 24 days after the administration of 10^4 cells. In contrast, only 20% of mice receiving 10^3 cells died at day 24, although mortality increased up to 90% at day 28 (Fig. 1C). Once verified that 10^2 cells is a cell number too small to assure tumor growth in the 100% of mice and taking into account that the mortality observed in week 3 increased markedly with the number of cells inoculated, we decided to inoculate 10^3 RM1 cells in order to allow behavioral studies during at least three weeks after inoculation.

The follow-up of tumor development by using an in vivo macroscopic image technique was performed in three mice receiving the intratibial inoculation of 10^3 Luc-RM1 cells. Whereas no signal was detected four days after inoculation, RM1 cell luminescence was already visible at day 8 at the proximal part of the tibia (Fig. 2). The signal measured at this time corresponds to a surface apparently larger than the tibial diameter, probably indicating the invasion of soft tissues surrounding the tibia, in coherence with the situation found in the microscopical analysis depicted below. The progressively greater luminescent surface measured in the following days fits well with the expansion of the tumor to more distal portions of the tibia and its surrounding tissues (Fig. 2). No signal of Luc-RM1 cells coming from other body regions, that could indicate the appearance of metastases, was observed.

Microscopic analysis of hematoxylin-eosin stained slices was performed 4, 8, 12, and 16 days after the inoculation of 10^3 RM1 cells (Fig. 3). Four days after cell inoculation, an important growth of tumoral RM1 spindle cells was observed in the bone medullary cavity accompanied in particular localisations by osteolityc injuries leading, in some cases, to microfractures that allowed tumoral cells to invade the external surface of bone and neighbour soft tissues (Fig. 3A). Tumoral growth progressed in such a way that a massive presence of tumoral cells appeared at muscle level at day 12 (Fig. 3B) and osteoblastic development in the external surface of the tibia was frequently observed at day 16 (Fig. 3C). From day 12 onwards, necrotic areas more clearly stained appeared inside the tumoral mass (Fig. 3D), as well as perineural infiltration (Fig. 3E). No clear inflammatory reaction appeared at any time studied and only a slight infiltration of PMN was seen at particular localisations.

Finally, we have checked that tumoral cells invading bone surrounding tissues 12 days after inoculation markedly express the respective epithelial and mesenchymal markers E-cadherin (Fig. 3F) and vimentin (Fig. 3G).

Immunohistochemical Expression of GFAP and Iba-1 in the Spinal Cord of Mice Intratibially Inoculated With 10^3 RM1 Cells

We have studied with immunohistochemical techniques if staining with markers of astroglial (GFAP) or

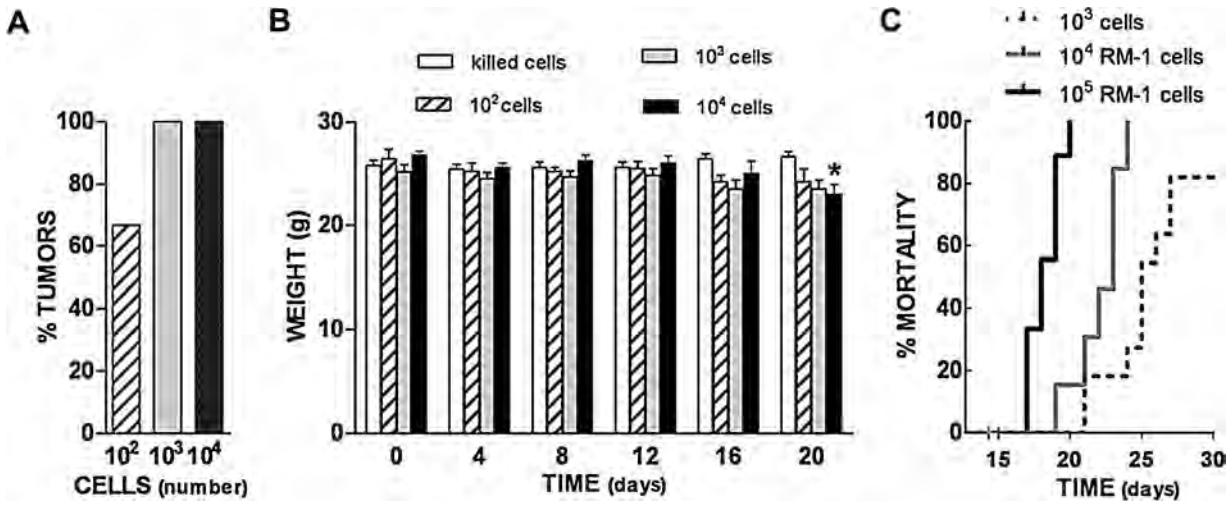

Fig. 1. (**A**) Percent of mice receiving the intratibial inoculation of 10^2–10^4 RM1 cells that develop tumors, as assessed by microscopic analysis 20 days after inoculation (N = 6); (**B**) Body weight of the same mice at different times after RM1 cell inoculation. Data are expressed as mean ± S.E.M. $^*P < 0.05$, compared with values obtained at the same time in the group inoculated with killed cells, Newman-Keuls test. (C) Percent of mice dead at different times after the inoculation of 10^2–10^5 RM1 cells (N = 9–13).

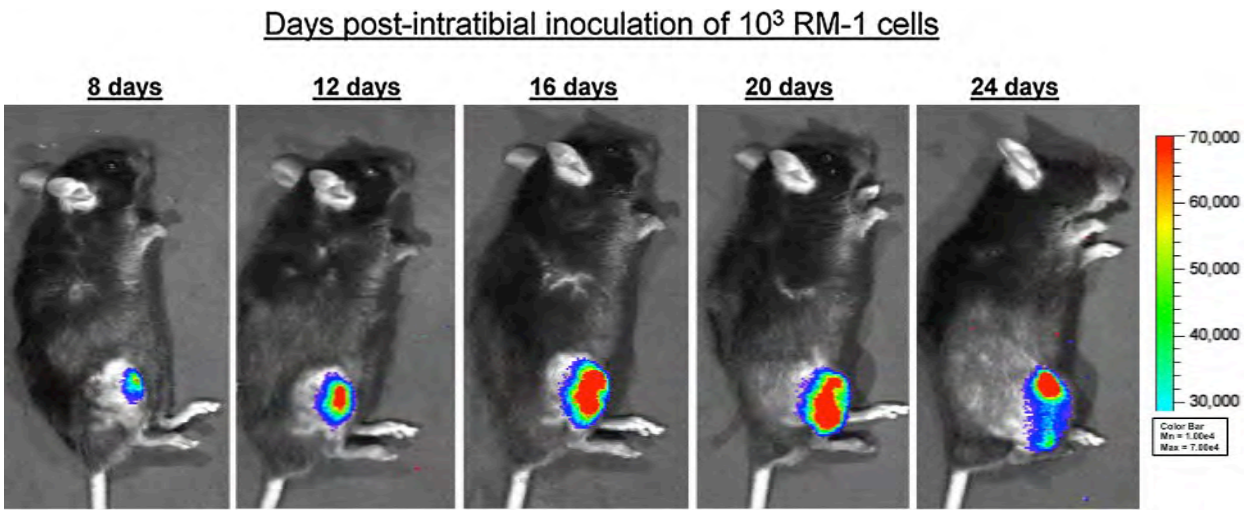

Fig. 2. Images of the luminiscence obtained at days 8, 12, 16, and 20 after the intratibial inoculation of 10^3 Luc-RMI cells by using an in vivo bioluminescence imaging system. The luminescence intensity scale is represented at the right hand side.

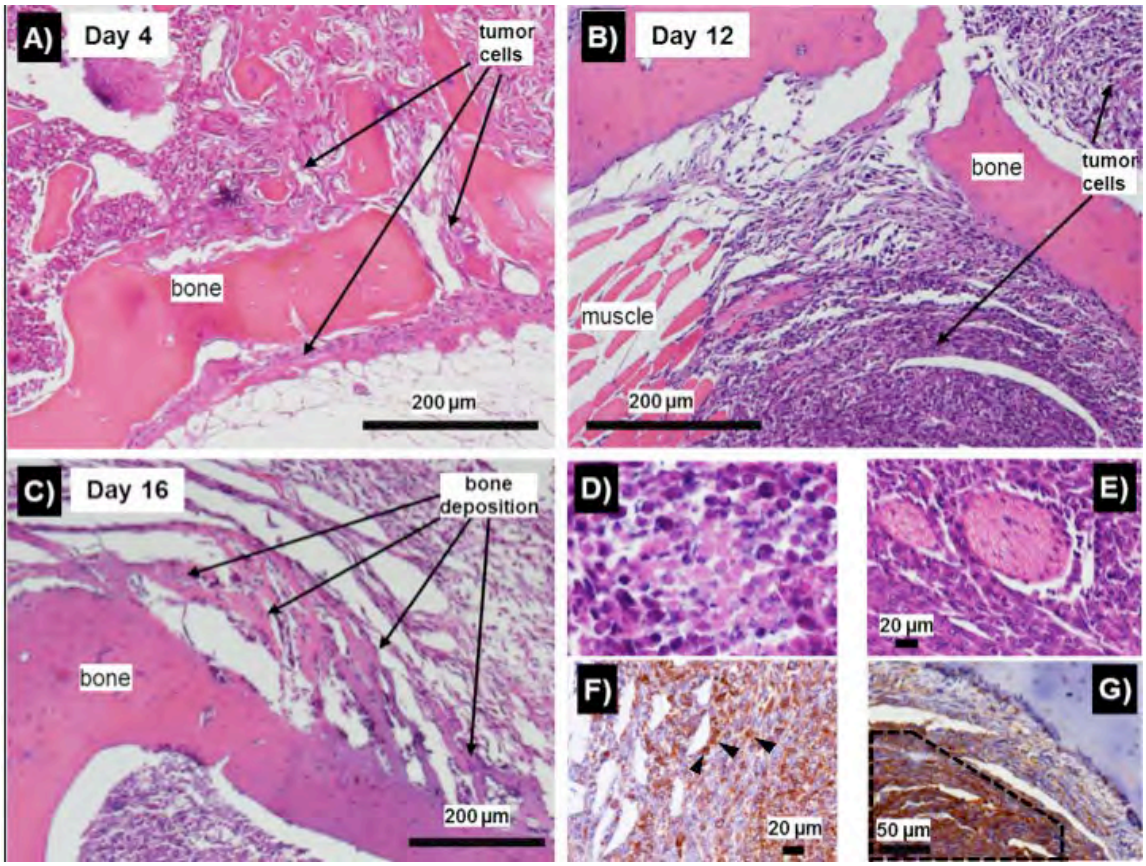

Fig. 3. Photomicrographs of hematoxylin and eosin-stained hind paw sections after the intratibial inoculation of 10^3 RMI cells in C57BL/6 mice. Four days after cell inoculation, cortical breaches allow cells to spread periostially (**A**). Twelve days after inoculation tumoral cells have invaded surrounding tissues (**B**). Marked periosteal bone deposition (**C**) clear stained necrotic areas inside the tumoral mass (**D**) and perineural infiltration (**E**) can be observed at day 16. Tumoral cells are stained with the anti-E-cadherin (**F**) and the anti-vimentin (**G**) antibodies 12 days after RMI inoculation. As shown, in (**F**), three clear examples of anti-E-cadherin staining are signaled by the arrow heads and in (**G**), the marked area corresponds to a very intense anti-vimentin staining.

microglial (Iba-1) activation could be increased in the spinal cord 12–16 days after the inoculation of 10^3 cells. As shown in Figure 4, GFAP immunoreactive surface was greater in the ipsilateral side of mice inoculated with live RM1 cells than in the corresponding contralateral side or in mice inoculated with killed cells. A more detailed analysis of GFAP distribution on the spinal laminae demonstrates that the greatest increase in GFAP staining occurs in laminae I–II, being also significant in laminae III–IV and in the ventral horn of mice inoculated with live RM1 cells (Fig. 4C). In contrast, the expression of the marker of microglial cells Iba-1 was not significantly enhanced in the spinal cord of mice inoculated intratibially with RM1 cells in any of the laminae studied (Fig. 5).

Assessment of Hyperalgesia and Allodynia After the Intratibial Inoculation of 10^3 RM1 Cells

Thermal and mechanical hyperalgesia as well as mechanical allodynia were assessed in mice intratibially inoculated with 10^3 RM1 cells 4, 8, 12, and 16 days after inoculation. The measurement of paw withdrawal latencies from the unilateral hot plate test revealed the establishment of significant thermal hyperalgesia four days after cell inoculation of live cells (Fig. 6A). This hyperalgesia increased slightly during the following days, reaching its maximum at days 12–14. No changes in withdrawal latencies were observed in mice inoculated with killed RM1 cells.

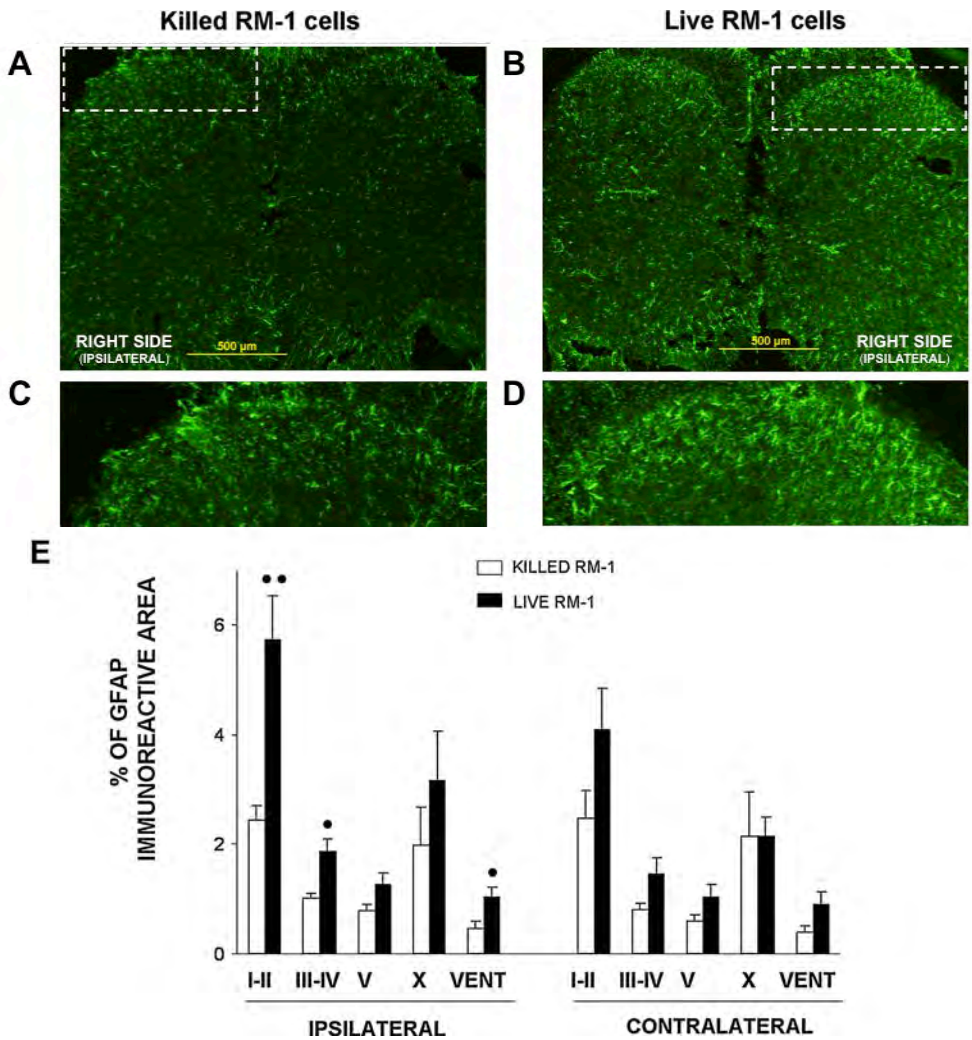

Fig. 4. Immunohistochemical staining obtained with an antibody against the astroglial marker GFAP in the lumbar spinal cord of mice inoculated with 10^3 killed (**A**) or live (**B**) RMI cells. In (**C**) and (**D**), the high-magnification insets of the corresponding areas marked with a dashed rectangle in (**A**) and (**B**) are shown. (**E**) Percent of GFAP immunoreactive area measured in five regions of the ipsilateral and contralateral spinal cords of mice inoculated either with killed (n = 6) or live (n = 9) RMI cells. Bars correspond to means and their SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with values obtained in the group inoculated with killed cells, Student's *t*-test.

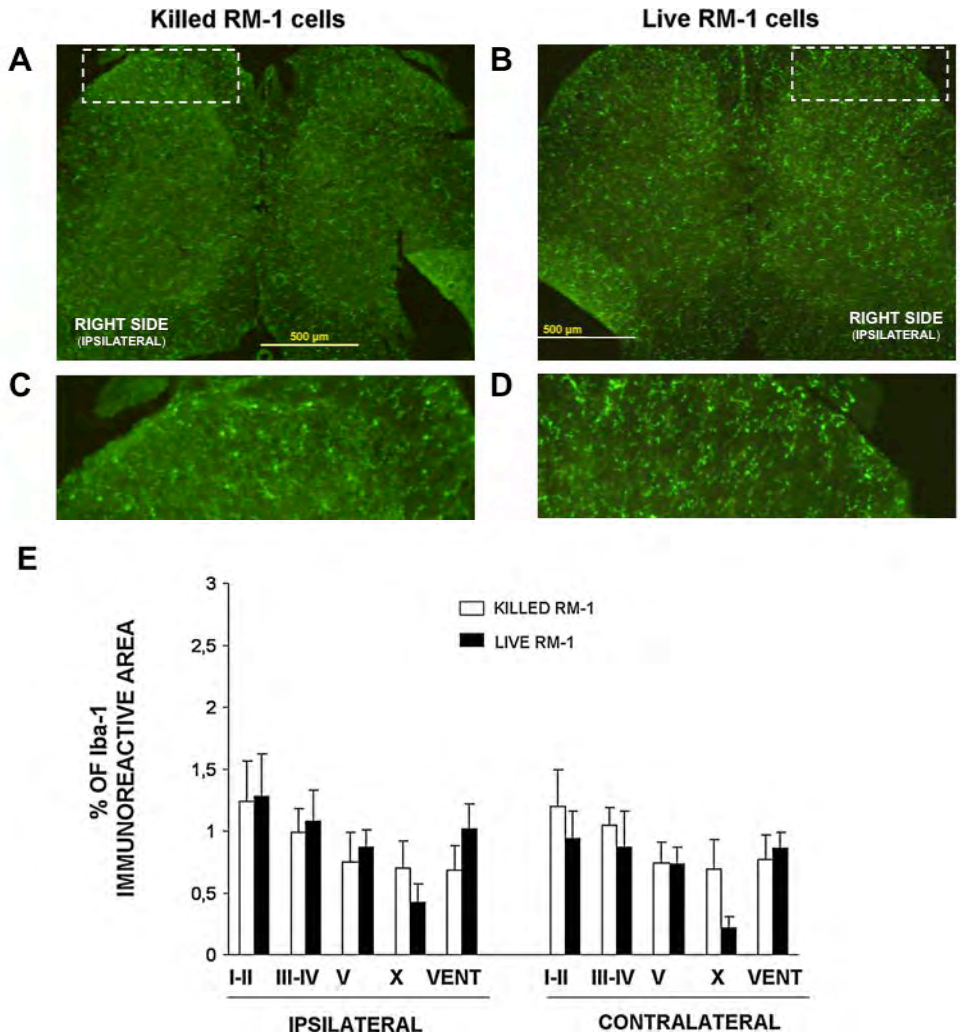

Fig. 5. Immunohistochemical staining obtained with an antibody against the microglial marker Iba-1 in the lumbar spinal cord of mice inoculated with 10^3 killed (**A**) or live (**B**) RMI cells. In (**C**) and (**D**), the high-magnification insets of the corresponding areas marked with a dashed rectangle in (**A**) and (**B**) are shown. (**E**) Percent of Iba-1 immunoreactive area measured in five regions of the ipsilateral and contralateral spinal cords of mice inoculated either with killed (n = 5) or live (n = 7) RMI cells. Bars correspond to means and their SEM.

A similar result was observed when measuring mechanical withdrawal latencies by a paw-pressure test since decreased withdrawal latencies were observed in the inoculated paws four days after inoculation and persisted until measurements finished, at day 16 (Fig. 6B). The latencies of mice inoculated with killed cells remained unaltered.

Finally, the measurement of the 50% mechanical thresholds with von Frey filaments showed that the values obtained in the paws inoculated with live RM1 cells were significantly lower than those obtained in contralateral paws or in those inoculated with killed cells, thus demonstrating the presence of mechanical allodynia from day 4 after cell inoculation and during all the period studied (Fig. 6C).

Antihyperalgesic and Antiallodynic Effect Evoked by Systemic Morphine and Zoledronic Acid in Mice Intratibially Inoculated With 10^3 RMI Cells

In order to assess the efficacy of two representative analgesic drugs used in the management of pain derived from bone metastasis of prostatic origin, we assessed the effects of morphine and the bisphosphonate zoledronic acid on thermal hyperalgesia and mechanical allodynia measured 12–16 days after the inoculation of 10^3 RM1 cells, a time at which although tumors are already in an advanced stage, there is still a considerable window period before mortality starts.

The s.c. administration of 0.1–1 mg/kg of morphine 30 min before testing dose-dependently inhibited ther-

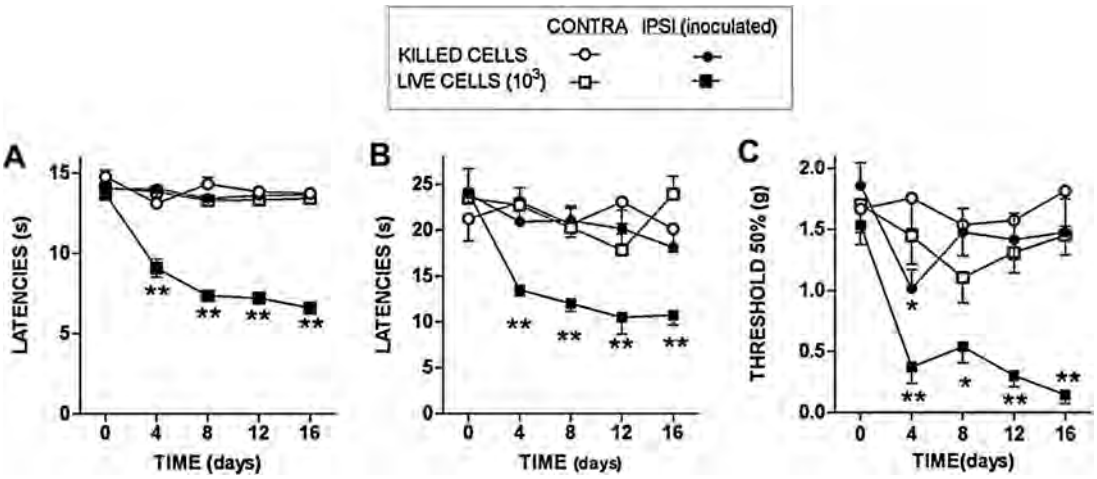

Fig. 6. Time course of thermal hyperalgesia measured in the unilateral hot plate test (**A**), mechanical hyperalgesia assessed by the paw pressure test (**B**) or mechanical allodynia measured in the von Frey test (**C**) in C57BL/6 mice receiving the intratibial inoculation of 10^3 live or killed RM1 cells in their right limbs. Data are expressed as mean ± S.E.M. (n = 6–10). In (**A**) and (**B**) $^{**}P < 0.01$, compared with values obtained in the corresponding group at day 0, Dunnett's *t*-test. In (**C**) $^{*}P < 0.05$, $^{**}P < 0.01$, compared with values obtained in the corresponding group at day 0, Dunn's test.

mal hyperalgesia, being the maximal antihyperalgesic effect produced after the administration of 1 mg/kg of this opiate receptor agonist (Fig. 7A). This dose did not evoke analgesia in mice inoculated with killed RM1 cells. Similarly, tumoral allodynia was also dose-dependently reduced after the s.c. administration of higher doses of morphine (1–5 mg/kg; Fig. 7B).

The acute s.c. administration of zoledronic acid 30 min before testing was also effective to reduce dose-dependently tumoral hypernociception, being again lower the doses necessary to counteract thermal hyperalgesia (0.3–1 mg/kg; Fig. 8A) than mechanical allodynia (1–3 mg/kg; Fig. 8B).

Release of CCL2 by RM1 Cells in Culture and Hind Paws Inoculated With RM1 Cells. Involvement of CCL2 and CCR2 in Hypernociception Evoked by RM1 Cells Inoculation

In order to check the ability of RM1 cells to release particular chemokines, the concentration of CCL2, CCL3, and CCL5 was measured by ELISA in culture

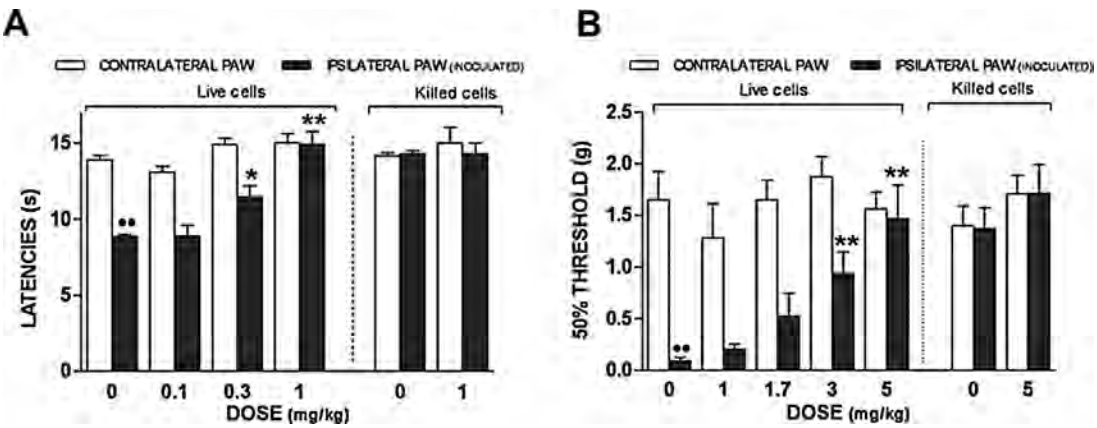

Fig. 7. Antinociceptive effects produced by the s.c. administration of morphine 30 min before testing 12–16 days after the intratibial inoculation of 10^3 killed or live RM1 cells by using the unilateral hot plate (**A**) or the von Frey (**B**) test. Data are expressed as means ± S.E.M. (n = 6–8). In (**A**) $^{\bullet\bullet}P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Student's *t* test; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with solvent-treated group, Dunnett's *t* test. In (**B**) $^{\bullet\bullet}P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Mann–Whitney's U test; $^{**}P < 0.01$, compared with solvent-treated group, Dunn's test.

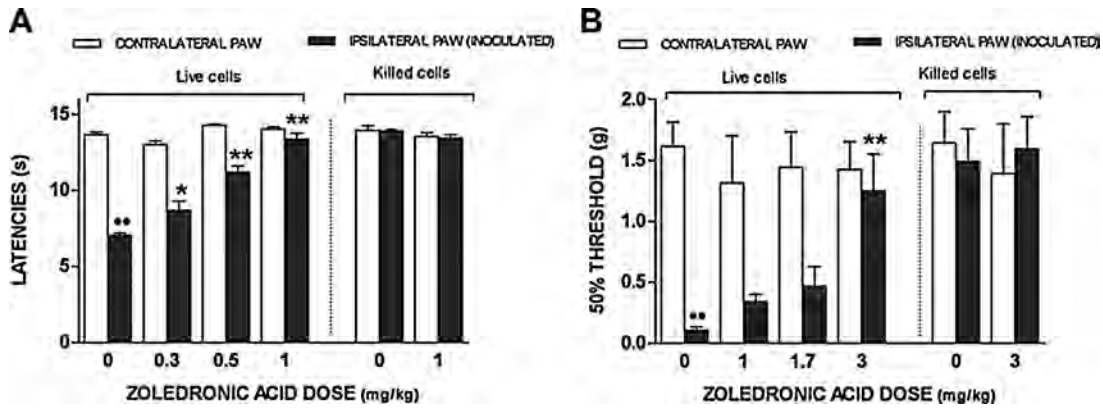

Fig. 8. Antinociceptive effects produced by the s.c. administration of zoledronic acid 30 min before testing 12–16 days after the intratibial inoculation of 10^3 killed or live RM1 cells by using the unilateral hot plate (**A**) or the von Frey test (**B**). Data are expressed as mean ± S.E.M. (n = 5–9). In (**A**) •• $P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Student's *t* test; * $P < 0.05$, ** $P < 0.01$ compared with solvent-treated group, Dunnett's *t*-test. In (**B**) •• $P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Mann–Whitney's U test; ** $P < 0.01$, compared with solvent-treated group, Dunn's test.

medium before and after RM1 cell incubation. None of these chemokines was detected in culture medium before cell culture, being their concentration below the threshold level of detection of the ELISA method used. RM1 cell incubation for two days did not evoke a detectable modification in CCL3 concentration and, in contrast, a consistent presence of CCL2 (101.4 ± 18 pg/ml; n = 6) and CCL5 (86.3 ± 13 pg/ml; n = 6) was measured after cell culture, thus demonstrating the ability of RM1 cells to release these chemokines.

ELISA assays were further performed in homogenates of the tibiae and surrounding tissues coming from hind paws of mice inoculated with either killed or live RM1 cells. A remarkable increase of CCL2 concentrations of more than 10 fold was observed in homogenates coming from limbs inoculated with live RM1 cells (Fig. 9A). Besides, whereas CCL3 levels were unaffected by tumor development (Fig. 9B), the presence of CCL5 was lower in mice inoculated with live cells, especially in homogenates of the ipsilateral limb (Fig. 9C).

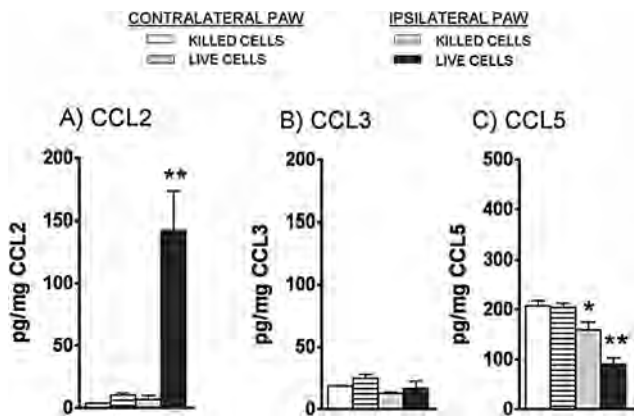

Fig. 9. Levels of CCL2 (**A**), CCL3 (**B**), and CCL5 (**C**) measured by ELISA assays in homogenates of contralateral or ipsilateral hind paws of mice intratibially inoculated with 10^3 live or killed RM1 cells 12–16 days before. Means and their corresponding standard errors are represented (n = 5–6). * $P < 0.05$, ** $P < 0.01$ compared with limbs of mice inoculated with killed cells, Newman-Keuls test.

Since the presence of increased levels of CCL2 in tumor-bearing paws made possible the involvement of this chemokine in tumoral hypernociception, we have next assayed if the blockade of CCR2 receptors by the selective antagonist RS 504393 could prevent it. Whereas a complete and dose-dependent inhibition of tumoral hyperalgesia was measured 30 min after the administration of RS504393 (0.3–3 mg/kg; Fig. 10A), the allodynic reaction evoked by tumor development was only slightly reduced after the administration of 10 mg/kg of the CCR2 antagonist (Fig. 10B).

DISCUSSION

In this study, we describe the nociceptive responses observed in C57BL/6 mice in response to the intratibial inoculation of RM1 cells, an androgen-independent prostate cancer cell line [13]. The presence of these cells induces the growth of a tumoral mass with an osteolytic and osteoblastic mixed pattern of bone injury. From four days after cell inoculation mice show mechanical and thermal hyperalgesia, as well as mechanical allodynia accompanied by astroglial activation in the spinal cord. Both hyperalgesia and allodynia evoked by RM1 cells were counteracted by the acute administration of morphine or zoledronic acid. Furthermore, the administration of the CCR2 antagonist RS 504393 dose-dependently antagonises

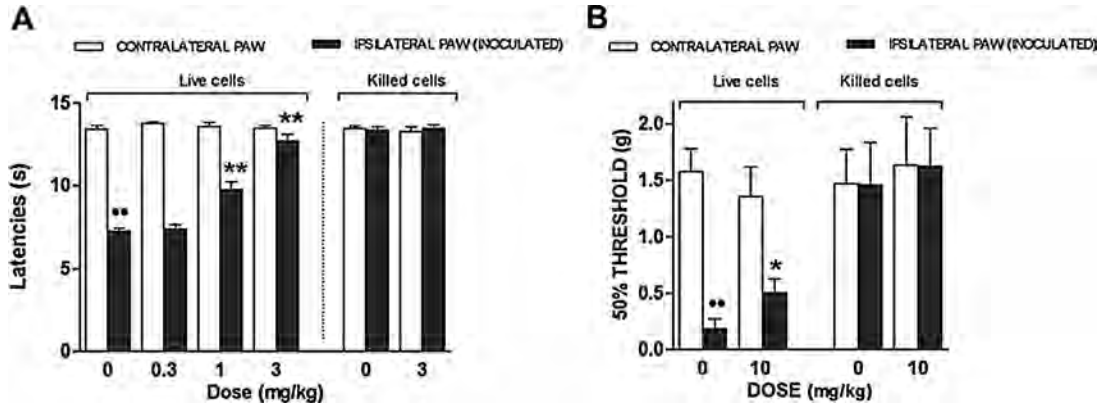

Fig. 10. (**A**) Antihyperalgesic effect produced by the s.c. administration of RS504393 30 min before testing 12–16 days after the intratibial inoculation of 10^3 killed or live RM1 cells by using the unilateral hot plate test. Means and their corresponding standard errors are represented (n = 5–7). •• $P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Student's *t*-test; ** $P < 0.01$ compared with solvent-treated group, Dunnett's *t*-test. (**B**) Effect of s.c. administered RS504393 on the allodynia measured 12–16 days after the intratibial inoculation of 10^3 killed or live RM1 cells by using the von Frey test. Means and their corresponding standard errors are represented (n = 10–11). •• $P < 0.01$ compared with its corresponding, contralateral, left paw of solvent group, Mann–Whitney's U test; *$P < 0.05$, compared with solvent-treated group, Dunn's test.

tumoral hyperalgesia probably by blocking the hyperalgesic effect of the increased levels of CCL2 present at tumor level due to the ability of RM1 cells to release this chemokine.

The properties of RM1 cells were rather similar to those described by other authors that also use RM1 cells provided by the laboratory led by Dr. Thompson in Texas, where they were originally developed [13]. Thus, in our hands the intratibial inoculation of 10^3 RM1 cells evoked the growth of bone tumors in the 100% of mice studied, as previously reported [14]. Moreover, our data showing 100% mortality 24 or 21 days after the inoculation of 10^4 or 10^5 cells, respectively, seem compatible with the 100% mortality 17 days after the s.c. inoculation of 10^4 RM1 cells previously reported [22]. In contrast, some recent studies describing the administration of a greater number of RM1 cells (10^6) reported either the performance of behavioral assays 21 days after their intrafemoral inoculation [16] or even a survival period of about 38 days after their s.c. inoculation [23]. When analyzing in detail the methodology described in these reports our impression was that RM1 cells coming from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences at Shanghai [16,23] could show lower aggressiveness than RM1 cells that, as in our case and others [14,22], were provided by Dr. Thompson laboratory. In any case, since the intratibial inoculation of 10^3 RM1 cells evoked tumor development allowing a survival of about three weeks, we designed further experiments after the inoculation of this cell number.

The time course of tumoral growth evoked by 10^3 RM1 cells was confirmed by measuring the luminescence following the intratibial inoculation of luciferase-expressing RM1 cells, an experimental approach previously used after s.c. administration [23]. Luminiscence in the tibial region proximal to the injection site started to be detected eight days after inoculation and progressively expanded along the tibia and adjacent tissues for the next three weeks. The lower luminescence measured at day 21 at the central part of the tumoral mass could be related to the development of tissue necrosis that was further confirmed by histological examination, as previously observed with other cell lines [24]. Microscopic analysis revealed intense osteoclastic activity together with periosteal bone deposition, in accordance with a previous description [14]. The appearance of both osteolytic and osteoblastic components resembles clinical settings of prostate cancer bone metastases in which markers of bone resorption are often increased and neoformation due to osteoblastic activity is also frequently found [25]. In agreement with previous reports [26], tumoral cells present in the inoculated limb showed positive staining for the epithelial and mesenchimal markers, E-cadherin and vimentin, respectively.

Aiming to elucidate whether the pathological injury provoked in the tibia by RM1 cells could promote nociceptive inputs into spinal cord, we next studied the possible activation of glial spinal cells. Experiments performed 12–16 days after the inoculation of 10^3 RM1 cells with an anti-GFAP antibody showed astroglial activation in the dorsal horn of the spinal cord ipsilateral to the inoculated limb, whereas the assays with the anti-Iba-1 antibody suggest that microglial activation does not occur at this time. Our results

related to astroglial cells agree with those previously described [16] after the intrafemoral inoculation of 10^6 RM1 cells, although these authors also mention, as data not shown, that they detect microglial activation 21 days after the inoculation of cells.

Confirming the painful nature of this bone tumor, nociceptive parameters were measured in behavioral experiments. Both thermal and mechanical hyperalgesia, as well as mechanical allodynia were already established four days after cell inoculation, being these parameters maintained or even increased during all the period studied (20 days). These nociceptive parameters were also reported after the intrafemoral inoculation of 10^6 RM1 cells [16], although nociceptive behaviors were absent during the first week. Previous studies performed in rats receiving the inoculation of tumoral prostate cells describe different nociceptive behaviors such as thermal and mechanical hyperalgesia or mechanical allodyinia depending on the type of cells [11].

To have a basic notion of the efficacy of some analgesic drugs prescribed in the clinical management of pain related to bone metastases from prostatic origin, we tested the effect of systemic morphine and zoledronic acid. As expected, the administration of systemic morphine counteracted thermal hyperalgesia as well as mechanical allodynia at doses similar to those reported in other models of bone cancer-induced pain [27]. Both hypernociceptive symptoms were also inhibited after the systemic administration of zoledronic acid. The analgesic effects of several bisphosphonates have been previously measured in different models of experimental bone cancer-induced pain, particularly after their chronic administration [28–30]. Although it seems logical to relate the analgesic effect of a bisphosphonate to their action on bone remodeling mechanisms, the fact that the antinociceptive effects here described occurred 30 min after the administration of a unique dose, excludes their dependence on bone remodeling. Several reports have described the ability of bisphosphonates to evoke acute analgesic effects in different tests in mice or rats [31–34] and even their efficacy to inhibit pathological pain, such as inflammatory [28,35] or neuropathic [28]. It seems possible that these effects could be related to other mechanisms unrelated to bone remodeling, such as the interference with neurotransmitter release [32] or the inhibition of P2X(2/3) receptors [33] or transient receptor potential channels [34]. The effects of zoledronic acid described in the present experiments demonstrate the acute analgesic effects of this drug in an experimental model of bone cancer and points the same direction than a previous report describing the efficacy of acutely administered ibandronate in mice inoculated with fibrosarcoma cells [12].

Chemokines are small molecules of peptidic nature that apart from promoting tumor development [36] have been also involved in the induction of pain [37]. We have made an attempt to know whether the chemokines CCL2, CCL3, and CCL5 could be involved in bone cancer-induced hypernociception evoked by RM1 cells. Our results, together with other previously obtained in mice receiving the intratibial inoculation of sarcoma or melanoma cells [18,20] reinforce the idea that the implication of particular chemokines in bone cancer pain seems dependent on the cell line responsible of tumor development. In particular, RM1 cells show a great ability to release CCL2 "in vivo" and "in vitro," whereas they only release CCL5 "in vitro" but not "in vivo" or even seem unable to release significant amounts of CCL3. The finding that CCL5 levels are decreased in the hind paws of mice inoculated with tumoral cells seems particularly surprising. We can not offer any explanation for this fact, although several possibilities, such as a decrease in its synthesis, an increased degradation or its scavenging by chemokine decoy receptors [38] could be considered. In any case, our results suggest that the blockade of CCR1, the main receptors on which CCL3 and CCL5 bind to, could not be a useful tool to inhibit the nociceptive symptoms derived from bone metastases of prostatic origin, contrasting with its efficacy in fibrosarcoma-bearing mice [20]. In contrast, the increased presence of CCL2 suggests that the administration of an antagonist of CCR2, the main receptor on which CCL2 acts, could be an accurate strategy to counteract RM1-evoked tumoral hypernociception. Supporting a functional role for this enhanced presence of CCL2, the administration of the CCR2 antagonist RS504393 produced the complete inhibition of thermal hyperalgesia evoked by the tumor and partially reduced mechanical allodynia evoked by RM1 cells. It has been previously reported that RS504393 can inhibit the hyperalgesia but not the allodynia evoked by the intratibial inoculation of fibrosarcoma cells [18], a finding that could be related to the predominance of spinal mechanisms in this nociceptive symptom [39]. Thus, although limited, the discrete antiallodynic effect obtained in mice inoculated with RM1 cells, can suggest that CCR2 antagonists could be more effective to relieve pain symptoms derived from prostate metastases. This may be particularly interesting due to the vast body of evidence suggesting a relationship between CCL2/CCR2 and the development of prostate tumors. An outstanding presence of this chemokine has been found both in experimental animals or patients with prostate cancer [36,40] and it has been proposed that CCL2 might act as a link between the loss of androgen receptor-mediated responses and the increased migration/invasion of prostate cells [41].

Although the administration of an anti-CCL2 antibody has been found ineffective in a phase II assay in patients with metastatic castration-resistant prostate cancer [42], the authors of the study describe that this result could be due to the lack of this antibody to effectively reduce serum CCL2 concentration and propose that CCR2 blockade could be a better strategy to impede tumor progression [42]. In this sense, it seems interesting to emphasize the possibility that a drug whose mechanism of action is envisaged as a putative strategy to treat prostate cancer could further act as an agent to treat some painful symptoms associated to bone metastases from prostatic origin.

CONCLUSION

Globally, our data contribute to characterize the nociceptive signature of this model of bone cancer-induced pain due to the presence of prostate tumoral cells in immunocompetent mice and suggest that CCL2 could play a role in the hypernociception evoked by bone prostate metastases. Thus, the blockade of the CCL2/CCR2 axis, proposed as a possible target to treat prostate tumors, could also be an attractive strategy to counteract some hypernociceptive symptoms derived from prostatic metastases.

ACKNOWLEDGMENTS

Grants were provided by MEC (SAF2012-36271). M.P. is recipient of a grant from the Instituto Universitario de Oncología (IUOPA). IUOPA is supported by Obra Social Cajastur-Asturias, Spain.

We appreciate the excellent technical assistance given by Marta Alonso Guervós and Ángel Martínez Nistal (Servicios Científico-Técnicos, Universidad de Oviedo) for fluorescence microscopy and imaging processing.

REFERENCES

1. Coleman R, Body JJ, Aapro M, Hadji P, Herrstedt J. On behalf of the ESMO Guidelines Working Group. Bone health in cancer patients: ESMO clinical practice guidelines. Ann Oncol 2014; [Epub ahead of print].
2. Currie GL, Delaney A, Bennett MI, Dickenson AH, Egan KJ, Vesterinen HM, Sena ES, Macleod MR, Colvin LA, Fallon MT. Animal models of bone cancer pain: Systematic review and meta-analyses. Pain 2013;154:917–926.
3. Lozano-Ondoua AN, Symons-Liguori AM, Vanderah TW. Cancer-induced bone pain: Mechanisms and models. Neurosci Lett 2013;557:52–59.
4. Soerjomataram I, Lortet-Tieulent J, Parkin DM, Ferlay J, Mathers C, Forman D, Bray F. Global burden of cancer in 2008: A systematic analysis of disability-adjusted life-years in 12 world regions. Lancet 2012;380:1840–1850.
5. Zustovich F, Fabiani F. Therapeutic opportunities for castration-resistant prostate cancer patients with bone metastases. Crit Rev Oncol Hematol 2014;91:197–209.
6. Lipton A. Implications of bone metastases and the benefits of bone targeted therapy. Semin Oncol 2010;37:S15–29.
7. Zhang RX, Liu B, Wang L, Ren K, Qiao JT, Berman BM, Lao L. Spinal glial activation in a new rat model of bone cancer pain produced by prostate cancer cell inoculation of the tibia. Pain 2005;118:125–136.
8. Kolosov A, Aurini L, Williams ED, Cooke I, Goodchild CS. Intravenous injection of leconotide, an omega conotoxin: Synergistic antihyperalgesic effects with morphine in a rat model of bone cancer pain. Pain Med 2011;12:923–941.
9. Muralidharan A, Wyse BD, Smith MT. Optimization and characterization of a rat model of prostate cancer-induced bone pain using behavioral, pharmacological, radiological, histological and immunohistochemical methods. Pharmacol Biochem Behav 2013;106:33–46.
10. De Ciantis PD, Yashpal K, Henry J, Singh G. Characterization of a rat model of metastatic prostate cancer bone pain. J Pain Res 2010;3:213–221.
11. Muralidharan A, Smith MT. Pathobiology and management of prostate cancer-induced bone pain: Recent insights and future treatments. Inflammopharmacology 2013;21:339–363.
12. Halvorson KG, Sevcik MA, Ghilardi JR, Sullivan LJ, Koewler NJ, Bauss F, Mantyh PW. Intravenous ibandronate rapidly reduces pain, neurochemical indices of central sensitization, tumor burden, and skeletal destruction in a mouse model of bone cancer. J Pain Symptom Manage 2008;36:289–303.
13. Baley PA, Yoshida K, Qian W, Sehgal I, Thompson TC. Progression to androgen insensitivity in a novel in vitro mouse model for prostate cancer. J Steroid Biochem Mol Biol 1995;52: 403–413.
14. McCabe NP, Madajka M, Vasanji A, Byzova TV. Intraosseous injection of RM1 murine prostate cancer cells promotes rapid osteolysis and periosteal bone deposition. Clin Exp Metastasis 2008;25:581–590.
15. Voeks DJ, Martiniello-Wilks R, Russell PJ. Derivation of MPR and TRAMP models of prostate cancer and prostate cancer metastasis for evaluation of therapeutic strategies. Urol Oncol 2002;7:111–118.
16. Xu J, Zhu MD, Zhang X, Tian H, Zhang JH, Wu XB, Gao YJ. NFκB-mediated CXCL1 production in spinal cord astrocytes contributes to the maintenance of bone cancer pain in mice. J Neuroinflammation 2014;11:38.
17. Hu JH, Zheng XY, Yang JP, Wang LN, Ji FH. Involvement of spinal monocyte chemoattractant protein-1 (MCP-1) in cancer-induced bone pain in rats. Neurosci Lett 2012;517:60–63.
18. Pevida M, González-Rodríguez S, Lastra A, Hidalgo A, Menéndez L, Baamonde A. CCL2 released at tumoral level contributes to the hyperalgesia evoked by intratibial inoculation of NCTC 2472 but not B16-F10 cells in mice. Naunyn Schmiedebergs Arch Pharmacol 2012;385:1053–1061.
19. Hang LH, Shao DH, Chen Z, Chen YF, Shu WW, Zhao ZG. Involvement of spinal CC chemokine ligand 5 in the development of bone cancer pain in rats. Basic Clin Pharmacol Toxicol 2013;113:325–328.
20. Pevida M, Lastra A, Meana A, Hidalgo A, Baamonde A, Menéndez L. The chemokine CCL5 induces CCR1-mediated hyperalgesia in mice inoculated with NCTC 2472 tumoral cells. Neuroscience 2014;259:113–125.

21. Pevida M, González-Rodríguez S, Lastra A, García-Suárez O, Hidalgo A, Menéndez L, Baamonde A. Involvement of spinal chemokine CCL2 in the hyperalgesia evoked by bone cancer in mice: A role for astroglia and microglia. Cell Mol Neurobiol 2014;34:143–156.
22. Chhikara M, Huang H, Vlachaki MT, Zhu X, Teh B, Chiu KJ, Woo S, Berner B, Smith EO, Oberg KC, Aguilar LK, Thompson TC, Butler EB, Aguilar-Cordova E. Enhanced therapeutic effect of HSV-tk + GCV gene therapy and ionizing radiation for prostate cancer. Mol Ther 2001;3:536–542.
23. Dong L, Zhang X, Yu C, Yu T, Liu S, Hou L, Fu L, Yi S, Chen W. Monitoring luciferase-labeled human prostate stem cell antigen-expressing tumor growth in a mouse model. Exp Ther Med 2013;6:1208–1212.
24. Lim E, Modi KD, Kim J. In vivo bioluminescent imaging of mammary tumors using IVIS spectrum. J Vis Exp 2009; pii: 1210.
25. Roodman GD. Mechanisms of bone metastasis. N Engl J Med 2004;350:1655–1664.
26. Power CA, Pwint H, Chan J, Cho J, Yu Y, Walsh W, Russell PJ. A novel model of bone-metastatic prostate cancer in immunocompetent mice. Prostate 2009;69:1613–1623.
27. González-Rodríguez S, Llames S, Hidalgo A, Baamonde A, Menéndez L. Potentiation of acute morphine-induced analgesia measured by a thermal test in bone cancer-bearing mice. Fundam Clin Pharmacol 2012;26:363–372.
28. Walker K, Medhurst SJ, Kidd BL, Glatt M, Bowes M, Patel S, McNair K, Kesingland A, Green J, Chan O, Fox AJ, Urban LA. Disease modifying and anti-nociceptive effects of the bisphosphonate, z oledronic acid in a model of bone cancer pain. Pain 2002;100:219–229.
29. Nagae M, Hiraga T, Yoneda T. Acidic microenvironment created by osteoclasts causes bone pain associated with tumor colonization. J Bone Miner Metab 2007;25:99–104.
30. Hald A, Nedergaard S, Hansen RR, Ding M, Heegaard AM. Differential activation of spinal cord glial cells in murine models of neuropathic and cancer pain. Eur J Pain 2009;13:138–145.
31. Goicoechea C, Porras E, Alfaro MJ, Martín MI. Alendronate induces antinociception in mice, not related with its effects in bone. Jpn J Pharmacol 1999;79:433–437.
32. Bonabello A, Galmozzi MR, Bruzzese T, Zara GP. Analgesic effect of bisphosphonates in mice. Pain 2001;91:269–275.
33. Kakimoto S, Nagakura Y, Tamura S, Watabiki T, Shibasaki K, Tanaka S, Mori M, Sasamata M, Okada M. Minodronic acid, a third-generation bisphosphonate, antagonizes purinergic P2X(2/3) receptor function and exerts an analgesic effect in pain models. Eur J Pharmacol 2008;589:98–101.
34. Kim S, Seiryu M, Okada S, Kuroishi T, Takano-Yamamoto T, Sugawara S, Endo Y. Analgesic effects of the non-nitrogen-containing bisphosphonates etidronate and clodronate, independent of anti-resorptive effects on bone. Eur J Pharmacol 2013;699:14–22.
35. Nagae M, Hiraga T, Wakabayashi H, Wang L, Iwata K, Yoneda T. Osteoclasts play a part in pain due to the inflammation adjacent to bone. Bone 2006;39:1107–1115.
36. Izhak L, Wildbaum G, Weinberg U, Shaked Y, Alami J, Dumont D, Friedman B, Stein A, Karin N. Predominant expression of CCL2 at the tumor site of prostate cancer patients directs a selective loss of immunological tolerance to CCL2 that could be amplified in a beneficial manner. J Immunol 2010;184:1092–1101.
37. Abbadie C, Bhangoo S, De Koninck Y, Malcangio M, Melik-Parsadaniantz S, White FA. Chemokines and pain mechanisms. Brain Res Rev 2009;60:125–134.
38. Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ, Horuk R, Sparre-Ulrich AH, Locati M, Luster AD, Mantovani A, Matsushima K, Murphy PM, Nibbs R, Nomiyama H, Power CA, Proudfoot AE, Rosenkilde MM, Rot A, Sozzani S, Thelen M, Yoshie O, Zlotnik A. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacol Rev 2013;66:1–79.
39. Kim YH, Back SK, Davies AJ, Jeong H, Jo HJ, Chung G, Na HS, Bae YC, Kim SJ, Kim JS, Jung SJ, Oh SB. TRPV1 in GABAergic interneurons mediates neuropathic mechanical allodynia and disinhibition of the nociceptive circuitry in the spinal cord. Neuron 2012;74:640–647.
40. Lu Y, Chen Q, Corey E, Xie W, Fan J, Mizokami A, Zhang J. Activation of MCP-1/CCR2 axis promotes prostate cancer growth in bone. Clin Exp Metastasis 2009;26:161–169.
41. Izumi K, Fang LY, Mizokami A, Namiki M, Li L, Lin WJ, Chang C. Targeting the androgen receptor with siRNA promotes prostate cancer metastasis through enhanced macrophage recruitment via CCL2/CCR2-induced STAT3 activation. EMBO Mol Med 2013;5:1383–1401.
42. Pienta KJ, Machiels JP, Schrijvers D, Alekseev B, Shkolnik M, Crabb SJ, Li S, Seetharam S, Puchalski TA, Takimoto C, Elsayed Y, Dawkins F, de Bono JS. Phase 2 study of carlumab (CNTO 888), a human monoclonal antibody against CC-chemokine ligand 2 (CCL2), in metastatic castration-resistant prostate cancer. Invest New Drugs 2013;31:760–768.

4. Resumen de resultados y discusión general

En los experimentos recogidos en este trabajo se ha estudiado la participación de los receptores CCR1 y CCR2 y de las quimiocinas endógenas capaces de activarlos en las respuestas hipernociceptivas observadas en dos modelos experimentales de inflamación, uno agudo y otro crónico, y en un modelo de dolor neoplásico experimental.

Como ha sido comentado en la introducción, el papel que desempeñan las quimiocinas en el desarrollo y mantenimiento del dolor representa un campo de estudio que se viene desarrollando con fuerza en un marco temporal relativamente reciente. En la actualidad, la información disponible sobre la participación de distintas moléculas de esta familia y de sus receptores en animales con dolor neuropático es bastante amplia (Ubogu, 2011; Liou et al., 2013; Biber y Boddeke, 2014; Knerlich-Lukoschus y Held-Feindt, 2015). Sin embargo, a pesar de la importante participación de las quimiocinas en la génesis de los procesos inflamatorios (Moser al., 2004; Griffith et al., 2014; Bachelerie et al., 2014) y del fuerte vínculo existente entre inflamación y dolor, la información relativa a la participación de las quimiocinas en los síntomas nociceptivos derivados de los procesos inflamatorios es más bien escasa. En algunos estudios previos se ha mostrado el aumento de la expresión en tejidos inflamados de diversas quimiocinas que estructuralmente pertenecen a la serie CC, como es el caso de la CCL2 (Monocyte Chemoattractant Protein 1, MCP-1) (Yang et al., 2007), la CCL3 (Macrophage Inflammatory Protein-1-alpha, MIP-1α) (Garcia-Ramallo et al., 2002) y la CCL5 (Regulated on Activation Normal T cell Expressed and Secreted, RANTES) (Dawes et al., 2013). Dichas quimiocinas actúan respectivamente sobre los receptores CCR2 y CCR1, que se expresan en diferentes localizaciones de las vías nociceptivas (Bodekke et al., 1999; Zhang et al., 2004; Jung et al., 2008; Jeon et al., 2011) y sufren regulación al alza en respuesta a daños nerviosos periféricos o espinales (Kiguchi et al., 2010a; Knerlich-Lukoschus et al., 2011; Knerlich-Lukoschus y Held-Feindt, 2015). Funcionalmente, la unión de las quimiocinas mencionadas a sus receptores correspondientes, CCL2 a CCR2 y CCL3 o CCL5 a CCR1, puede provocar respuestas hipernociceptivas en roedores (Eijkelkamp et al., 2010; Kiguchi et al., 2010a; Baamonde et al., 2011; Pevida et al., 2014b). En nuestro laboratorio tenemos experiencia en el estudio de la analgesia producida por antagonistas de

receptores CCR2 y CCR1 en ratones con dolor neoplásico (Pevida et al., 2012; 2014a; 2014b) o secundario a la administración de antineoplásicos (Pevida et al., 2013) y sobre esta base se diseñó el presente estudio relativo a la posible eficacia de estos fármacos sobre el dolor inflamatorio. Así, el objetivo general planteado en las **publicaciones 1 y 2** fue valorar en ratones inflamados de forma aguda con carragenina o crónica con coadyuvante de Freund (CFA), la posible eficacia analgésica derivada del bloqueo de los receptores CCR1 y CCR2. Además de realizar ensayos de comportamiento, se midieron los niveles de las quimiocinas correspondientes mediante ensayos de ELISA y la expresión de su mRNA por PCR cuantitativa (qPCR). Asimismo, se realizaron estudios inmunohistoquímicos para determinar su localización en los tejidos inflamados.

A diferencia de las dos primeras, en la **publicación 3** se trabajó con ratones inoculados con células tumorales de próstata, siguiendo un esquema similar al que se había utilizado previamente en nuestro laboratorio para explorar la implicación de quimiocinas en otros modelos de dolor neoplásico experimental (Pevida et al., 2012; 2014a; 2014b). El conocimiento del papel jugado por las quimiocinas en el dolor neoplásico es aún bastante limitado, a pesar de que el número de publicaciones relacionadas con este tema ha ido en aumento durante los últimos años (Guo y Gao, 2015; Zhou et al., 2015). El estudio del dolor neoplásico constituye un problema complejo en sí mismo debido a los diversos factores etiológicos que lo determinan, como el tipo y grado de lesión ósea, el componente neuropático o inflamatorio asociado y la posible liberación de diferentes mediadores capaces de sensibilizar nociceptores, entre los que pueden estar diversas quimiocinas (Mantyh, 2006; Falk y Dickenson, 2014). De hecho, podría llegar a considerarse que cada tipo de dolor tumoral representa una entidad con características diferentes, por lo que el desarrollo de modelos experimentales de dolor neoplásico óseo que mimeticen en el mayor grado posible las características observadas en la clínica resulta de gran interés. Los modelos utilizados previamente en nuestro laboratorio estaban basados en la inoculación por vía intratibial de dos líneas diferentes de células tumorales, las células NCTC 2472 derivadas de fibrosarcoma (Menéndez et al., 2003) y las B16-F10 derivadas de melanoma (Curto-Reyes et al., 2008), capaces de provocar el crecimiento de tumores óseos cuyo desarrollo conlleva la producción de

hiperalgesia y alodinia. En estos estudios previos, se demostró la implicación tanto a nivel periférico (Pevida et al, 2012) como central (Pevida et al., 2014a) de la quimiocina CCL2 y su receptor CCR2 así como la participación de la quimiocina CCL5 a través del receptor CCR1 (Pevida et al., 2014b) en el modelo de fibrosarcoma. Por el contrario, en el modelo de dolor tumoral por inoculación de células B16-F10 no se observó la participación de ninguna de estas quimiocinas (Pevida et al., 2012, 2014b). Precisamente debido a la observación de la diferente participación de quimiocinas en función de las células tumorales inoculadas, en los experimentos recogidos en la publicación 3 se trató de desarrollar un modelo experimental de tumor óseo que fuese más próximo a la clínica que los dos precedentes. Una limitación de los dos modelos utilizados previamente es su menor semejanza con la clínica humana, debido a la baja incidencia con que aparecen en la población los tumores óseos primarios del tipo del osteosarcoma (Mirabello et al., 2009) y a que el melanoma es un tipo de tumor con mayor tendencia a invadir órganos como el hígado o el cerebro, lo que frecuentemente compromete la supervivencia antes de que se observen metástasis óseas (Tas, 2012). Considerando la elevada prevalencia de metástasis óseas dolorosas cuyo origen es prostático (Coleman, 2006), se seleccionaron las células tumorales RM1 derivadas de cáncer de próstata, capaces de provocar el crecimiento de tumores óseos en ratones inmunocompetentes (McCabe et al., 2008), para estudiar las características del tumor resultante y dilucidar la participación de las quimiocinas mencionadas.

Respecto a los experimentos recogidos en la **publicación 1** (Llorián-Salvador et al., Fundam Clin Pharmacol 2016;30:235-247), en ellos se estudió la implicación en la hiperalgesia térmica y la alodinia mecánica del receptor CCR2 y de su ligando principal, la quimiocina CCL2, en ratones con inflamación aguda producida por administración intraplantar de carragenina o crónica, producida por inyección de CFA.

Inicialmente, se observó un marcado aumento en las concentraciones de CCL2 presentes tanto 6 h después de la inyección de carragenina como 7 días tras la administración de CFA. En consonancia con el carácter crónico de la inflamación producida por la inyección de CFA (Miranda et al., 2014), este aumento persistió

al menos durante 4 semanas. Los estudios inmunohistoquímicos permitieron determinar que tanto en la inflamación aguda como la crónica provocaban expresión de CCL2 fundamentalmente localizada en macrófagos y, en menor medida, en células residentes como fibroblastos.

El estudio del efecto de la administración del antagonista de receptores CCR2, RS504393 (Mirzadegan et al., 2000), sobre la hiperalgesia térmica y la alodinia mecánica provocadas por la inflamación demostró la capacidad de dicho antagonista para revertir la hiperalgesia térmica secundaria a la inyección de carragenina y CFA de manera dependiente de la dosis. Este efecto desapareció 1 h tras su administración, en concordancia con la corta semivida descrita para compuestos análogos al RS504393 (Abel et al., 2009; Mitchell et al., 2013). La inhibición de la hiperalgesia tras la administración de dosis bajas de RS504393 por vía i.pl. sugiere que los CCR2 implicados en este efecto están localizados en el tejido inflamado y estaría de acuerdo con la sobreexpresión de estos receptores descrita en DRG de roedores con osteoartritis (Miller et al., 2012) o cistitis (Arms et al., 2013) experimentales e incluso tras la administración i.pl. de CFA (Abbadie et al., 2003). Además, la inhibición de la hiperalgesia inflamatoria observada tras la administración de un anticuerpo anti-CCL2 sugiere que esta quimiocina es la que actúa como agonista endógeno de los receptores CCR2 localizados en el tejido inflamado. Ciertamente, este resultado no descarta la posibilidad teórica de que otras quimiocinas que presentan afinidad por los receptores CCR2 como la CCL7, la CCL8 o la CCL11 (Charo et al., 2016), también pudieran estar implicadas en esta respuesta, aunque esta posibilidad parece improbable puesto que no hay resultados previos que apoyen su participación en el procesamiento nociceptivo.

Al contrario de lo que ocurrió con la hiperalgesia, la administración de RS504393 no modificó la alodinia mecánica presente en ratones con inflamación aguda ni crónica incluso a dosis superiores a las antihiperalgésicas. Este resultado no se debe a una supuesta incapacidad de la CCL2 para producir alodinia, ya que se comprobó que la administración i.pl. de CCL2 reduce el umbral en el test de von Frey, aunque a dosis mucho mayores a las necesarias para producir hiperalgesia, al igual que se describió en un trabajo previo (Abbadie et al., 2003). El menor protagonismo de la participación de la CCL2 en la alodinia que en la hiperalgesia

está de acuerdo con datos que muestran cómo los ratones que sobreexpresan CCL2 presentan respuestas hiperalgésicas aumentadas en respuesta al CFA mientras que la alodinia mecánica no se ve modificada (Menetski et al., 2007). La interpretación de los datos no es sencilla, ya que hay ensayos previos en los que el bloqueo de receptores CCR2 resultó eficaz para revertir la alodinia mecánica en animales con inflamación visceral (Arms et al., 2013), dolor neuropático (Van Steenwinckel et al., 2011) o neoplásico (Ren et al., 2015). A falta de argumentos más sólidos, sólo podemos proponer que, dada la amplia variedad de mecanismos que participan en el desarrollo de la alodinia en procesos patológicos (Lolignier et al., 2015), pueda darse el caso de que en el desarrollo de la alodinia inflamatoria tengan un protagonismo especial mediadores diferentes a la CCL2. En este contexto, podría pensarse que la importante participación de los canales TRPV1 en la transducción nociceptiva térmica (Caterina et al., 1997) y su colocalización con CCR2 (Jung et al., 2008) pudiera justificar que la acción de los receptores CCR2 estuviese particularmente ligada a la acción sobre neuronas que expresen TRPV1, que estarían preferentemente implicadas en la hiperalgesia térmica pero no en alodinia mecánica. En cualquier caso, hay varios estudios de nuestro laboratorio que parecen indicar que los receptores CCR2 y CCR1 participan de manera más relevante en la hiperalgesia que en la alodinia tanto en lo que respecta al dolor canceroso como al inflamatorio. Así, la administración de un antagonista CCR2 en ratones con tumores producidos por células NCTC 2472 produjo también efecto antihiperalgésico pero no antialodínico (Pevida et al., 2012) y lo mismo ocurrió tras la administración de un antagonista CCR1 en ratones con el mismo tipo de tumor (Pevida et al., 2014b). Los resultados descritos la publicación 2, en la que se ensayó el efecto de un antagonista CCR1 en ratones con inflamación aguda, vuelven a describir una situación en la que el fármaco es más eficaz como antihiperalgésico que como antialodínico.

Al margen de la acción hiperalgésica aquí descrita, la participación del sistema CCL2/CCR2 en el desarrollo del propio proceso inflamatorio es un hecho bien descrito, de modo que incluso se ha propuesto que su bloqueo pudiera ser una diana terapéutica para reducir la inflamación de la artritis reumatoide o incluso de la osteoartritis (Taylor et al., 2000; Lin et al., 2012; Xu et al., 2015; Talbot et al., 2015). Experimentalmente, hay datos que avalan esta posibilidad, como el

aumento de los síntomas inflamatorios que ocurre tras la administración de CFA en animales que sobreexpresan CCL2 (Menetski et al., 2007), la menor celularidad observada en un modelo experimental de cistitis autoinmune en ratones knockout de CCR2 y de CCL2 (Bicer et al., 2015) o la eficacia de un antagonista de CCR2 y de la deleción del gen *ccr2* en un modelo de lesión nerviosa inflamatoria (Yuan et al., 2014). En base a estos datos y a pesar de que en nuestro caso el efecto analgésico evocado por el antagonista CCR2 ocurrió tras una única administración, consideramos la posibilidad teórica de que la acción antihiperalgésica del RS504393 pudiera ser el reflejo de una hipotética inhibición de la reacción inflamatoria secundaria al bloqueo de receptores CCR2. Para valorarlo, se estudió el efecto de la administración de RS504393 sobre las alteraciones tisulares inflamatorias y el grado de tumefacción de la pata inflamada. Ni la administración del antagonista de CCR2 30 min antes, tal y como se hizo en los ensayos de comportamiento, ni la inyección repetida del RS504393 en un intervalo de 6 h produjo ninguna modificación ni en la sección de la pata inflamada con carragenina o con CFA ni en la intensidad de la reacción inflamatoria valorada a través de la infiltración de macrófagos y polimorfonucleares. De manera similar, se ha descrito que la inflamación producida por formalina (Abbadie et al., 2003) o la derivada de la cauterización corneal (Oshima et al., 2006) no se modifica en ratones carentes del gen *ccr2.* En nuestro contexto, los resultados indican que, aunque no sería descartable que una dosis mayor o, sobre todo, un tratamiento más mantenido con RS504393 pudiese producir efecto antiinflamatorio, la acción antihiperalgésica evocada por el antagonista de receptores CCR2 en nuestros experimentos ocurre de un modo independiente a un supuesto efecto antiinflamatorio.

En resumen, los resultados recogidos en esta publicación muestran que el aumento local en la concentración de CCL2 tras una inflamación aguda o crónica contribuye al desarrollo de la hiperalgesia pero no de la alodinia inflamatorias. De acuerdo con ello, la hiperalgesia puede ser inhibida tanto a través del antagonismo de CCR2 como de la neutralización de la CCL2 sin que esa acción dependa de un efecto antiinflamatorio paralelo.

Continuando con el estudio de la participación de quimiocinas de la serie CC en el dolor inflamatorio, en los experimentos recogidos en la **publicación 2** (Llorián-Salvador et al., Basic Clin Pharmacol Toxicol. 2015. En prensa) se exploró la eficacia analgésica derivada del bloqueo del receptor CCR1. Algunos trabajos previos describen la participación de este receptor en procesos relacionados con la inflamación (White et al., 2013; Bachelerie et al., 2014) y el dolor (Kiguchi et al., 2010a; 2010b; Pevida et al., 2014b). Siguiendo el mismo planteamiento del trabajo anterior, se estudió en ratones con inflamación aguda y crónica si el bloqueo de receptores CCR1 podía provocar efectos analgésicos en ratones inflamados y si estos pudieran ser secundarios a una posible acción antiinflamatoria. Asimismo, se trató de determinar qué ligandos endógenos de este receptor podrían participar, centrando el estudio en aquellos cuya participación en procesos nociceptivos ha sido previamente descrita, como ocurre con la CCL3 y la CCL5, (Eijkelkamp et al., 2010; Pevida et al., 2014b; Liou et al., 2012; Li et al., 2015).

Hasta el momento, aunque se había descrito el efecto analgésico debido al bloqueo de receptores CCR1 en modelos de dolor neuropático (Kiguchi et al., 2010b) o neoplásico (Pevida et al., 2014b), la información referida al dolor inflamatorio era muy limitada. Así, sólo se disponía de un trabajo previo que mostraba un efecto analgésico parcial debido al bloqueo de receptores CCR1 producido por administración de un antagonista y a la deleción del mismo en una prueba de retorcimiento ("writhing") inducido por la inyección de ácido acético en ratones y el antagonismo de CCR1 en ratas inflamadas con CFA (Lewis et al., 2014). En este contexto, nuestro trabajo se diseñó para tratar de obtener una información más completa referida tanto a inflamación aguda como crónica y a hiperalgesia como a alodinia, intentando dilucidar si el bloqueo de CCR1 puede sólo producir efectos parciales como sugería la publicación mencionada o puede llegar a alcanzarse un efecto analgésico completo en ratones con inflamación.

La administración sistémica del antagonista de receptores CCR1 J113863 (Naya y Saeki, 2001) inhibió de manera total y dependiente de la dosis la hiperalgesia térmica observada en ratones con inflamación tanto aguda producida por carragenina como crónica producida por CFA, mostrando la capacidad de este

mecanismo de producir un efecto antihiperalgésico completo. Además, a diferencia de lo que ocurrió cuando se ensayó el efecto de un antagonista de receptores CCR2 (Llorián-Salvador et al., 2016), el bloqueo de receptores CCR1 produjo efecto antialodínico, aunque sólo sobre la alodinia mecánica producida por inflamación crónica. Para comprobar que la diferencia en la eficacia antialodínica se debía a la presencia de inflamación aguda o crónica más que al agente inflamatorio utilizado para generarla (carragenina o CFA), se realizaron experimentos en ratones en los que se estudiaron los efectos del fármaco un tiempo breve tras el CFA (6h) o un tiempo largo tras la carragenina (5 días). Los datos obtenidos confirmaron que el efecto antialodínico sólo ocurría en procesos inflamatorios largos y no en reacciones inflamatorias agudas, independientemente del agente inflamatorio utilizado. Puesto que, como se comentará a continuación, en ambos modelos se encontró una elevación similar de quimiocinas en el tejido inflamado, inicialmente nos planteamos la posibilidad de que quizá la mayor sensibilización de neuronas espinales que podría darse en ratones con inflamación crónica (Basbaum et al., 2009) pudiera explicar que una supuesta acción espinal del J113863 actuase sólo sobre la alodinia observada en respuesta a la inflamación crónica, y no aguda. Sin embargo, el hecho de que los niveles de CCL3 y CCL5 en médula espinal tampoco se modificaron en ratones inflamados, junto con la escasa capacidad del J113863 para atravesar la barrera hematoencefálica, dada su estructura de amonio cuaternario (Amat et al., 2006), nos hizo descartar esta posibilidad.

Como ocurría en el caso de los receptores CCR2, algunos ensayos previos describían un efecto antiinflamatorio derivado de la administración de antagonistas de receptores CCR1 tanto tras su administración aguda (Garcia-Ramallo et al., 2002) como crónica (Amat et al., 2006). Por ello, una vez demostrado el efecto antinociceptivo del antagonista CCR1, se trató de medir si la acción analgésica podría relacionarse con un posible efecto antiinflamatorio. Al igual que se observó en los experimentos con el antagonista CCR2, se pudo comprobar cómo, con la pauta de administración utilizada en nuestros ensayos e incluso haciendo dos administraciones de la máxima dosis ensayada, no hubo ninguna reducción de la reacción inflamatoria indicando que el efecto analgésico del J113863 no parece derivarse de una supuesta acción antiinflamatoria.

La existencia de un efecto antihiperalgésico tras la administración de J113863 sugería que debía darse un aumento en la expresión de alguna quimiocina capaz de activar los receptores CCR1. Entre los ligandos endógenos del este receptor, sólo las quimiocinas CCL3, CCL5 y CCL7 habían sido relacionadas con el procesamiento de señales nociceptivas. Sin embargo, en el caso de esta última, el único trabajo que describía su implicación en respuestas dolorosas, vinculaba esta respuesta a la acción sobre receptores CCR2 (Imai et al., 2013), lo que en principio la descarta como candidato a actuar como algógeno a través de CCR1. Por tanto, se cuantificaron los niveles de CCL3 y CCL5 tanto en homogeneizados de tejido inflamatorio como en la médula espinal de ratones tratados con carragenina o CFA. La expresión de CCL3 y CCL5 en la médula fue similar en controles y ratones inflamados, sugiriendo que, aunque se ha demostrado la existencia de CCR1 en células microgliales de la médula espinal (Boddeke et al., 1999), probablemente los mecanismos medulares no sean relevantes en el efecto del antagonista de receptores CCR1. Por el contrario, tanto en los homogeneizados de las patas inflamadas con carragenina como con CFA, se detectóun aumento de la concentración de CCL3 en ensayos de ELISA, apoyado en ambos casos con la sobreexpresión de mRNA de CCL3 a este nivel. En estudios inmunohistoquímicos pudimos comprobar cómo la expresión de CCL3 se localizaba preferentemente en macrófagos y neutrófilos presentes en tejidos inflamados. Estos resultados están de acuerdo con trabajos previos que describían un aumento en los niveles de CCL3 medidos 4–6 h tras la administración de carragenina en el espacio subcutáneo de la región dorsal de ratones (García-Ramallo et al., 2002). Asimismo, el hallazgo de CCL3 en macrófagos y neutrófilos coincide con datos que describen que algunos mediadores inflamatorios como el TNF- α en macrófagos (Leichtle et al., 2010) o la IL-1 en neutrófilos (Paulsson et al., 2012) pueden provocar un aumento de la expresión de CCL3. Sin embargo, contrastando con datos previos obtenidos en las articulaciones de ratas inflamadas con yodoacetato sódico (Dawes et al., 2013), en nuestros experimentos no observamos que la inflamación produjese ninguna alteración en los niveles locales de CCL5.

Apoyando la implicación funcional de la CCL3 en la hiperalgesia mediada por receptores CCR1, se comprobó cómo la administración de un anticuerpo contra

dicha quimiocina conseguía revertir eficazmente tanto la hiperalgesia en ambos tipos de inflamación como, a dosis mayores, la alodinia en el caso de la inflamación crónica por CFA. Este resultado está de acuerdo con trabajos previos que muestran el potencial de la quimiocina CCL3 para inducir hiperalgesia térmica (Qin et al., 2005; Jung et al., 2008), una propiedad a la que pueden contribuir varios mecanismos, como su capacidad para inducir la expresión de IL-1β en el nervio ciático (Kiguchi et al., 2010b) y en las neuronas de los DRG, sensibilizar los TRPV1 (Zhang et al., 2005), desensibilizar receptores opioides μ (Zhang et al., 2004), activar la proteinkinasa C (PKC) o modular la actividad de canales de sodio (Gold et al., 1996).

En conjunto, nuestros datos indican que mediante el bloqueo de los receptores CCR1 se pueden contrarrestar la hiperalgesia y la alodinia inflamatorias sin que este efecto sea debido a un efecto antiinflamatorio. En esta situación particular de inflamación aguda o crónica, producidas por carragenina o CFA respectivamente, la inhibición de la sensibilidad dolorosa tras la administración de un antagonista CCR1 parece deberse al antagonismo de la acción nociceptiva derivada de un aumento en la expresión de CCL3.

Finalmente, en la **publicación 3** (Llorián-Salvador et al., Prostate 75:70-83, 2015) se describen las respuestas nociceptivas medidas en ratones inmunocompetentes C57BL/6 tras la inoculación intratibial de células RM1 derivadas de una línea de cáncer de próstata así como la implicación de las quimiocinas CCL2, CCL3 y CCL5 en este modelo experimental de dolor tumoral.

Las células RM1, procedentes de tumores de próstata insensibles a andrógenos, se seleccionaron debido a la descripción previa del crecimiento de tumores en ratones C57BL/6 tras su inyección subcutánea (Power et al., 2009) y por las alteraciones que producen en el estroma óseo tras su inoculación intratibial (McCabe et al., 2008). Siguiendo el procedimiento habitualmente utilizado en nuestro laboratorio, se inocularon las células en la cavidad medular de la tibia de los ratones y, tras ensayar el efecto de distintas cantidades de células (100-100.000) se seleccionó la inoculación de 1.000 células como el número más adecuado para realizar los estudios posteriores. El 100% de los ratones inoculados con este número de células desarrolló tumor, no sufrió pérdida de

peso significativa y sufrió una mortalidad inferior al 20% 3 semanas tras la inoculación. El estudio de la evolución del desarrollo tumoral *in vivo*, realizado tras la inoculación de células RM1 transfectadas con el gen de la luciferasa, demostró una expansión gradual de la señal de bioluminiscencia desde una región próxima al sitio de inoculación en la tibia hacia partes más distales y tejidos adyacentes, que disminuyó sólo a partir del día 21, cuando se observó menor intensidad en la parte central de la masa tumoral, probablemente debida a la aparición de tejido necrótico (Lim et al., 2009; Khalil et al., 2013). El análisis histológico realizado en paralelo demostró la presencia de lesiones osteolíticas tempranas en el hueso cortical capaces de permitir la salida de células tumorales a los tejidos adyacentes, una invasión que resultó muy evidente 12 días tras la inoculación de las células. Sobre el día 16 se comenzaron a apreciar depósitos óseos en la superficie externa del periostio, reflejando la naturaleza mixta osteoblástica-osteolítica de las lesiones óseas provocadas por las células RM1, tal como había sido descrito previamente (McCabe et al., 2008). Además, se produjo la infiltración y compresión de fibras nerviosas, así como un aumento progresivo de la masa tumoral, reflejo de la invasión de tejidos adyacentes.

Posteriormente, a fin de explorar la naturaleza nociceptiva de estas lesiones, se midió la hiperalgesia térmica mediante el test de la placa caliente unilateral, la hiperalgesia mecánica con un test de aplicación de presión sobre la pata y la alodinia mecánica mediante la aplicación de los filamentos de von Frey. A partir del día 4 se comenzó a detectar hiperalgesia y alodinia, que alcanzaron un máximo entre los días 12 y 16, periodo que fue seleccionado para los estudios posteriores. Tanto la administración sistémica aguda de morfina como del bisfosfonato, ácido zoledrónico, inhibió la hiperalgesia térmica y la alodinia mecánica de forma dependiente de la dosis. Aunque la eficacia analgésica de los bisfosfonatos tras administración crónica ha sido descrita en diversas situaciones dolororsas asociado a una disminución de la actividad osteoclástica (Le Goff et al., 2010; Pappagallo et al., 2014; Yao et al., 2016), el hecho de que el efecto antihiperalgésico del zoledronato aparezca 30 min tras su administración descarta la posibilidad de que se deba a alteraciones en el remodelado óseo. En este sentido, la acción analgésica aguda producida por este grupo de fármacos de un modo independiente de alteraciones en el hueso se ha descrito previamente

en modelos experimentales de dolor inflamatorio (Nagae et al., 2006; Segawa et al., 2013) o neuropático (Walker et al., 2002). Se han propuesto diversos mecanismos moleculares a través de los cuales podría ocurrir este efecto agudo como el posible antagonismo de los receptores P2X3/4 (Kakimoto et al., 2008) y TRPs, como TRPV3, TRPV1 (Bang et al., 2011) y TRPV4 (Bang et al., 2012; Kim et al., 2013), la interferencia con la liberación de neurotransmisores (Bonabello et al., 2001) o la acción a través de canales de potasio dependientes de ATP (Kawabata et al., 2006) como posibles responsables.

La activación de células gliales de la médula espinal es una característica que frecuentemente contribuye al mantenimiento y desarrollo de los procesos dolorosos crónicos (Ji et al., 2013). En los ratones inoculados con células RM1 se detectó activación de la astroglía en las láminas superficiales I y II mientras que la activación microglial no fue modificada significativamente. Estos datos se corresponden con los hallados en otros modelos de dolor neoplásico experimental en relación a la astrogliosis (Hald et al., 2009; Doré-Savard et al., 2010; Pevida et al., 2014a; Xu et al., 2014), mientras que no existe uniformidad en los datos referentes a la activación de la microglía en este tipo de situaciones dolorosas, ya que existe una gran variabilidad en función del origen de las células tumorales y de la especie estudiada (Honore et al., 2000; Hald et al., 2009; Pevida et al., 2014a; Ducorneau et al., 2014)

Al igual que ocurrió con otras líneas celulares (Pevida et al., 2012; Khasavoba et al., 2007; Schiller et al., 2009), las células RM1 fueron capaces de liberar CCL2 directamente en su medio de cultivo. Además, de un modo semejante al observado en el modelo de fibrosarcoma, pero no en el de melanoma (Pevida et al., 2012*)*, se detectó un aumento de CCL2 en los tumores provocados por células RM1. Dada la capacidad de la CCL2 de producir efectos nociceptivos a través del receptor CCR2, se ensayó el efecto de la administración de un antagonista de dichos receptores para inhibir la hiperalgesia tumoral. El bloqueo de receptores CCR2 revirtió la hiperalgesia térmica y parcialmente la alodinia mecánica medida en ratones inoculados con células RM1, al igual que se observó previamente tras el inóculo de células de fibrosarcoma pero no de melanoma (Pevida et al., 2012) y células Walker 256 de cáncer de mama (Ren et al., 2015).

Por el contrario, las células RM1 no mostraron capacidad de liberar CCL3 ni *in vitro* ni *in vivo* y, respecto a la CCL5, se observó un aumento de su expresión en cultivo mientras que, curiosamente, en los tumores se apreció un descenso en sus niveles. Este resultado, que parece descartar la importancia de la CCL5 en la hiperalgesia tumoral, podría deberse a la internalización y degradación de esta quimiocina por receptores atípicos de quimiocinas, como el ACKR2, que se encuentra expresado en ciertos tipos de tumor, incluyendo el de próstata (Yu et al., 2015) y ejerce ese efecto en otros tipos de cáncer (Wu et al., 2008; Wu et al., 2013; Massara et al., 2016).

Coincidiendo con la finalización de los experimentos de nuestro trabajo, se publicó un estudio similar al nuestro (Xu et al., 2014) en el que se inoculaban en el fémur de ratones C57BL/6 10^6 células RM1 procedentes del Cell Bank of Type Culture Collection of Chinese Academy Sciences de Shanghái. Aunque en principio parece una variación del mismo modelo que empleamos nosotros, los datos recogidos en esa publicación presentan importantes discrepancias con los nuestros. Por ejemplo, Xu et al., (2014) muestran la existencia de lesiones exclusivamente osteolíticas frente al componente mixto observado en nuestro trabajo así como en la publicación previa que nos sirvió de referencia (McCabe et al., 2008). Asimismo, los datos obtenidos mostrando el 100% de mortalidad unos 20 días tras la inoculación de más de 10.000 células es compatible con la mortalidad total que aparece 17 días tras la inoculación de 10.000 células en experimentos de otro laboratorio que utiliza células RM1 de la misma procedencia que las nuestras (Chhikara et al., 2001). Ello contrasta con las publicaciones basadas en la utilizan de células RM1 procedentes del banco de células de la Chinese Academy Sciences de Shanghái en el que la mortalidad es mucho menor. Así, se ha descrito una supervivencia media de 38 días tras la inoculación via s.c. de 10^6 células (Dong et al., 2013) y, en el estudio mencionado de Xu *et al.* (2014), los ensayos de comportamiento se realizan 21 días tras la inoculación intrafemoral de 10^6 de células RM1. Estas discrepancias nos hacen pensar que la línea celular procedente de Shanghái podría presentar alguna diferencia que les confiere menor agresividad que la de las células usadas en nuestro caso, de la Universidad de Texas donde originalmente se desarrollaron (Baley et al., 1995; McCabe et al., 2008; Power et al., 2009).

En conjunto, nuestros resultados describen la presencia de hiperalgesia térmica y alodinia mecánica en ratones inmunocompetentes inoculados con células RM1 de cáncer de próstata. Sus características histopatológicas con un patrón mixto osteoblástico-osteoclástico, su grado de desarrollo así como la presencia de astrogliosis en la médula o su respuesta a morfina o ácido zoledrónico le confieren utilidad como modelo para el estudio del dolor neoplásico experimental. La implicación de la CCL2 en sus respuestas hipernociceptivas abre la posibilidad al empleo de antagonistas CCR2 para inhibirlas.

Globalmente, en el trabajo recogido en esta tesis, se exploran nuevas estrategias analgésicas relacionadas con el bloqueo de los receptores CCR1 y CCR2 en el contexto de dolor inflamatorio y neoplásico. El aumento en la expresión de determinadas quimiocinas de la serie CC, como la CCL2 y la CCL3, en tejidos inflamados o tumorales y el efecto antinociceptivo derivado del empleo de los anticuerpos correspondientes apoya su participación en la hiperalgesia térmica asociada a estas situaciones. Los ensayos sobre el efecto antinociceptivo del bloqueo de los receptores CCR1 y CCR2 se realizaron tras la administración aguda de los antagonistas correspondientes lo que, en los modelos inflamatorios, se produjo a dosis que no indujeron efecto antiinflamatorio. Se dispondrá de una visión más completa de la posible utilidad de este tipo de fármacos cuando se ensaye el efecto producido por su administración crónica y se hayan evaluado convenientemente los posibles efectos adversos que puedan ocasionar.
Actualmente se dispone de fármacos antagonistas de CCR1 o de CCR2 en fase de ensayos clínicos y hay datos que muestran cómo la administración crónica de un antagonista del receptor CCR1 puede ser eficaz para reducir síntomas de la artritis reumatoide en humanos, incluyendo algunas manifestaciones dolorosas, y aparentemente sin efectos adversos reseñables (Tak et al., 2013). El desarrollo y la valoración del posible uso clínico de los antagonistas CCR2 se encuentra bastante avanzado y algunos de estos fármacos diseñados para ser administrados por vía oral (Xue et al., 2011; Padi et al., 2012; Cherney et al., 2014) se han propuesto como una posible alternativa para el tratamiento de

diversos procesos como la artritis, la esclerosis múltiple, el daño vascular, la obesidad o la diabetes tipo 2. Alguno de ellos se encuentra en fase de ensayos clínicos para el tratamiento de la diabetes tipo 2 (Di Prospero et al., 2014). En particular, la detección en nuestros experimentos de niveles elevados de CCL2 en un proceso tumoral relacionado con la presencia de células de cáncer próstata así como la analgesia producida por su inhibición adquiere un interés especial teniendo en cuenta la relevancia que posee CCL2 en el desarrollo del propio cáncer de próstata (Guilloton et al., 2012), en el que la presencia de esta quimiocina parece relacionarse con un peor pronóstico (Izumi et al., 2013; Lim et al., 2016). De hecho, existe un anticuerpo anti-CCL2 (Carlumab) que está en fase de ensayos clínicos como tratamiento adyuvante antitumoral en tumores sólidos (Brana et al., 2015; Lim et al., 2016). En este contexto, nuestros resultados parecen indicar que el bloqueo del eje CCL2/CCR2 en el cáncer de próstata podría ser útil no sólo para paliar el dolor tumoral sino también para inhibir el propio crecimiento del tumor.

5. Conclusiones

1. La inflamación aguda y crónica en ratones produce un aumento de la concentración de CCL2 principalmente debido a su liberación desde los macrófagos atraídos al foco inflamatorio. Tanto el bloqueo agudo de los receptores CCR2, sobre los que actúa esta quimiocina, como la neutralización de la misma con un anticuerpo, inhiben la hiperalgesia inflamatoria, pero no la alodinia, y este efecto que no se debe a una reducción de la inflamación.

2. Los tejidos afectados por inflamación aguda o crónica presentan un aumento de la concentración de CCL3 liberada fundamentalmente desde neutrófilos y macrófagos. La acción de esta quimiocina sobre los receptores CCR1 contribuye a la hiperalgesia inflamatoria así como a la alodinia mecánica observada en ratones con inflamación crónica pero no aguda. El efecto analgésico producido por el antagonista CCR1 en ratones inflamados se debe al bloqueo de estos receptores a nivel periférico y no espinal, es independiente de una supuesta acción antiinflamatoria y no parece relacionado con el bloqueo de la acción de otras quimiocinas agonistas de CCR1, como la CCL5.

3. La inoculación intratibial de células RM1 en ratones C57BL/6 provoca el desarrollo de tumores óseos y la posterior invasión de los tejidos adyacentes. Estos tumores muestran algunos rasgos propios de las metástasis óseas de origen prostático en humanos ya que presentan un patrón mixto osteoblástico/osteoclástico y producen síntomas dolorosos que pueden inhibirse con morfina o ácido zoledrónico.

4. Tanto las células RM1 en cultivo como los tumores producidos tras su inoculación intraósea poseen capacidad de liberar CCL2 y la administración de un antagonista de CCR2 reduce la hiperalgesia tumoral. Este resultado, junto a los obtenidos en otros laboratorios describiendo la reducción del crecimiento de tumores prostáticos mediante la inhibición de la acción de la CCL2, sugiere que el bloqueo del sistema CCL2/CCR2 podría ser una diana interesante en el tratamiento de las metástasis de origen prostático.

6. Bibliografía

Abbadie C, Lindia JA, Cumiskey AM, Peterson LB, Mudgett JS, Bayne EK, *et al*. Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. Proc Natl Acad Sci U S A. 2003;100:7947–7952.

Abbadie C, Bhangoo S, De Koninck Y, Malcangio M, Melik-Parsadaniantz S, White FA. Chemokines and pain mechanisms. Brain Res Rev. 2009;60:125-134.

Abel S, Back DJ, Vourvahis M. Maraviroc: pharmacokinetics and drug interactions. Antivir Ther. 2009;14:607–618.

Aldinucci D, Colombatti A. The inflammatory chemokine CCL5 and cancer progression. Mediators Inflamm. 2014;2014:292376.

Alexander SPH, Catterall WA, Kelly E, Marrion N, Peters JA, Benson HE *et al.* The Concise Guide to PHARMACOLOGY 2015/16: Voltage-gated ion channels. Br J Pharmacol. 2015;172: 5904-5941.

Alfonso Romero-Sandoval E, Sweitzer S. Nonneuronal central mechanisms of pain: glia and immune response. Prog Mol Biol Transl Sci. 2015;131:325-358.

Alloui A, Zimmermann K, Mamet J, Duprat F, Noël J, Chemin J, et al. TREK-1, a K+ channel involved in polymodal pain perception. EMBO J. 2006;25:2368-2376.

Amat M, Benjamim CF, Williams LM, Prats N, Terricabras E, Beleta J, *et al.* Pharmacological blockade of CCR1 ameliorates murine arthritis and alters cytokine networks in vivo. Br J Pharmacol. 2006;149:666–675.

Arms L, Girard BM, Malley SE, Vizzard MA. Expression and function of CCL2/CCR2 in rat micturition reflexes and somatic sensitivity with urinary bladder inflammation. Am J Physiol Renal Physiol. 2013;305:F111-122.

Arnoux I, Audinat E. Fractalkine Signaling and Microglia Functions in the Developing Brain. Neural Plast. 2015;2015:689404

Baamonde A, Lastra A, Villazón M, Bordallo J, Hidalgo A, Menéndez L. Involvement of endogenous endothelins in thermal and mechanical inflammatory hyperalgesia in mice. Naunyn Schmiedebergs Arch Pharmacol. 2004; 369:245–251.

Baamonde A, Curto-Reyes V, Juarez L, Meana A, Hidalgo A, Menéndez L. Antihyperalgesic effects induced by the IL-1 receptor antagonist anakinra and increased IL-1beta levels in inflamed and osteosarcoma-bearing mice. Life Sci. 2007;81:673–682.

Baamonde A, Hidalgo A, Menéndez L. Involvement of glutamate NMDA and AMPA receptors, glial cells and IL-1β in the spinal hyperalgesia evoked by the chemokine CCL2 in mice. Neurosci. Lett. 2011;502:178-181.

Bachelerie F, Ben-Baruch A, Burkhardt AM, Combadiere C, Farber JM, Graham GJ *et al*. International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. Pharmacol Rev. 2014;66:1-79.

Bachelerie F, Ben-Baruch A, Combadiere C, Farber JM, Graham GJ, Horuk R, *et al*. Chemokine receptors. Accessed on 12/04/2016. IUPHAR/BPS Guide to PHARMACOLOGY, http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=14.

Bagal SK, Chapman ML, Marron BE, Prime R, Storer RI, Swain NA. Recent progress in sodium channel modulators for pain. Bioorg Med Chem Lett. 2014;24:3690-9.

Baley PA, Yoshida K, Qian W, Sehgal I, Thompson TC. Progression to androgen insensitivity in a novel in vitro mouse model for prostate cancer. J Steroid Biochem Mol Biol. 1995;52:403-413.

Baliki MN, Apkarian AV. Nociception, Pain, Negative Moods, and Behavior Selection. Neuron. 2015;87:474–91

Bang S, Yoo S, Yang TJ, Cho H, Hwang SW. Isopentenyl pyrophosphate is a novel antinociceptive substance that inhibits TRPV3 and TRPA1 ion channels. Pain. 2011;152:1156-1164.

Bang S, Yoo S, Yang TJ, Cho H, Hwang SW. Nociceptive and proinflammatory effects of dimethylallyl pyrophosphate via TRPV4 activation. Br J Pharmacol. 2012; 166:1433–1443.

Basbaum AI, Bautista DM, Scherrer G, Julius D. Cellular and molecular mechanisms of pain. Cell. 2009;139:267-284.

Basbaum AI and Jessel T. The perception of pain. In principles of Neuroscience., E.R Kandel, J. Schwartz and T. Jessell, eds (New York: Appleton and Lange) 2000, pp.472-491.

Bautista DM, Siemens J, Glazer JM, Tsuruda PR, Basbaum AI, Stucky CL *et al.* The menthol receptor TRPM8 is the principal detector of environmental cold. Nature. 2007;448:204-208.

Beggs S, Trang T, Salter MW. P2X4R+ microglia drive neuropathic pain. Nat Neurosci. 2012;15: 1068-1073.

Belkouch M, Dansereau MA, Réaux-Le Goazigo A, Van Steenwinckel J, Beaudet N, Chraibi A, et al. The chemokine CCL2 increases Nav1.8 sodium channel activity in primary sensory neurons through a Gβγ-dependent mechanism. J Neurosci. 2011;31:18381-18390.

Benamar K, Geller EB, Adler MW. Elevated level of the proinflammatory chemokine, RANTES/CCL5, in the periaqueductal grey causes hyperalgesia in rats. Eur J Pharmacol. 2008;592:93-95.

Bhangoo S, Ren D, Miller RJ, Henry KJ, Lineswala J, Hamdouchi C, et al. Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors. Mol Pain. 2007;3:38

Biber K, Boddeke E. Neuronal CC chemokines: the distinct roles of CCL21 and CCL2 in neuropathic pain. Front Cell Neurosci. 2014;8:210.

Bicer F, Altuntas CZ, Izgi K, Ozer A, Kavran M, Tuohy VK, *et al.* Chronic pelvic allodynia is mediated by CCL2 through mast cells in an experimental autoimmune cystitis model. Am J Physiol Renal Physiol. 2015;308:F103-113.

Boddeke EW, Meigel I, Frentzel S, Gourmala NG, Harrison JK, Buttini M, *et al.* Cultured rat microglia express functional beta-chemokine receptors. J Neuroimmunol. 1999;98:176-184.

Bonabello A, Galmozzi MR, Bruzzese T, Zara GP. Analgesic effect of bisphosphonates in mice. Pain. 2001;91:269-275.

Brana I, Calles A, LoRusso PM, Yee LK, Puchalski TA, Seetharam S *et al.* Carlumab, an anti-C-C chemokine ligand 2 monoclonal antibody, in combination with four chemotherapy regimens for the treatment of patients with solid tumors: an open-label, multicenter phase 1b study. Target Oncol. 2015;10:111-123.

Brack A, Rittner HL, Machelska H, Leder K, Mousa SA, Schäfer M et al. Control of inflammatory pain by chemokine-mediated recruitment of opioid-containing polymorphonuclear cells. Pain. 2004a;112:229–238.

Brack, A, Rittner HL, Machelska H, Shaqura M, Mousa SA, Labuz D, et al. Endogenous peripheral antinociception in early inflammation is not limited by the number of opioid-containing leukocytes but by opioid receptor expression. Pain. 2004b;108:67–75.

Braz JM, Nassar MA, Wood JN, Basbaum AI. Parallel "pain" pathways arise from subpopulations of primary afferent nociceptor. Neuron. 2005;47:787-793.

Braz JM, Solorzano C, Wang X, Basbaum AI. Transmitting pain and itch messages: a contemporary view of the spinal cord circuits that generate gate control. Neuron 2014;82: 522-536

Brito R, Sheth S, Mukherjea D, Rybak LP, Ramkumar V. TRPV1: A Potential Drug Target for Treating Various Diseases. Cells. 2014;3:517-45.

Bu H, Shu B, Gao F, Liu C, Guan X, Ke C, et al. Spinal IFN-γ-induced protein-10 (CXCL10) mediates metastatic breast cancer-induced bone pain by activation of microglia in rat models. Breast Cancer Res Treat. 2014;143:255–263.

Budai D, Wilcox GL, Larson AA. Effects of nitric oxide availability on responses of spinal wide dynamic range neurons to excitatory amino acids. Eur J Pharmacol. 1995;278:39-47.

Burnstock G. Purinergic mechanisms and pain--an update. Eur J Pharmacol. 2013;716:24-40.

Burnstock G. Physiopathological roles of P2X receptors in the central nervous system. Curr Med Chem. 2015;22:819-44.

Cancellieri C, Vacchini A, Locati M, Bonecchi R, Borroni EM. Atypical chemokine receptors: from silence to sound. Biochem Soc Trans 2013;41(1):231–6.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. Nature. 1997;389:816-24.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, Julius D. Impaired nociception and pain sensation in mice lacking the capsaicin receptor. Science. 2000;288:306-13.

Caterina MJ, Julius D. The vanilloid receptor: a molecular gateway to the pain pathway. Annu Rev Neurosci. 2001;24:487-517.

Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. N Engl J Med. 2006;354:610-621.

Charo IF, Hills R, Horuk R, Matsushima K, Murphy PM, Oppenheim JJ. Chemokine receptors: CCR2. Last modified on 23/02/2016. Accessed on 13/05/2016. IUPHAR/BPS Guide to PHARMACOLOGY, http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=59.

Cherney RJ, Mo R, Yang MG, Xiao Z, Zhao Q, Mandlekar S, *et al.* Alkylsulfone-containing trisubstituted cyclohexanes as potent and bioavailable chemokine receptor 2 (CCR2) antagonists. Bioorg Med Chem Lett. 2014;24:1843-1845.

Chhikara M, Huang H, Vlachaki MT, Zhu X, Teh B, Chiu KJ, et al., Enhanced therapeutic effect of HSV-tk+GCV gene therapy and ionizing radiation for prostate cancer. Mol Ther. 2001;3:536-542.

Cohen MR, Moiseenkova-Bell VY. Structure of thermally activated TRP channels. Curr Top Membr. 2014;74:181-211.

Cohen SP, Mao J. Neuropathic pain: mechanisms and their clinical implications. BMJ. 2014;348:f7656.

Coleman RE. Clinical features of metastasic bone disease and risk of skeletal morbidity. Clin Cancer Res 2006; 12:6243s-6249s.

Coleman R, Body JJ, Aapro M, Hadji P, Herrstedt J; ESMO Guidelines Working Group. Bone health in cancer patients: ESMO Clinical Practice Guidelines. Ann Oncol. 2014 Sep;25 Suppl 3:iii124-37.

Cunha, F. Q., Ferreira, S. H. Peripheral hyperalgesic cytokines. Adv. Exp. Med. Biol. 2003; 521, 22–39.

Cunha TM, Verri Jr WA, Silva JR, Poole S, Cunha FQ, Ferreira SH. A cascade of cytokines mediates mechanical inflammatory hypernociception Proc. Natl. Acad. Sci. USA. 2005; 102:1755–1760.

Cunha FQ, Lorenzetti BB, Poole S, Ferreira SH. Interleukin-8 as a mediator of sympathetic pain. Br J Pharmacol. 1991;104:765-767.

Curto-Reyes V, Juárez L, García-Pérez E, Fresno MF, Hidalgo A, Menéndez L, et al. Local loperamide inhibits thermal hyperalgesia but not mechanical allodynia induced by intratibial inoculation of melanoma cells in mice. Cell Mol Neurobiol. 2008;28:981-990.

Curto-Reyes V, Llames S, Hidalgo A, Menéndez L, Baamonde A. Spinal and peripheral analgesic effects of the CB2 cannabinoid receptor agonist AM1241 in two models of bone cancer-induced pain. Br J Pharmacol. 2010;160:561-573

Dairaghi DJ, Oyajobi BO, Gupta A, McCluskey B, Miao S, Powers J *et al.* CCR1 blockade reduces tumor burden and osteolysis in vivo in a mouse model of myeloma bone disease. Blood. 2012. 16;120:1449-1457.

Davies AN. Breakthrough cancer pain. Curr Pain Headache Rep. 2014;18:420.

Davis KD, Moayedi M. Central mechanisms of pain revealed through functional and structural MRI. J Neuroimmune Pharmacol. 2013;8:518-534.

Davis MP. Cannabinoids in pain management: CB1, CB2 and non-classic receptor ligands. Expert Opin Investig Drugs. 2014;23:1123-40.

Dawes JM, Kiesewetter H, Perkins JR, Bennett DL, McMahon SB. Chemokine expression in peripheral tissues from the monosodium iodoacetate model of chronic joint pain. Mol Pain. 2013;9:57.

Dawes JM, McMahon SB. Chemokines as peripheral pain mediators. Neurosci Lett. 2013;557 Pt A:1-8.

de Miguel M, Kraychete DC, Meyer Nascimento RJ. Chronic pain: cytokines, lymphocytes and chemokines. Inflamm Allergy Drug Targets. 2014;13:339-349.

Delaunay A, Gasull X, Salinas M, Noël J, Friend V, Lingueglia E, Deval E. Human ASIC3 channel dynamically adapts its activity to sense the extracellular pH in both acidic and alkaline directions. Proc Natl Acad Sci U S A. 2012;109:13124-13129.

Deshmane SL, Kremlev S, Amini S, Sawaya BE: Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009;29:313-326.

D'Haese JG, Demir IE, Friess H, Ceyhan GO. Fractalkine/CX3CR1: why a single chemokine-receptor duo bears a major and unique therapeutic potential. Expert Opin Ther Targets. 2010;14:207-219.

Di Prospero NA, Artis E, Andrade-Gordon P, Johnson DL, Vaccaro N, Xi L, *et al*. CCR2 antagonism in patients with type 2 diabetes mellitus: a randomized, placebo-controlled study. Diabetes Obes Metab. 2014;16:1055-1064.

Djouhri L, Lawson SN. Abeta-fiber nociceptive primary afferent neurons: a review of incidence and properties in relation to other afferent A-fiber neurons in mammals. Brain Res Brain Res Rev. 2004;46:131-145.

D'Mello R, Dickenson AH. Spinal cord mechanisms of pain. Br J Anaesth. 2008;101:8-16.

Dong L, Zhang X, Yu C, Yu T, Liu S, Hou L, *et al*. Monitoring luciferase-labeled human prostate stem cell antigen-expressing tumor growth in a mouse model. Exp Ther Med. 2013;6:1208-1212.

Doré-Savard L, Otis V, Belleville K, Lemire M, Archambault M, Tremblay L *et al.* Behavioral, medical imaging and histopathological features of a new rat model of bone cancer pain. PLoS One. 2010;5:e13774.

Du X, Gamper N. Potassium channels in peripheral pain pathways: expression, function and therapeutic potential. Curr Neuropharmacol. 2013;11:621-640.

Dubin AE, Patapoutian A. Nociceptors: the sensors of the pain pathway. J Clin Invest. 2010;120:3760-3772.

Ducourneau VR, Dolique T, Hachem-Delaunay S, Miraucourt LS, Amadio A, Blaszczyk L *et al.* Cancer pain is not necessarily

correlated with spinal overexpression of reactive glia markers. Pain. 2014;155:275-291.

Eijkelkamp N, Heijnen CJ, Willemen HL, Deumens R, Joosten EA, Kleibeuker W *et al.* GRK2: a novel cell-specific regulator of severity and duration of inflammatory pain. J Neurosci. 2010;30:2138-49.

Ellis A, Bennett Dl. Neuroinflammation and the generation of neuropathic pain. Br J Anaesth. 2013;111:26-37.

Falk S, Dickenson AH. Pain and nociception: mechanisms of cancer-induced bone pain. J Clin Oncol. 2014;32:1647-54.

Franceschini A. and Adinolfi E. P2X receptors: New players in cancer pain. World J Biol Chem. 2014; 5:429-436.

Fuchs PN, Peng YB, Boyette-Davis JA, Uhelski ML. The anterior cingulate cortex and pain processing. Front Integr Neurosci. 2014;8:35.

Gao YJ, Ji RR. Chemokines, neuronal-glial interactions, and central processing of neuropathic pain. Pharmacol Ther. 2010;126:56-68.

Garcia-Ramallo E, Marques T, Prats N, Beleta J, Kunkel SL, Godessart N. Resident Cell Chemokine Expression Serves as the Major Mechanism for Leukocyte Recruitment During Local Inflammation. J Immunol. 2002; 169:6467-6473.

Gerszten RE, Garcia-Zepeda EA, Lim Y-C, et al. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. Nature 1999; 398:718-23.

Ghilardi JR, Freeman KT, Jiménez-Andrade JM, Mantyh WG, Bloom AP, Kuskowski MA *et al.* Administration of a tropomyosin receptor kinase inhibitor attenuates sarcoma-induced nerve sprouting, neuroma formation and bone cancer pain. Mol Pain. 2010;6:87.

Gibofsky A. Overview of epidemiology, pathophysiology, and diagnosis of rheumatoid arthritis. Am J Manag Care. 2012;18(13 Suppl):S295-302.

Giordano J. The neurobiology of nociceptive and anti-nociceptive systems. Pain Physician. 2005;8:277-90.

Gold MS, Gebhart GF. Nociceptor sensitization in pain pathogenesis. Nat Med. 2010;16:1248-57.

Gold MS, Reichling DB, Shuster MJ, Levine JD. Hyperalgesic agents increase a tetrodotoxin-resistant Na+ current in nociceptors. Proc Natl Acad Sci U S A. 1996;93:1108-12.

González-Rodríguez S, Pevida M, Roques BP, Fournié-Zaluski MC, Hidalgo A, Menéndez L *et al.* Involvement of enkephalins in the inhibition of osteosarcoma-induced thermal hyperalgesia evoked by the blockade of peripheral P2X3 receptors. Neurosci Lett. 2009;465:285-289.

González-Rodríguez S, Hidalgo A, Baamonde A, Menéndez L. Spinal and peripheral mechanisms involved in the enhancement of morphine analgesia in acutely inflamed mice. Cell Mol Neurobiol. 2010a;30:113-121.

González-Rodríguez S, Hidalgo A, Baamonde A, Menéndez L. Involvement of Gi/o proteins and GIRK channels in the potentiation of morphine-induced spinal analgesia in acutely inflamed mice. Naunyn Schmiedebergs Arch Pharmacol. 2010b;381:59-71.

González-Rodríguez S, Llames S, Hidalgo A, Baamonde A, Menéndez L. Potentiation of acute morphine-induced analgesia measured by a thermal test in bone cancer-bearing mice. Fundam Clin Pharmacol. 2012;26:363-72.

Grace PM, Maier SF, Watkins LR. Opioid-induced central immune signaling: implications for opioid analgesia. Headache. 2015;55:475-489.

Gregory NS, Harris AL, Robinson CR, Dougherty PM, Fuchs PN, Sluka KA. An overview of animal models of pain: disease models and outcome measures. J Pain. 2013;14:1255-69.

Griffith JW, Sokol CL, Luster AD. Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 2014;32:659–702.

Groom JR, Richmond J, Murooka TT, Sorensen EW, Sung JH, Bankert K et al. CXCR3 chemokine receptor-ligand interactions in the lymph node optimize CD4+ T helper 1 cell differentiation. Immunity. 2012;37:1091-1103.

Guan XH, Fu QC, Shi D, Bu HL, Song ZP, Xiong BR *et al.* Activation of spinal chemokine receptor CXCR3 mediates bone cancer pain through an Akt-ERK crosstalk pathway in rats. Exp Neurol. 2015 Jan;263:39-49.

Guilloton F, Caron G, Ménard C, Pangault C, Amé-Thomas P, Dulong J *et al.* Mesenchymal stromal cells orchestrate follicular lymphoma cell niche through the CCL2-dependent recruitment and polarization of monocytes. Blood. 2012;119:2556–2567.

Guan XH, Fu QC, Shi D, Bu HL, Song ZP, Xiong BR *et al.* Activation of spinal chemokine receptor CXCR3 mediates bone cancer pain through an Akt-ERK crosstalk pathway in rats. Exp Neurol. 2015 Jan;263:39-49.

Guo G, Gao F. CXCR3: latest evidence for the involvement of chemokine signaling in bone cancer pain. Exp Neurol.

2015;265:176-179.

Guindon J, Hohmann AG. The endocannabinoid system and pain. CNS Neurol Disord Drug Targets. 2009;8:403-421.

Hains BC, Waxman SG. Activated microglia contribute to the maintenance of chronic pain after spinal cord injury. J Neurosci. 2006;26:4308-4317.

Hald A, Nedergaard S, Hansen RR, Ding M, Heegaard AM. Differential activation of spinal cord glial cells in murine models of neuropathic and cancer pain. Eur J Pain. 2009;13:138-145.

Halvorson KG, Kubota K, Sevcik MA, Lindsay TH, Sotillo JE, Ghilardi JR, *et al.* A blocking antibody to nerve growth factor attenuates skeletal pain induced by prostate tumor cells growing in bone. Cancer Res. 2005;65:9426-9435.

Hang LH, Shao DH, Chen Z, Chen YF, Shu WW, Zhao ZG. Involvement of spinal CC chemokine ligand 5 in the development of bone cancer pain in rats. Basic Clin Pharmacol Toxicol. 2013;113:325-328.

Hang LH, Li SN, Luo H, Shu WW, Mao ZM, Chen YF, *et al.* Connexin 43 Mediates CXCL12 Production from Spinal Dorsal Horn to Maintain Bone Cancer Pain in Rats. Neurochem Res. 2016;41:1200-1208.

Honoré P, Rogers SD, Schwei MJ, Salak-Johnson JL, Luger NM, Sabino MC, *et al*. Murine models of inflammatory, neuropathic and cancer pain each generates a unique set of neurochemical changes in the spinal cord and sensory neurons. Neuroscience. 2000;98:585-98.

Hu JH, Zheng XY, Yang JP, Wang LN, Ji FH.Involvement of spinal monocyte chemoattractant protein-1 (MCP-1) in cancer-induced bone pain in rats.Neurosci Lett. 2012a ;517:60-63.

Hu JH, Yang JP, Liu L, Li CF, Wang LN, Ji FH, *et al.* Involvement of CX3CR1 in bone cancer pain through the activation of microglia p38 MAPK pathway in the spinal cord. Brain Res; 2012b;1465:1–9.

Hu JH, Wu MY, Tao M, Yang JP. Changes in protein expression and distribution of spinal CCR2 in a rat model of bone cancer pain. Brain Res. 2013;1509:1-7.

Hu XM, Liu YN, Zhang HL, Cao SB, Zhang T, Chen LP, Shen W. CXCL12/CXCR4 chemokine signaling in spinal glia induces pain hypersensitivity through MAPKs-mediated neuroinflammation in bone cancer rats. J Neurochem. 2015;132:452-463.

Imai S, Narita M, Ikegami D, Yamashita A, Shimizu T, Narita M, *et al.* Epigenetic transcriptional activation of monocyte chemotactic protein 3 contributes to long-lasting neuropathic pain. Brain. 2013;136:828–843.

Ito Y, Ishiguro H, Kobayashi N, Hasumi H, Watanabe M, Yao M, *et al.* Adipocyte-Derived Monocyte Chemotactic Protein-1 (MCP-1) Promotes Prostate Cancer Progression Through the Induction of MMP-2 Activity. Prostate. 2015;1019:1009–1019.

Izhak L, Wildbaum G, Jung S, Stein A, Shaked Y, Karin N. Dissecting the autocrine and paracrine roles of the CCR2-CCL2 axis in tumor survival and angiogenesis. PLoS One. 2012;7:e28305.

Izumi K, Fang LY, Mizokami A, Namiki M, Li L, Lin WJ *et al.* Targeting the androgen receptor with siRNA promotes prostate cancer metastasis through enhanced macrophage recruitment via CCL2/CCR2-induced STAT3 activation. EMBO Mol Med. 2013;5:1383-1401.

Izumi K, Mizokami A, Lin HP, Ho HM, Iwamoto H, Maolake A *et al.* Serum chemokine (CC motif) ligand 2 level as a diagnostic, predictive, and prognostic biomarker for prostate cancer. Oncotarget. 2015 Dec 19. [Epub ahead of print].

Jeon SM, Lee KM, Park ES, Jeon YH, Cho HJ. Monocyte chemoattractant protein-1 immunoreactivity in sensory ganglia and hindpaw after adjuvant injection. Neuroreport.2008;19:183–186.

Jeon SM, Sung JK, Cho HJ. Expression of monocyte chemoattractant protein-1 and its induction by tumor necrosis factor receptor 1 in sensory neurons in the ventral rhizotomy model of neuropathic pain. Neuroscience. 2011;190:354-366.

Ji RR, Baba H, Brenner GJ, Woolf CJ. Nociceptive-specific activation of ERK in spinal neurons contributes to pain hypersensitivity. Nat Neurosci 1999; 2:1114–1119.

Ji RR, Berta T, Nedergaard M. Glia and pain: Is chronic pain a gliopathy?. Pain. 2013;154:S10-S28.

Jiménez-Andrade JM, Ghilardi JR, Castañeda-Corral G, Kuskowski MA, Mantyh PW. Preventive or late administration of anti-NGF therapy attenuates tumor-induced nerve sprouting, neuroma formation, and cancer pain. Pain. 2011;152:2564-2574.

Jin D, Yang JP, Hu JH, Wang LN, Zuo JL. MCP-1 stimulates spinal microglia via PI3K/Akt pathway in bone cancer pain. Brain Res. 2015;1599:158-167.

Julius D. TRP channels and pain. Annu Rev Cell Dev Biol. 2013;29:355-384.

Jung, H, Toth PT, White FA, Miller RJ. Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root

ganglia neurons. J Neurochem. 2008;104: 254-263.

Jung H, Bhangoo S, Banisadr G, Freitag C, Ren D, White FA, Miller RJ. Visualization of chemokine receptor activation in transgenic mice reveals peripheral activation of CCR2 receptors in states of neuropathic pain. J Neurosci. 2009;29:8051-8062.

Kakimoto S, Nagakura Y, Tamura S, Watabiki T, Shibasaki K, Tanaka S *et al.* Minodronic acid, a third-generation bisphosphonate, antagonizes purinergic P2X(2/3) receptor function and exerts an analgesic effect in pain models. Eur J Pharmacol. 2008;589:98-101.

Kane CM, Hoskin P, Bennett MI. Cancer induced bone pain. BMJ. 2015;350:h315.

Kawabata A, Kawao N, Hironaka Y, Ishiki T, Matsunami M, Sekiguchi F. Antiallodynic effect of etidronate, a bisphosphonate, in rats with adjuvant-induced arthritis: involvement of ATP-sensitive K+ channels. Neuropharmacology. 2006;51:182-190.

Kellenberger S, Schild L. International Union of Basic and Clinical Pharmacology. XCI. structure, function, and pharmacology of acid-sensing ion channels and the epithelial Na+ channel. Pharmacol Rev. 2015;67:1-35.

Kennedy J, Kelner GS, Kleyensteuber S, Schall TJ, Weiss MC, Yssel H *et al*. Molecular cloning and functional characterization of human lymphotactin. J Immunol. 1995;155:203–209.

Khalil AA, Jameson MJ, Broaddus WC, Lin PS, Dever SM, Golding SE, Rosenberg E *et al.* The Influence of Hypoxia and pH on Bioluminescence Imaging of Luciferase-Transfected Tumor Cells and Xenografts. Int J Mol Imaging. 2013;2013:287697.

Khasabova IA, Stucky CL, Harding-Rose C, Eikmeier L, Beitz AJ, Coicou LG, Hanson AE, Simone DA, Seybold VS. Chemical interactions between fibrosarcoma cancer cells and sensory neurons contribute to cancer pain. J Neurosci. 2007;27:10289-10298.

Kiguchi N, Kobayashi Y, Maeda T, Saika F, Kishioka S. Chemokine MIP-1α in the spinal cord contributes to nerve injury-induced neuropathic pain. Neurosci Lett. 2010a;484:17-21.

Kiguchi N, Maeda T, Kobayashi Y, Fukazawa Y, Kishioka S. Macrophage inflammatory protein-1alpha mediates the development of neuropathic pain following peripheral nerve injury through interleukin-1beta up-regulation. Pain. 2010b;149:305–315.

Kiguchi N, Kobayashi Y, Kishioka S. Chemokines and cytokines in neuroinflammation leading to neuropathic pain. Curr Opin Pharmaco.2012;12:55–61.

Kim S, Seiryu M, Okada S, Kuroishi T, Takano-Yamamoto T, Sugawara S, *et al*. Analgesic effects of the non-nitrogen-containing bisphosphonates etidronate and clodronate, independent of anti-resorptive effects on bone. Eur J Pharmacol. 2013;699:14-22.

Kim YH, Back SK, Davies AJ, Jeong H, Jo HJ, Chung G *et al.* TRPV1 in GABAergic interneurons mediates neuropathic mechanical allodynia and disinhibition of the nociceptive circuitry in the spinal cord. Neuron. 2012; 74:640–647.

Knerlich-Lukoschus F, von der Ropp-Brenner B, Lucius R, Mehdorn HM, Held-Feindt J. Spatiotemporal CCR1, CCL3 (MIP-1α), CXCR4, CXCL12(SDF-1α) expression patterns in a rat spinal cord injury model of posttraumatic neuropathic pain. J Neurosurg Spine. 2011;14:583-597.

Knerlich-Lukoschus F, Held-Feindt J. Chemokine-ligands/receptors: multiplayers in traumatic spinal cord injury. Mediators Inflamm. 2015;2015:486758.

Kotrych D, Dziedziejko V, Safranow K, Drozdzik M, Pawlik A. CXCL9 and CXCL10 gene polymorphisms in patients with rheumatoid arthritis. Rheumatol Int. 2015;35:1319-1323.

Kuang Y, Wu Y, Jiang H, Wu D. Selective G protein coupling by C-C chemokine receptors. J Biol Chem. 1996;271:3975-3978.

Kuner R. Central mechanisms of pathological pain. Nat Med. 2010;16:1258-1266.

Le Goff B, Berthelot JM, Maugars Y, Romas E. Alternative use of bisphosphonate therapy for rheumatic disease. Curr Pharm Des. 2010;16:3045-3052.

Lebre MC, Vergunst CE, Choi IY, Aarrass S, Oliveira AS, Wyant T *et al.* Why CCR2 and CCR5 blockade failed and why CCR1 blockade might still be effective in the treatment of rheumatoid arthritis. PLoS One. 2011;6:e21772.

Lee YC, Cui J, Lu B, Frits ML, Iannaccone CK, Shadick NA *et al.* Pain persists in DAS28 rheumatoid arthritis remission but not in ACR/EULAR remission: a longitudinal observational study. Arthritis Res Ther. 2011;13:R83.

Lee EY, Lee ZH, Song YW. The interaction between CXCL10 and cytokines in chronic inflammatory arthritis. Autoimmun Rev. 2013;12:554-557.

Leichtle A, Hernandez M, Ebmeyer J, Yamasaki K, Lai Y, Radek K *et al.* CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF-/- mice. J Immunol 2010;184:3087–3097.

Lewis ND, Muthukumarana A, Fogal SE, Corradini L, Stefanopoulos DE, Adusumalli P *et al.* CCR1 plays a critical role in modulating pain through hematopoietic and non-hematopoietic cells. PLoS One. 2014;9:e105883.

Li X, Wang XW, Feng XM, Zhou WJ, Wang YQ, Mao-Ying QL. Stage-dependent anti-allodynic effects of intrathecal Toll-like receptor 4 antagonists in a rat model of cancer induced bone pain. J Physiol Sci. 2013;63:203-209.

Li BT, Wong MH, Pavlakis N. Treatment and Prevention of Bone Metastases from Breast Cancer: A Comprehensive Review of Evidence for Clinical Practice. J Clin Med. 2014;3:1-24.

Li N, Zhang L, Shu R, Ding L, Wang Z, Wang H, Yu Y, Wang G. Involvement of CCL3/CCR5 Signaling in Dorsal Root Ganglion in Remifentanil-induced Hyperalgesia in Rats. Clin J Pain. 2015 Nov 6. [Epub ahead of print].

Li D, Chen H, Luo XH, Sun Y, Xia W, Xiong YC. CX3CR1-Mediated Akt1 Activation Contributes to the Paclitaxel-Induced Painful Peripheral Neuropathy in Rats. Neurochem Res. 2016 Mar 9. [Epub ahead of print].

Lim E, Modi KD, Kim J. In vivo bioluminescent imaging of mammary tumors using IVIS spectrum. J Vis Exp. 2009 Apr 29;(26). pii: 1210.

Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ. Targeting the CCL2-CCR2 signaling axis in cancer metastasis. Oncotarget. 2016 Feb 14. [Epub ahead of print].

Lin YM, Hsu CJ, Liao YY, Chou MC, Tang CH. The CCL2/CCR2 axis enhances vascular cell adhesion molecule-1 expression in human synovial fibroblasts. PLoS One. 2012;7:e49999.

Lindsay TH, Jonas BM, Sevcik MA, Kubota K, Halvorson KG, Ghilardi JR *et al.* Pancreatic cancer pain and its correlation with changes in tumor vasculature, macrophage infiltration, neuronal innervation, body weight and disease progression. Pain. 2005;119:233-246.

Linley JE, Rose K, Ooi L, Gamper N. Understanding inflammatory pain: ion channels contributing to acute and chronic nociception. Pflugers Arch. 2010;459:657-669.

Liou JT, Yuan HB, Mao CC, Lai YS, Day YJ. Absence of C-C motif chemokine ligand 5 in mice leads to decreased local macrophage recruitment and behavioral hypersensitivity in a murine neuropathic pain model. Pain. 2012;153:1283-1291.

Liu Y, Shen W, Hu X, Chen L, Cao S, Zhang T. Role of chemokine CXCL12 in spinal cord in development of bone cancer pain in rats: relationship with microglial activation. Chin. J. Anesthesiol. 2014;34:40–42.

Liou JT, Lee CM, Day YJ. The immune aspect in neuropathic pain: role of chemokines. Acta Anaesthesiol Taiwan. 2013;51:127-132.

Loeser JD, Treede RD. The Kyoto protocol of IASP Basic Pain Terminology. Pain. 2008;137:473-477.

Lolignier S, Eijkelkamp N, Wood JN. Mechanical allodynia. Pflugers Arch. 2015;467:133-139.

Lopes AH, Brandolini L, Aramini A, Bianchini G, Silva RL, Zaperlon AC *et al.* DF2755A, a novel non-competitive allosteric inhibitor of CXCR1/2, reduces inflammatory and post-operative pain. Pharmacol Res. 2016;103:69-79.

Lucarini R, Tozatti MG, Silva ML, Gimenez VM, Pauletti PM, Groppo M. Antibacterial and anti-inflammatory activities of an extract, fractions, and compounds isolated from Gochnatia pulchra aerial parts. Braz J Med Biol Res. 2015;48:822-830.

Luo X, Tai WL, Sun L, Pan Z, Xia Z, Chung SK *et al*. Crosstalk between astrocytic CXCL12 and microglial CXCR4 contributes to the development of neuropathic pain. Mol Pain. 2016;12: pii: 1744806916636385.

Malinow R, Schulman H, Tsien RW. Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science. 1989;245:862-866.

Manjavachi MN, Quintão NL, Campos MM, Deschamps IK, Yunes RA, Nunes RJ *et al.* The effects of the selective and non-peptide CXCR2 receptor antagonist SB225002 on acute and long-lasting models of nociception in mice. Eur J Pain. 2010;14:23-31.

Mantyh PW. Cancer pain and its impact on diagnosis, survival and quality of life. Nat Rev Neurosci. 2006;7:797-809.

Mantyh PW. Bone cancer pain: from mechanism to therapy. Curr Opin Support Palliat Care. 2014;8:83-90.

Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. Nat Rev Neurosci. 2005;6:521-532.

Masi L and Brandi ML Calcitonin and calcitonin receptors .Clin Cases Miner Bone Metab. 2007; 4: 117–122.

Massara M, Bonavita O, Mantovani A, Locati M, Bonecchi R. Atypical chemokine receptors in cancer: friends or foes? J

Leukoc Biol. 2016 Feb 23. [Epub ahead of print].

Mathie A, Veale EL. Two-pore domain potassium channels: potential therapeutic targets for the treatment of pain. Pflugers Arch. 2015;467:931-943.

Matsushita K, Tozaki-Saitoh H, Kojima C, Masuda T, Tsuda M, Inoue K *et al.* Chemokine (C-C motif) receptor 5 is an important pathological regulator in the development and maintenance of neuropathic pain. Anesthesiology. 2014;120:1491-1503.

Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004;36:1882-1886.

McCabe NP, Madajka M, Vasanji A, Byzova TV. Intraosseous injection of RM1 murine prostate cancer cells promotes rapid osteolysis and periosteal bone deposition. Clin Exp Metastasis. 2008;25:581-590.

McCarson KE. Models of Inflammation: Carrageenan- or Complete Freund's Adjuvant (CFA)-Induced Edema and Hypersensitivity in the Rat. Curr Protoc Pharmacol. 2015;70:5.4.1-9.

McCorvy JD, Roth BL. Structure and function of serotonin G protein-coupled receptors. Pharmacol Ther. 2015;150:129-142.

McMahon SB, La Russa F, Bennett DL. Crosstalk between the nociceptive and immune systems in host defence and disease. Nat Rev Neurosci. 2015;16:389-402.

Mélik Parsadaniantz S, Rivat C, Rostène W, Réaux-Le Goazigo A. Opioid and chemokine receptor crosstalk: a promising target for pain therapy? Nat Rev Neurosci. 2015;16:69-78.

Menéndez L, Lastra A, Fresno MF, Llames S, Meana A, Hidalgo A *et al.* Initial thermal heat hypoalgesia and delayed hyperalgesia in a murine model of bone cancer pain. Brain Res. 2003;969:102-109.

Menéndez L, Lastra A, Hidalgo A, Baamonde A. The analgesic effect induced by capsaicin is enhanced in inflammatory states. Life Sci. 2004;74:3235-3244.

Menéndez L, Juárez L, García E, García-Suárez O, Hidalgo A, Baamonde A. Analgesic effects of capsazepine and resiniferatoxin on bone cancer pain in mice. Neurosci Lett. 2006;393:70-73.

Menetski J, Mistry S, Lu M, Mudgett JS, Ransohoff RM, Demartino JA *et al.* Mice overexpressing chemokine ligand 2 (CCL2) in astrocytes display enhanced nociceptive responses. Neuroscience. 2007;149:706-714.

Mercadante S, Fulfaro F. Management of painful bone metastases. Curr Opin Oncol. 2007;19:308–314.

Mercadante S. Cancer pain. Curr Opin Support Palliat Care. 2013;7:139-143.

Meuser T, Pietruck C, Radbruch L, Stute P, Lehmann KA, Grond S. Symptoms during cancer pain treatment following WHO-guidelines: a longitudinal follow-up study of symptom prevalence, severity and etiology. Pain. 2001;93:247-257.

Miller R.E., Tran P.B., Das R. et al. CCR2 chemokine receptor signaling mediates pain in experimental osteoarthritis. Proc Natl Acad Sci USA.2012; 109: 20602–20607.

Milligan ED, Zapata V, Chacur M, Schoeniger D, Biedenkapp J, O'Connor KA, et al. Evidence that exogenous and endogenous fractalkine can induce spinal nociceptive facilitation in rats. Eur J Neurosci. 2004;20:2294-2302.

Mirabello L, Troisi RJ, Savage SA. Osteosarcoma incidence and survival rates from 1973 to 2004: data from the Surveillance, Epidemiology, and End Results Program. Cancer. 2009;115:1531-1543.

Miranda HF, Romero MA, Puig MM. Antinociceptive and anti-exudative synergism between dexketoprofen and tramadol in a model of inflammatory pain in mice. Fundam Clin Pharmacol. 2012;26:373–382.

Mirzadegan T, Diehl F, Ebi B, Bhakta S, Polsky I, McCarley D *et al.* Identification of the binding site for a novel class of CCR2b chemokine receptor antagonists: binding to a common chemokine receptor motif within the helical bundle. J Biol Chem. 2000;275:25562-25571.

Mitchell LA, Hansen RJ, Beaupre AJ, Gustafson DL, Dow SW. Optimized dosing of a CCR2 antagonist for amplification of vaccine immunity. Int Immunopharmacol. 2013;15:357-363.

Mizumura K, Murase S. Role of nerve growth factor in pain. Handb Exp Pharmacol. 2015;227:57-77.

Moriyama T, Higashi T, Togashi K, Iida T, Segi E, Sugimoto Y, Tominaga T *et al.* Sensitization of TRPV1 by EP1 and IP reveals peripheral nociceptive mechanism of prostaglandins. Mol Pain. 2005;1:3.

Moser B, Wolf M, Walz A, Loetscher P. Chemokines: Multiple levels of leukocyte migration control. Trends Immunol. 2004;25:75–84.

Muley MM, Krustev E, McDougall JJ. Preclinical Assessment of Inflammatory Pain. CNS Neurosci Ther. 2016;22:88-101.

Nagae M, Hiraga T, Wakabayashi H, Wang L, Iwata K, Yoneda T. Osteoclasts play a part in pain due to the inflammation adjacent to bone. Bone. 2006;39:1107-1115.

Naya A, Saeki T. J-113863. Chemokine CCR1 receptor antagonist. Drugs future. 2001;26: 121–127.

Noël J, Sandoz G, Lesage F. Molecular regulations governing TREK and TRAAK channel functions. Channels (Austin). 2011;5:402-409.

Ogura N, Satoh K, Akutsu M, Tobe M, Kuyama K, Kuboyama N *et al.* MCP-1 production in temporomandibular joint inflammation. J Dent Res. 2010;89:1117-1122.

Oh SB, Tran PB, Gillard SE, Hurley RW, Hammond DL, Miller RJ. Chemokines and glycoprotein120 produce pain hypersensitivity by directly exciting primary nociceptive neurons. J Neurosci. 2001;21:5027-5035.

Okamoto M, Suzuki T, Watanabe N. Modulation of inflammatory pain in response to a CCR2/CCR5 antagonist in rodent model. J Pharmacol Pharmacother. 2013;4: 208–210.

Oshima T, Sonoda KH, Tsutsumi-Miyahara C, Qiao H, Hisatomi T, Nakao S *et al.* Analysis of corneal inflammation induced by cauterisation in CCR2 and MCP-1 knockout mice. Br J Ophthalmol. 2006;90:218-22.

Osikowicz M, Mika J y Przewlocka B The glutamatergic system as a target for neuropathic pain relief. Exp Physiol. 2013;98:372–384.

Padi SS, Shi XQ, Zhao YQ, Ruff MR, Baichoo N, Pert CB, *et al*. Attenuation of rodent neuropathic pain by an orally active peptide, RAP-103, which potently blocks CCR2- and CCR5-mediated monocyte chemotaxis and inflammation. Pain. 2012;153:95-106.

Paoletti P. Molecular basis of NMDA receptor functional diversity. Eur J Neurosci. 2011 Apr;33(8):1351-65.

Pan Z, Zhu LJ, Li YQ, Hao LY, Yin C, Yang JX *et al.* Epigenetic modification of spinal miR-219 expression regulates chronic inflammation pain by targeting CaMKIIγ. J Neurosci. 2014;34:9476-9483.

Pappagallo M, Breuer B, Lin HM, Moberly JB, Tai J, Noto C *et al*. A pilot trial of intravenous pamidronate for chronic low back pain. Pain. 2014;155:108-117.

Paulsson JM, Moshfegh A, Dadfar E, Held C, Jacobson SH, Lundahl J. In-vivo extravasation induces the expression of interleukin 1 receptor type 1 in human neutrophils. Clin Exp Immunol 2012;168:105–112.

Pedersen SF, Owsianik G, Nilius B. TRP channels: an overview. Cell Calcium. 2005 Sep-Oct;38(3-4):233-52

Perrot S, Dieudé P, Pérocheau D, Allanore Y. Comparison of pain, pain burden, coping strategies, and attitudes between patients with systemic sclerosis and patients with rheumatoid arthritis: a cross-sectional study. Pain Med. 2013;14:1776-1785.

Peters CM, Ghilardi JR, Keyser CP, Kubota K, Lindsay TH, Luger NM *et al*. Tumor-induced injury of primary afferent sensory nerve fibers in bone cancer pain. Exp Neurol. 2005;193:85-100.

Pevida M, González-Rodríguez S, Lastra A, Hidalgo A, Menéndez L, Baamonde A. CCL2 released at tumoral level contributes to the hyperalgesia evoked by intratibial inoculation of NCTC 2472 but not B16-F10 cells in mice. Naunyn Schmiedebergs Arch Pharmacol. 2012;385:1053-1061.

Pevida M, Lastra A, Hidalgo A, Baamonde A, Menéndez L. Spinal CCL2 and microglial activation are involved in paclitaxel-evoked cold hyperalgesia. Brain Res Bull. 2013;95:21-27.

Pevida M, González-Rodríguez S, Lastra A, García-Suárez O, Hidalgo A, Menéndez L *et al.* Involvement of spinal chemokine CCL2 in the hyperalgesia evoked by bone cancer in mice: a role for astroglia and microglia. Cell Mol Neurobiol. 2014a;34:143-156.

Pevida M, Lastra A, Meana Á, Hidalgo A, Baamonde A, Menéndez L. The chemokine CCL5 induces CCR1-mediated hyperalgesia in mice inoculated with NCTC 2472 tumoral cells. Neuroscience. 2014b;259:113-125.

Pircio AW, Fedele CT, Bierwagen ME. A new method for the evaluation of analgesic activity using adjuvant-induced arthritis in the rat. Eur J Pharmacol.1975; 31:207-215.

Power CA, Pwint H, Chan J, Cho J, Yu Y, Walsh W *et al.* A novel model of bone-metastatic prostate cancer in immunocompetent mice. Prostate. 2009; 69:1613–1623.

Premkumar LS, Abooj M. TRP channels and analgesia. Life Sci. 2013;92:415-424.

Qin X, Wan Y, Wang X. CCL2 and CXCL1 trigger calcitonin gene-related peptide release by exciting primary nociceptive neurons. J Neurosci Res. 2005;82:51-62.

Reale C, Turkiewicz AM, Reale CA. Antalgic treatment of pain associated with bone metastases. Crit Rev Oncol Hematol. 2001;37:1–11.

Reichling DB, Green PG, Levine JD. The fundamental unit of pain is the cell. Pain. 2013;154 Suppl 1:S2-9.

Ren F, Jiao H, Cai H. Analgesic Effect of Intrathecal Administration of Chemokine Receptor CCR2 Antagonist is Related to Change in Spinal NR2B, nNOS, and SIGIRR Expression in Rat with Bone Cancer Pain. Cell Biochem Biophys. 2015 Feb 5. [Epub ahead of print]

Rittner HL, Machelska H, Stein C. Leukocytes in the regulation of pain and analgesia. J Leukoc Biol. 2005;78:1215-1222

Roudier MP, Bain SD, Dougall WC. Effects of the RANKL inhibitor, osteoprotegerin, on the pain and histopathology of bone cancer in rats. Clin Exp Metastasis. 2006;23:167-175.

Roy I, Evans DB, Dwinell MB. Chemokines and chemokine receptors: update on utility and challenges for the clinician. Surgery. 2014;155:961-973.

Saika F, Kiguchi N, Kobayashi Y, Fukazawa Y, Kishioka S. CC-chemokine ligand 4/macrophage inflammatory protein-1β participates in the induction of neuropathic pain after peripheral nerve injury. Eur J Pain. 2012;16:1271-1280.

Salter MW, Beggs S. Sublime microglia: Expending roles for the guardians of the CNS. Cell. 2014; 158:15-24.

Satish L. Chemokines as Therapeutic Targets to Improve Healing Efficiency of Chronic Wounds. Adv Wound Care (New Rochelle). 2015;4:651-659.

Schiller KR, Zillhardt MR, Alley J, Borjesson DL, Beitz AJ, Mauro LJ. Secretion of MCP-1 and other paracrine factors in a novel tumor-bone coculture model. BMC Cancer. 2009;9:45.

Schmelz M, Schmidt R, Weidner C, Hilliges M, Torebjork HE, Handwerker HO. Chemical response pattern of different classes of C-nociceptors to pruritogens and algogens. J Neurophysiol. 2003;89:2441-2448

Schmidt BL, Pickering V, Liu S, Quang P, Dolan J, Connelly ST *et al.* Peripheral endothelin A receptor antagonism attenuates carcinoma-induced pain. Eur J Pain. 2007;11:406-414.

Schwei MJ, Honoré P, Rogers SD, Salak-Johnson JL, Finke MP, Ramnaraine ML *et al.* Neurochemical and cellular reorganization of the spinal cord in a murine model of bone cancer pain. J Neurosci. 1999;19:10886–10897.

Segawa T, Miyakoshi N, Kasukawa Y, Aonuma H, Tsuchie H, Shimada Y. Analgesic effects of minodronate on formalin-induced acute inflammatory pain in rats. Biomed Res. 2013;34:137-141.

Sevcik MA, Ghilardi JR, Peters CM, Lindsay TH, Halvorson KG, Jonas BM, et al . Anti-NGF therapy profoundly reduces bone cancer pain and the accompanying increase in markers of peripheral and central sensitization. Pain. 2005a;115(1-2):128-41.

Sevcik MA, Ghilardi JR, Halvorson KG, Lindsay TH, Kubota K, Mantyh PW. Analgesic efficacy of bradykinin B1 antagonists in a murine bone cancer pain model. J Pain. 2005b;6(11):771-5.

Shen W, Hu XM, Liu YN, Han Y, Chen LP, Wang CC *et al.* CXCL12 in astrocytes contributes to bone cancer pain through CXCR4-mediated neuronal sensitization and glial activation in rat spinal cord. J Neuroinflammation. 2014; 11:75.

Slade GD, Conrad MS, Diatchenko L, Rashid NU, Zhong S, Smith S *et al.* Cytokine biomarkers and chronic pain: association of genes, transcription, and circulating proteins with temporomandibular disorders and widespread palpation tenderness. Pain. 2011;152:2802-2812.

Slosky LM, Largent-Milnes TM, Vanderah TW. Use of Animal Models in Understanding Cancer-induced Bone Pain. Cancer Growth Metastasis. 2015;8(Suppl 1):47-62.

Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016 May 3. pii: S0140-6736(16)30173-8. [Epub ahead of print]

Snider WD, McMahon SB. Tackling pain at the source: new ideas about nociceptors. Neuron. 1998;20:629-632.

Souza GR, Talbot J, Lotufo CM, Cunha FQ, Cunha TM, Ferreira SH. Fractalkine mediates inflammatory pain through activation of satellite glial cells. Proc Natl Acad Sci U S A. 2013;110:11193-11198.

Sozzani S, Del Prete A, Bonecchi R, Locati M. Chemokines as effector and target molecules in vascular biology. Cardiovasc Res. 2015;107:364-372.

Staniland AA, et al. Reduced inflammatory and neuropathic pain and decreased spinal microglial response in fractalkine receptor (CX3CR1) knockout mice. J Neurochem. 2010;114:1143–1157.

Svensson CI, Brodin E. Spinal astrocytes in pain processing: non-neuronal cells as therapeutic targets. Mol Interv. 2010;10:25-38.

Szekanecz Z, Koch AE, Tak PP. Chemokine and chemokine receptor blockade in arthritis, a prototype of immune-mediated inflammatory diseases. Neth J Med. 2011;69:356-366.

Szekanecz Z, Koch AE. Successes and failures of chemokine-pathway targeting in rheumatoid arthritis. Nat Rev Rheumatol. 2016;12:5–13.

Tak PP, Balanescu A, Tseluyko V, Bojin S, Drescher E, Dairaghi D *et al.* Chemokine receptor CCR1 antagonist CCX354-C treatment for rheumatoid arthritis: CARAT-2, a randomised, placebo controlled clinical trial. Ann Rheum Dis. 2013;72:337-344

Talbot J, Bianchini FJ, Nascimento DC, Oliveira RDR, Souto FO, Pinto LG *et al.* CCR2 expression in neutrophils plays a critical role in their migration into the joints in rheumatoid arthritis. Arthritis Rheumatol. 2015;67:1751–1759.

Tarakanov AO, Fuxe K. The triplet puzzle theory indicates extensive formation of heteromers between opioid and chemokine receptor subtypes. J Neural Transm (Vienna). 2015;122:1509-1514.

Tas F. Metastatic behavior in melanoma: timing, pattern, survival, and influencing factors. J Oncol. 2012;2012:647684.

Taylor PC, Peters AM, Paleolog E, Chapman PT, Elliott MJ, McCloskey R *et al.* Reduction of chemokine levels and leukocyte traffic to joints by tumor necrosis factor alpha blockade in patients with rheumatoid arthritis. Arthritis Rheum. 2000;43:38–47.

Than, Y. X., Li, L., Hasan, R., y Zhang, X. Excitation and modulation of TRPA1, TRPV1, and TRPM8 channel-expressing sensory neurons by the pruritogen chloroquine. J Biol Chem. 2013;288: 12818-12827.

Todd AJ. Neuronal circuitry for pain processing in the dorsal horn Nat Rev Neurosci . 2010;11: 823–836.

Tracey I, Johns E. The pain matrix: reloaded or reborn as we image tonic pain using arterial spin labelling. Pain. 2010;148:359-360.

Tsaur I, Noack A, Makarevic J, Oppermann E, Waaga-Gasser AM, Gasser M. CCL2 Chemokine as a Potential Biomarker for Prostate Cancer: A Pilot Study. Cancer Res Treat. 2015;47:306-312.

Tsuda M. Microglia in the spinal cord and neuropathic pain. J Diabetes Investig. 2016;7:17-26.

Turner MD, Nedjai B, Hurst T, Pennington DJ. Cytokines and chemokines: At the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta. 2014;1843:2563-2582.

Ubogu EE, Cossoy MB, Ransohoff RM. The expression and function of chemokines involved in CNS inflammation. Trends Pharmacol Sci. 2006;27:48-55.

Ubogu EE. Chemokine receptors as specific anti-inflammatory targets in peripheral nerves. Endocr Metab Immune Disord Drug Targets. 2011;11:141-153.

Vacchini A, Locati M, Borroni EM. Overview and potential unifying themes of the atypical chemokine receptor family. J Leukoc Biol. 2016 Jan 6. pii: jlb.2MR1015-477R. [Epub ahead of print]

Van Steenwinckel J, Reaux-Le Goazigo A, Pommier B, Mauborgne A, Dansereau M-A, Kitabgi P *et al*. CCL2 released from neuronal synaptic vesicles in the spinal cord is a major mediator of local inflammation and pain after peripheral nerve injury. J Neurosci.2011;31:5865–5875.

Vit JP, Ohara PT, Tien DA, Fike JR, Eikmeier L, Beitz A *et al.* The analgesic effect of low dose focal irradiation in a mouse model of bone cancer is associated with spinal changes in neuro-mediators of nociception. Pain. 2006;120:188-201.

Voscopoulos C, Lema M. When does acute pain become chronic? Br J Anaesth. 2010 Dec;105 Suppl 1:i69-85.

Wacnik PW, Eikmeier LJ, Ruggles TR, Ramnaraine ML, Walcheck BK, Beitz AJ *et al.* Functional interactions between tumor and peripheral nerve: morphology, algogen identification, and behavioral characterization of a new murine model of cancer pain. J Neurosci. 2001;21:9355-9366.

Wacnik PW, Kehl LJ, Trempe TM, Ramnaraine ML, Beitz AJ, Wilcox GL. Tumor implantation in mouse humerus evokes movement-related hyperalgesia exceeding that evoked by intramuscular carrageenan. Pain. 2003;101:175-186.

Walder RY, Radhakrishnan R, Loo L, Rasmussen LA, Mohapatra DP, Wilson SP *et al.* TRPV1 is important for mechanical and heat sensitivity in uninjured animals and development of heat hypersensitivity after muscle inflammation. Pain. 2012;153:1664–1672.

Walker K, Medhurst SJ, Kidd BL, Glatt M, Bowes M, Patel S *et al.* Disease modifying and anti-nociceptive effects of the bisphosphonate, zoledronic acid in a model of bone cancer pain. Pain 2002;100:219–229.

Wainger BJ, Buttermore ED, Oliveira JT, Mellin C, Lee S, Saber WA *et al.* Modeling pain in vitro using nociceptor neurons reprogrammed from fibroblasts. Nat Neurosci. 2015;18:17-24.

Wang Y, Wei D, Lai Z, Le Y. Triptolide inhibits CC chemokines expressed in rat adjuvant-induced arthritis. Int Immunopharmacol. 2006;6:1825–1832.

Wang XM, Hamza M, Wu TX, Dionne RA. Upregulation of IL-6, IL-8 and CCL2 gene expression after acute inflammation: Correlation to clinical pain. Pain. 2009;142:275-283.

Wang XM, Lehky TJ, Brell JM, Dorsey SG. Discovering cytokines as targets for chemotherapy-induced painful peripheral neuropathy. Cytokine. 2012;59:3-9.

Wang ZL, Du TT, Zhang RG. JNK in spinal cord facilitates bone cancer pain in rats through modulation of CXCL1. J Huazhong Univ Sci Technolog Med Sci. 2016;36:88-94.

Wemmie JA, Taugher RJ, Kreple CJ. Acid-sensing ion channels in pain and disease. Nat Rev Neurosci. 2013;14:461–471.

White FA, Wilson NM. Chemokines as pain mediators and modulators. Curr Opin Anaesthesiol. 2008;21:580-585.

White GE, Iqbal AJ, Greaves DR. CC Chemokine Receptors and Chronic Inflammation — Therapeutic Opportunities and Pharmacological Challenges. 2013;65:47–89.

Whittle SL, Richards BL, van der Heijde DM, Buchbinder R. The efficacy and safety of opioids in inflammatory arthritis: a Cochrane systematic review. J Rheumatol Suppl. 2012;90:40-46.

Wigerblad G, Bas DB, Fernades-Cerqueira C, Krishnamurthy A, Nandakumar KS, Rogoz K *et al*. Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism. Ann Rheum Dis. 2016;75:730-738.

Woodhams SG, Sagar DR, Burston JJ, Chapman V. The role of the endocannabinoid system in pain. Handb Exp Pharmacol. 2015;227:119-143.

Woolf CJ, Ma Q. Nociceptors--noxious stimulus detectors. Neuron. 2007;55:353-364.

World Health Organization. Cancer pain relief and palliative care. Expert Committee Report. Technical Series 804. Geneva: World Health Organization; 1990.

World Health Organization. Cancer pain relief, 2nd edition with a guide to opioid availability. Geneva: World Health Organization; 1996.

Wu FY, Ou ZL, Feng LY, Luo JM, Wang LP, Shen ZZ *et al.* Chemokine decoy receptor d6 plays a negative role in human breast cancer. Mol Cancer Res. 2008;6:1276-1288.

Wu FY, Fan J, Tang L, Zhao YM, Zhou CC. Atypical chemokine receptor D6 inhibits human non-small cell lung cancer growth by sequestration of chemokines. Oncol Lett. 2013;6:91-95.

Xu J, Zhu MD, Zhang X, Tian H, Zhang JH, Wu XB *et al.* NFκB-mediated CXCL1 production in spinal cord astrocytes contributes to the maintenance of bone cancer pain in mice.J Neuroinflammation. 2014;11:38.

Xu YK, Ke Y, Wang B, Lin JH. The role of MCP-1-CCR2 ligand-receptor axis in chondrocyte degradation and disease progress in knee osteoarthritis. Biol Res. 2015;48:64.

Xue CB, Wang A, Han Q, Zhang Y, Cao G, Feng H *et al.* Discovery of INCB8761/PF-4136309, a Potent, Selective, and Orally Bioavailable CCR2 Antagonist. ACS Med Chem Lett. 2011;2:913-918.

Yaksh TL, Woller SA, Ramachandran R, Sorkin LS. The search for novel analgesics: targets and mechanisms. F1000Prime Rep. 2015;7:56.

Yamamoto J, Kawamata T, Niiyama Y, Omote K, Namiki A. Down-regulation of mu opioid receptor expression within distinct subpopulations of dorsal root ganglion neurons in a murine model of bone cancer pain. Neuroscience. 2008;151:843-853.

Yang HYT, Mitchell K, Keller JM, Iadarola MJ. Peripheral inflammation increases Scya2 expression in sensory ganglia and cytokine and endothelial related gene expression in inflamed tissue. J Neurochem. 2007;103:1628–1643.

Yao Y, Tan YH, Light AR, Mao J, Hoi Yu AC, Fu KY. Alendronate attenuates spinal microglial activation and neuropathic pain. J Pain. 2016 Apr 5. pii: S1526-5900(16)30005-0. [Epub ahead of print].

Ye D, Bu H, Guo G, Shu B, Wang W, Guan X, et al. Activation of CXCL10/CXCR3 signaling attenuates morphine analgesia: involvement of Gi protein. J Mol Neurosci. 2014;53:571-579.

Yin Q, Cheng W, Cheng MY, Fan SZ, Shen W. Intrathecal injection of anti-CX3CR1 neutralizing antibody delayed and attenuated pain facilitation in rat tibial bone cancer pain model. Behav Pharmacol. 2010;21:595-601.

Yu KD, Wang X, Yang C, Zeng XH, Shao ZM. Host genotype and tumor phenotype of chemokine decoy receptors integrally affect breast cancer relapse. Oncotarget. 2015;6:26519-26527.

Yuan F, Yosef N, Lakshmana Reddy C, Huang A, Chiang SC, Tithi HR *et al.* CCR2 gene deletion and pharmacologic blockade ameliorate a severe murine experimental autoimmune neuritis model of Guillain-Barré syndrome. PLoS One. 2014;9:e90463.

Zeilhofer HU: Loss of glycinergic and GABAergic inhibition in chronic pain-contributions of inflammation and microglia. Int Immunopharmcol 2008, 8:182-187.

Zeppetella G. Breakthrough pain in cancer patients. Clin Oncol (R Coll Radiol). 2011;23:393-398.

Zhang N, Rogers TJ, Caterina M, Oppenheim JJ. Proinflammatory chemokines, such as C-C chemokine ligand 3, desensitize mu-opioid receptors on dorsal root ganglia neurons. J Immunol. 2004;173:594-599.

Zhang N, Oppenheim JJ. Crosstalk between chemokines and neuronal receptors bridges immune and nervous systems. J Leukoc Biol. 2005;78:1210-1214

Zhang N, Inan S, Cowan A, Su R, Wang JM, Rogers TJ *et al.* A proinflammatory chemokine, CCL3, sensitizes the heat-and capsaicin-gated ion channel TRPV1. Proc Natl Acad Sci USA. 2005; 102:4536-4541.

Zhang J, Shi XQ, Echeverry S, Mogil JS, De Koninck Y, Rivest S. Expression of CCR2 in both resident and bone marrow-derived microglia plays a critical role in neuropathic pain. J Neurosci. 2007;27:12396-12406.

Zhang J, Patel L, Pienta KJ. CC chemokine ligand 2 (CCL2) promotes prostate cancer tumorigenesis and metastasis. Cytokine Growth Factor Rev. 2010a;21:41-48.

Zhang J, Lu Y, Pienta KJ. Multiple roles of chemokine (C-C motif) ligand 2 in promoting prostate cancer growth. J Natl Cancer Inst. 2010b;102:522–528.

Zhang H, Yoon SY, Zhang H, Dougherty PM. Evidence that spinal astrocytes but not microglia contribute to the pathogenesis of paclitaxel-induced painful neuropathy. Journal of Pain. 2012a;13:293–303.

Zhang R, Lao L. A new rat model of bone cancer pain. Methods Mol Biol. 2012;851:261-73.

Zhang ZJ, Dong YL, Lu Y, Cao S, Zhao ZQ, Gao YJ. Chemokine, CCL2 and its receptor CCR2 in the medullary dorsal horn are involved in trigeminal neuropathic pain. J Neuroinflammation. 2012b;9:136.

Zhao C, Wacnik PW, Tall JM, Johns DC, Wilcox GL, Meyer RA, Raja SN. Analgesic effects of a soy-containing diet in three murine bone cancer pain models. J Pain. 2004;5:104-10.

Zheng FY, Xiao WH, Bennett GJ. The response of spinal microglia to chemotherapy-evoked painful peripheral neuropathies is distinct from that evoked by traumatic nerve injuries. Neuroscience. 2011;176:447–454.

Zhou D, Chen ML, Zhang YQ, Zhao ZQ. Involvement of spinal microglial P2X7 receptor in generation of tolerance to morphine analgesia in rats. J Neurosci. 2010; 30:8042–8047.

Zhou YQ, Gao HY, Guan XH, Yuan X, Fang GG, Chen Y *et al.* Chemokines and Their Receptors: Potential Therapeutic Targets for Bone Cancer Pain. Curr Pharm Des. 2015;21:5029-5033.

Zhu H, Yu Y, Zheng L, Wang L, Li C, Yu J *et al.* Chronic inflammatory pain upregulates expression of P2Y2 receptor in small-diameter sensory neurons. Metab Brain Dis. 2015;30:1349-1358.

Zhuang ZY, Kawasaki Y, Tan PH, Wen YR, Huang J, Ji RR. Role of the CX3CR1/ p38 MAPK pathway in spinal microglia for the development of neuropathic pain following nerve injury-induced cleavage of fractalkine. Brain Behav Immun 2007;21:642-651.

Zöllner C, Stein C. Opioids. Handb Exp Pharmacol. 2007;177:31-63.