Central inhibition of granulocyte-macrophage colony-stimulating factor is analgesic in experimental neuropathic pain

Louise S.C. Nicol, Peter Thornton, Jon P. Hatcher, Colin P. Glover, Carl I. Webster, Matthew Burrell, Kessia Hammett, Clare A. Jones, Matthew A. Sleeman, Andrew Billinton, Iain Chessell

1. Introduction

Granulocyte-macrophage colony-stimulating factor is a small secreted hematopoietic growth factor required for the proliferation and differentiation of bone marrow precursor cells into distinct colonies comprised of granulocytes and macrophages. The GM-CSF receptor is a heterodimer that consists of a major ligand binding α-subunit and a common signal-transduction β-chain subunit, which in humans is shared with the interleukin (IL)-3 and IL-5 receptor. The α-subunit binds with high specificity and low affinity, and the β-chain subunit is responsible for Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K) pathway. Despite initially entering clinical practice for the treatment of chemotherapy-induced neutropenia, GM-CSF has since been proven to possess a broader range of biological activities within both innate and adaptive immunity. Pharmacological studies examining anti-ligand or anti-receptor antibodies in rodent models of inflammatory disease have shown profound effects on disease severity and progression.

GM-CSF to the hind paw was painful. Pretreatment with GM-CSF potentiated lipopolysaccharide-induced release of proinflammatory cytokines. In summary, our data indicate that GM-CSF is a proinflammatory cytokine that contributes to nociceptive signalling through driving spinal glial cell secretion of proinflammatory mediators. In addition, we report a successful approach to accessing CNS pain targets, providing promise for central compartment delivery of analgesics.

Keywords: Granulocyte-macrophage colony-stimulating factor, Neuropathic pain, Inflammation, Microglia, Astrocyte, Blood–brain barrier, Cytokine

Abstract

With less than 50% of patients responding to the current standard of care and poor efficacy and selectivity of current treatments, neuropathic pain continues to be an area of considerable unmet medical need. Biological therapeutics such as monoclonal antibodies (mAbs) provide better intrinsic selectivity; however, delivery to the central nervous system (CNS) remains a challenge. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is well described in inflammation-induced pain, and early-phase clinical trials evaluating its antagonism have exemplified its importance as a peripheral pain target. Here, we investigate the role of this cytokine in a murine model of traumatic nerve injury and show that deletion of the GM-CSF receptor or treatment with an antagonizing mAb alleviates pain. We also demonstrate enhanced analgesic efficacy using an engineered construct that has greater capacity to penetrate the CNS. Despite observing GM-CSF receptor expression in microglia and astrocytes, the glosis response in the dorsal horn was not altered in nerve injured knockout mice compared with wild-type littermate controls as evaluated by ionized calcium binding adapter molecule 1 (Iba1) and glial fibrillary acidic protein, respectively. Functional analysis of glial cells revealed that pretreatment with GM-CSF potentiated lipopolysaccharide-induced release of proinflammatory cytokines. In summary, our data indicate that GM-CSF is a proinflammatory cytokine that contributes to nociceptive signalling through driving spinal glial cell secretion of proinflammatory mediators. In addition, we report a successful approach to accessing CNS pain targets, providing promise for central compartment delivery of analgesics.
via prostaglandin.53 This notion is supported by reports of diverse ligand and receptor expression in the plasma, synovial fluid, and tissue of patients with rheumatoid arthritis (RA) as well as on circulating mononuclear cells,1,5,55 where it is believed that GM-CSF–driven modulatory effects on innate immune cells and other cell types induces cytokine secretion and the formation of proinflammatory cytokine networks that contribute to neuronal sensitization.25,32 Unsurprisingly, results from clinical trials targeting GM-CSF or its receptor in RA patients show significant analgesic efficacy, demonstrating its role as a pronociceptive cytokine.25 Granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR) expression has also been reported on peripheral nerves of healthy individuals and pancreatic cancer patients, as well as on peripheral nerves dispersed in the perineuroma and throughout the bone matrix and in dorsal root ganglia sections of mice bearing calcaneal bone tumors, which suggests that GM-CSF may directly activate sensory neurons.45

To date, few studies have examined the role of GM-CSF in neuropathic pain and the potential mechanisms involved remain unclear.36,45 Here, we used an anti–GM-CSFR inhibitory mAb and GM-CSFR β-chain knockout (KO) mice to elucidate the role of the ligand in neuropathic pain pathways with a focus on the contribution of spinal glial cells.

2. Methods

2.1. In vivo study subjects

Studies were conducted on 8-week-old GM-CSFR β-chain deficient female mice on a C57/BL6 background, kindly provided by Jackson Laboratory (Bar Harbor, MA). Mice were housed in small groups in standard environmental conditions (12 hours light/dark cycle) with ad libitum access to food and water. Animal husbandry and the procedures used were in accordance with the guidelines of the AstraZeneca Animal Care Committee and complied with the Animals (Scientific Procedures) Act, 1986. All mice underwent insertion of transponders for identification purposes 5 days before the start of the study. For all studies, the experimenter was blinded to genotype and treatment, and animals were block randomised to treatment groups.

2.2. Paw pressure

In lightly restrained alert mice, noxious mechanical thresholds were examined using an Analgesymeter (7200; Ugo Basile, Monvalle VA, Italy).41 As previously described, the plantar surface of the hind paw was placed on a pedestal with a probe resting on the dorsal surface and increasing pressure was applied through the probe. The force at which a withdrawal response was observed was taken as the nociceptive threshold.41 Data were expressed as ipsilateral/contralateral ratios in grams.

2.3. Neuropathic pain model

Animals were anesthetised with 2% to 3% isoflurane (Abbott Animal Health, Maidenhead, United Kingdom), and the left hind paw was secured, shaved, and sterilized. A small incision was made midway of the left thigh to carefully expose and isolate approximately 1 cm of the sciatic nerve from neighbouring connective tissue. A 9-0 Virgin Silk suture (Ethicon, Livingston, United Kingdom) was inserted through the dorsal third of the nerve and tied tightly, as previously described.46 The incision was then closed using Vetbond, and the mice were allowed to recover for at least 3 days before commencement of testing. Sham-operated mice underwent the same procedure but the nerve was not ligated and the mice were sutured and allowed to recover.

Mice were tested on days 7 and 10 after surgery and were subsequently randomly allocated into several treatment groups with approximately equal ipsilateral/contralateral ratios as follows: sham + phosphate buffered saline (PBS), partial sciatic nerve ligation (PNL) + PBS, PNL + CAT004, PNL + CAM3003 (anti-GM-CSFR–neutralising mAb, previously described in25), and PNL + Bbbt-CAM3003 (blood–brain barrier technology conjugated anti-GM-CSFR mAb).

Mice were administered with either the isotype control, CAT004 (30 mg/kg subcut and 50 µg/5 µL intrathecal), anti-GM-CSFR mAb, CAM3003 (30 or 75 mg/kg subcut and 50 µg/5 µL intrathecal), Bbbt-CAM3003 (25, 50 or 100 mg/kg subcut), or with PBS vehicle on day 13 and were retested for changes in mechanical hyperalgesia 4 hours and on day 1, 2, 4, and 7 after dose. Intrathecal injections were performed under isoflurane anesthesia, and antibody or PBS was administered into the lumbar region of the spine (between the L5 and L6 vertebrae) using a 26 G 3/8-inch needle connected to a 25-µL Hamilton syringe.

2.4. Tissue preparation and immunohistochemistry

On completion of behavioural testing, mice were anesthetized with a lethal dose of sodium pentobarbital (Euthatal, Merial Animal Health Ltd, Woking, United Kingdom) and underwent transcardiac perfusion with saline followed by fixation with 4% paraformaldehyde (PFA; WVR, Leicestershire, United Kingdom). Lumbosacral spinal cords were extracted and postfixed for 2 hours in PFA and cryoprotected in sucrose before embedding in optimum cutting temperature medium (WWR) and snap freezing with liquid nitrogen. Transverse spinal cord sections of the L4 and L5 lumbar region were cut on the cryostat and thaw mounted onto Super Frost plus microscope slides (WWR) and left to air dry. As shown before, spinal cord sections were incubated overnight with primary antibody solution raised against Iba1 (rabbit anti-Iba1, 1:1000; Wako Chemicals, Neuss, Germany) to assess microgliosis or glial fibrillary acidic protein (GFAP) (rabbit anti-GFAP, 1:1000; DakoCytomation, Glostrup, Denmark) to assess astrogliosis followed by 2 hours incubation with the appropriate secondary antibody solution (1:1000; IgG conjugated Alexa Fluor 488 or 546; Invitrogen, United Kingdom). Slides were then cover slipped with vectashield mounting medium with DAPI (Vector Laboratories, Peterborough, United Kingdom), sealed with nail varnish and dried.9

2.5. Quantification of immunoreactivity

Images were visualised and captured on an Olympus microscope (Olympus KeyMed, Southend-On-Sea, United Kingdom). Analysis of Iba1 and GFAP immunoreactivity (IR) was performed by counting the number of positive profiles or measuring the IR signal, respectively, within 3 fixed 1 × 104 µm² boxes in the lateral, central, and medial areas of the dorsal horn (DH). The nuclear marker DAPI was used to assist the identification of positive cell profiles, as previously described.8 Quantification was performed blind, and 3 sections per animal were assessed to obtain a mean value for both the ipsilateral and contralateral DHs.

2.6. Isolation and culture of neonatal glial cells

Primary cultures of mixed murine glial cells were prepared as previously described using a modified protocol.30,42 Briefly, brains from C57/BL6 P2 neonatal pups (purchased from Charles River, Saffron Walden, United Kingdom) were extracted, rolled across sterile filter paper to
remove vasculature and meninges, and were mechanically dissociated. Cells were resuspended in 40 mL per 175 cm² flask (Coming, Amsterdam, Netherlands) and were maintained in Dulbecco’s Modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Invitrogen, Sigma Aldrich, Gillingham, United Kingdom) and incubated at 37°C (5% CO₂/95% O₂). A week later, 5 ng/mL of murine GM-CSF (R&D systems, Abingdon, United Kingdom) in fresh media was added to the flasks to expand yields, and the cells were maintained for a further week. Microglia were harvested by overnight shaking in an orbital shaker incubator (37°C, 180 rev/min), and the supernatant was collected from the flask to obtain the dissociated microglia. Trypsin was subsequently added to lift the astrocytic layer. Microglia and astrocytes were plated as required for further experiments.

2.7. Verification of glial cell culture purity

2.7.1. Flow cytometry assessment

Microglia were collected from either a mixed glia T175 culture flask or purified by shaking as described above. Cell pellets were resuspended in FACS buffer (3% FCS/PBS) containing Mouse BD Fc Block (BD Biosciences, Wokingham, United Kingdom) with FITC rat anti-mouse CD11b or FITC rat IgG2b isotype control (BD Biosciences) and incubated for 30 minutes on ice. All antibodies had a final concentration of 1:50. Cells were washed by adding FACS buffer before being centrifuged (400g, 3 minutes) and analysed on the BD FACScanto II system.

2.7.2. Immunocytochemistry assessment

As described above, microglia and astrocytes were harvested and plated into 24 well plates on glass slides and left to rest for 24 to 48 hours in DMEM with 10% FBS and 1% pen/strep at 37°C. Cells were fixed with 4% PFA for 10 minutes, washed with PBS, and incubated for 2 hours with primary antibody solution for the microglia marker lba1 (rabbit anti-Iba1, 1:1000) or the astrocyte marker GFAP (rabbit anti-GFAP, 1:1000). Cells were then washed and incubated with the appropriate secondary antibody solution (1:1000; IgG conjugated Alexa Fluor 546) for 45 minutes. Glass slides were carefully mounted with Vectashield Mounting Medium with DAPI, and images were visualised and captured on an Olympus microscope (Olympus KeyMed). Positive cell profiles were identified by costaining with the nuclear marker DAPI.

2.8. Stimulation of neonatal glial cells and detection of cytokine release

Microglia and astrocytes were plated in 96 well plates at a density of 1 × 10⁵ cells/well in DMEM with 10% FBS and 1% pen/strep. After 24 hours, the medium was replaced with FBS-free medium for a duration of 3 hours followed by 24 hours stimulation with increasing concentrations of lipopolysaccharide (LPS; Sigma Aldrich) +/- GM-CSF (5 ng/mL). Supernatants were collected and snap frozen with liquid nitrogen and stored at −80°C until further processing. The release of IL-1β, TNF-α, IL-6, and IL-10 was measured by an electrochemiluminescence immunoassay technique using the V-PLEX proinflammatory panel 1 mouse kit and analysed on a Sector Imager 6000 (Meso Scale Discovery, MD) according to manufacturer’s guidelines.

2.9. Stimulation of neonatal glial cells and detection of phosphorylated extracellular signal-regulated kinase

Granulocyte-macrophage colony-stimulating factor-induced phosphorylated extracellular signal-regulated kinase (p-ERK) signals generated in neonatal microglia and astrocytes were measured using the p-ERK1/2 (Thr202/Tyr204) assay kit (Cisbio, Cedex, France) according to the 2-plate assay protocol detailed in the manufacturer’s guide. Briefly, cells were stimulated for either 5, 15, or 30 minutes with GM-CSF (5 ng/mL) and the reaction was stopped by removing the media and adding supplemented lysosome buffer to each well for 30 minutes at RT under shaking. After homogenisation, cell lysates were transferred to 384 well small volume white SV plates and premixed antibody solutions were added to each well and incubated for 2 hours. Between 2 and 24 hours, the fluorescence emission signal was read at 665 and 620 nm on an Envision plate reader (Perkin Elmer, Beaconsfield, United Kingdom). Phorbol 12-myristate 13-acetate (PMA, 100 ng/mL; Sigma Aldrich) was used as a positive control.

2.10. Development of the blood–brain barrier transmigrating anti-granulocyte-macrophage colony-stimulating factor receptor antibody

Development of the BBB-transporting antibody, Bbbt, is described in a separate article that is in preparation. Briefly, Bbbt was generated by phage display to select for single-chain variable fragments (scFvs) that bind mouse brain endothelial cells and cross the BBB in vitro and in vivo models. The bispecific Bbbt-CAM3003 antibody was expressed using a plasmid encoding the Bbbt antibody as a scFv fused using a (Gly₄Ser₂) linker to the heavy chain of CAM3003 IgG1, that was cotransfected with a plasmid encoding the CAM3003 light chain. Antibodies were expressed as chimeric mouse IgG1 molecules in transiently transfected Chinese hamster ovary cells in serum-free media as described previously. Binding of CAM3003 mouse IgG1 and the Bbbt-CAM3003 bispecific to GM-CSFR was confirmed using a Biacore T100 system (GE Healthcare, Amersham, United Kingdom).

2.11. RNA extraction and quantification

Microglia and astrocytes cells were cultured as described above, and total RNA was purified from cell pellets using the Qiagen RNeasy plus mini kit according to manufacturer’s instructions (RNeasy; Qiagen, Manchester, United Kingdom). After purification, total RNA was eluted using RNAse-free water, and the concentration and purity were measured using a NanoDrop ND-100 Spectrophotometer (Thermo Fisher Scientific, United Kingdom). In accordance with manufacturer’s instructions, eluted RNA was subsequently reverse transcribed using the Life Tech VILO mastermix (Thermo Fisher Scientific). Relative mRNA expression levels were evaluated by Taqman real-time PCR using TaqMan Gene Expression Master Mix (Applied Biosystems, CA) and the Taqman Gene Expression assays for GM-CSF α-chain (Mm00438331_g1), β-chain (Mm00665745_m1), and eukaryotic 18S rRNA endogenous control (Thermo Fisher Scientific). Relative transcript levels were calculated using the ∆∆CT method, normalising against 18S. Control reactions using RNAse-free water as template or heat-inactivated reverse transcriptase produced no amplification signal.

2.12. Data and statistical analysis

All data were analysed using PRISM software. For multiple comparisons, one-way (immunohistochemical [IHC] data) or 2-way (behavioural and in vitro data) analysis of variance was used, followed by Tukey’s or Sidak’s post hoc test to determine individual group differences. In all cases, the data are presented as the mean ± SEM, and P < 0.05 was set as the level of statistical significance.
3. Results

3.1. Inhibition of granulocyte-macrophage colony-stimulating factor receptor signalling using an antireceptor mAb is analgesic in the partial sciatic nerve ligation model of neuropathic pain

To date, published literature has focused primarily on the peripheral inflammatory activity of GM-CSF in pain, and there has been limited exploration of a potential role in neuropathic pain.36,63 In light of this research gap and several studies showing expression of GM-CSF in the CNS,16,63 we wanted to investigate the contribution of centrally mediated GM-CSF signalling in a nerve injury model of neuropathic pain.

It is well documented that the PNL model produces a robust mechanical and thermal hypersensitivity in the ipsilateral but not the contralateral hind paw, which may serve as a control. In agreement with this, nerve injured mice exhibited significant reductions in mechanical thresholds on days 7 and 10 compared with sham-operated controls (Fig. 1A). To test whether mechanical hyperalgesia induced by nerve injury was dependent on GM-CSF signalling, we investigated the effect of peripheral and central delivery of the inhibitory anti-mouse GM-CSFR mAb, CAM3003, previously described in.23 Peripheral administration of CAM3003 (Fig. 1A) had no effect at doses shown to be previously efficacious in the complete Freund’s adjuvant model of inflammatory pain (data not shown) but significantly reversed mechanical hyperalgesia in nerve injured mice at 4 hours, 1 and 2 days after intrathecally (Fig. 1B), increasing the ipsi-contra ratio from 53% to approximately 73% at 4 hours after dose and returning to baseline levels (~58%) by day 4. The administration of isotype control, CAT004, and PBS vehicle, closed subcutaneously or intrathecally, showed no reversal of this hyperalgesia at any time point, nor was there a reduction observed in the sham-operated groups or contralateral thresholds (Supplementary Figs. 1A and B, available online as supplemental digital content at http://links.lww.com/PAIN/A516) compared with pretreatment baseline levels, thus indicating that animal handling and drug administration procedures did not alter the response (Figs. 1A and B).

There is a growing agenda to enhance BBB penetration of CNS-targeted therapeutics and thus provide greater efficacy for a longer duration of time.52 To achieve delivery of a pharmacologic payload to the CNS, we generated a BBB-transporting antibody, Bbbt, which we have shown accumulates in the brain at up to 3% of the injected peripheral dose as opposed to non-BBB-targeted antibodies that typically accumulate at <0.1% of the injected dose (manuscript in preparation). Here, we are the first to re-engineer an anti-GM-CSF mAb to increase its capacity to enter the CNS and have shown that subcutaneous administration of Bbbt-CAM3003 (100 mg/kg) strikingly alleviated mechanical hyperalgesia equivalent to that of intrathecal administration of CAM3003 (Fig. 1C). The ipsi/contra ratio was seen to increase from around 54% to 78% at 4 hours after dose and eventually return to 60% at day 4. As expected, lower doses of Bbbt-CAM3003 (25 and 50 mg/kg) had less analgesic efficacy for a shorter duration of time with mechanical thresholds beginning to decrease by day 2. Neither antibody nor PBS vehicle effected contralateral thresholds (Supplementary Fig. 1C, available online as supplemental digital content at http://links.lww.com/PAIN/A516).

As subcutaneous administration of CAM3003 lacked efficacy in contrast to Bbbt-CAM3003, these data suggest a centrally mediated mechanism for GM-CSF signalling in the neuropathic pain model as opposed to a peripherally mediated mechanism in the inflammation-induced pain model. However, a potential role at the level of the dorsal root ganglia cannot be unequivocally ruled out. In addition, we have demonstrated an exciting new approach to increase drug delivery access to the CNS and achieve greater therapeutic efficacy.

3.2. Nerve injured granulocyte-macrophage colony-stimulating factor receptor β-chain knockout mice exhibit attenuated mechanical hyperalgesia, which is not associated with an altered gliosis response

To consolidate the role of GM-CSFR-mediated signalling in neuropathic pain, we tested the mechanical thresholds of GM-CSF β-chain KO mice after PNL surgery. Although the β-chain is still present, these mice show no signalling through the family of cognate cytokines, including GM-CSF, IL-3, and IL-5.21 Nerve injured β-chain KO mice exhibited significantly attenuated mechanical hyperalgesia compared with wild-type (WT) controls from day 3 to 14 with the ipsi/contra ratio averaging around 67% in the KO compared with 52% in the WT over the 12 testing days (Fig. 1D). The magnitude of the analgesic effect was notably consistent to that observed after intrathecal delivery of CAM3003 (Fig. 1B) and indicates that GM-CSFR deletion impairs the development and maintenance of behavioural hypersensitivity. These findings are in accordance with our mAb data and thus support our hypothesis of a pronociceptive role of GM-CSF. Notably, both genotypes exhibited equivalent baseline mechanical thresholds, and so GM-CSF may not be involved in steady-state nociception. There were no significant effects seen on contralateral thresholds (Supplementary Fig. 1D, available online as supplemental digital content at http://links.lww.com/PAIN/A516).

To determine, which cell types could be driving the centrally mediated pronociceptive effects of GM-CSF observed in our in vivo data, we profiled mRNA expression of its cognate receptor in glial cell cultures. In line with previous reports,52 Tagm data revealed both α- and β-chain subunit expression in neonatal microglia and astrocyte cultures (Figs. 2A and B).

Since the role of microglia and astrocytes in pain pathways are well established29,56 and we saw glial expression of GM-CSFR, we sought to characterise nerve injury–induced gliosis in the KO mouse to determine whether this is altered with GM-CSF receptor deletion. Quantification of the Iba1 IR signal 14 days post PNL revealed that both genotypes showed a significant increase in Iba1-positive cells in the ipsilateral DH of the spinal cord compared with the contralateral DH (WT ipsi: 10.6 ± 1.2, KO ipsi: 9.7 ± 1.1 vs WT contra: 4.5 ± 0.8, and KO contra: 4.6 ± 0.5, Figs. 3A and B). There were no significant differences between genotypes, suggesting that microgliosis after nerve injury does not require GM-CSF signalling. Notably, KO microglia exhibited typical denaturated and amoeboid morphology in response to injury to that seen in the WT.

Quantification of the GFAP IR signal 14 days post PNL revealed that both genotypes exhibited a similar astrocytic response with a trend towards a higher ipsilateral signal compared with the contralateral DH. There were no significant differences between genotypes (WT ipsi: 1340.9 ± 189.7, KO ipsi: 1283 ± 119.4 vs WT contra: 1228.2 ± 157.0, and KO contra: 1198.1 ± 110.2, Figs. 3C and D). In summary, these data indicate that GM-CSF signalling contributes to pain-associated behaviour that is independent of a gliosis response. However, we must note that our evaluation was limited to the examination of 2 pan glial cell markers that only provide information about the number and morphology of the cells and so, based on these data alone, we cannot exclude the contribution of microglia and astrocytes.
3.3. Granulocyte-macrophage colony-stimulating factor receptor is functionally expressed in glial cells and is associated with an upregulation of lipopolysaccharide-induced cytokine release through a phosphorylated extracellular signal-regulated kinase pathway

As we found no immunophenotypic differences in the KO glial response, we sought to investigate whether there was a functional impact of GM-CSFR deletion by assessing the ability of microglia and astrocytes to launch an inflammatory response. This was based on the notion that an altered cytokine profile may correspond to an altered nerve injury–induced inflammatory response in the KO.

We, first, verified the purity of our primary microglia and astrocyte cultures through flow cytometry and immunocytochemistry for the glial-specific markers, CD11b/Iba1, and GFAP, respectively. In accordance with the literature, we demonstrated that our cultures consistently exceeded a purity of at least 95% (Supplementary Fig. 2, available online as supplemental digital content at http://links.lww.com/PAIN/A516). Activation of GM-CSFR is known to stimulate the JAK-STAT, MAPK, and PI3K signalling pathways. As p-ERK is a key MAPK involved in the transduction of nociceptive signalling and has been documented to be expressed in both microglia and astrocytes,30 we examined GM-CSF–induced p-ERK responses in these cells to confirm functional expression of the receptor. Phospho-ERK activation was demonstrated in neonatal microglial cultures (EC50 = 0.6 ng/mL; Fig. 4A), which was dose-dependently inhibited by CAM3003 (IC50 = 240 pM; Fig. 4C). Thus, confirming functional expression of GM-CSFR and antibody-dependent inhibition of signalling in microglia in accordance with our Taqman data. Isotype control, CAT004, had no significant effect in this system. In contrast, GM-CSF...
stimulation did not induce a p-ERK signal from neonatal astrocytes and although the baseline levels of p-ERK seemed high in these cultures, the reason for the lack of a GM-CSF effect remains unclear (Fig. 4B).

There are several reports on the effects of GM-CSF on the release of mediators that may contribute directly or indirectly to nociceptive transduction.\textsuperscript{25,26} Based on this evidence, we screened the effects of GM-CSF on glial cell release of several putative cytokines in vitro. Cotreatment with recombinant mouse GM-CSF (5 ng/mL) significantly potentiated LPS-induced IL-1β (1.7-fold), TNFα (1.7 fold), and IL-10 (1.3-fold) release from microglia as well as IL-6 release across several doses of LPS (0.06 μg/mL: 20.1-fold; 0.25 μg/mL: 2.0-fold; and 1 μg/mL: 1.6-fold; Fig. 5A), as previously shown.\textsuperscript{39} Granulocyte-macrophage colony-stimulating factor did not exhibit a significant effect on LPS-induced IL-1β, TNFα, and IL-10 release from astrocyte cultures but significantly potentiated IL-6 release by 1.6-fold (1 μg/mL LPS; Fig. 5B). Together these data support our hypothesis that GM-CSF promotes the release of proinflammatory cytokines that can directly and indirectly drive nociceptive transmission, which may be absent in the KO.

Figure 2. Verification of GM-CSFR mRNA expression. Taqman profiles confirm expression of both GM-CSFR α-chain (A) and common β-chain (B) subunits in astrocytes and microglia. GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor.

Figure 3. Nerve injury–induced gliosis is normal in GM-CSFR β-chain KO mice. At 14 days after PNL, there was a significant increase in Iba1-positive cells in the ipsilateral (Ipsi) DH of WT PNL and KO PNL mice, compared with the contralateral (Contra) DH (A), quantified in (B). There was bilateral expression of GFAP-positive cells in the DH of nerve injured WT and KO mice (C), quantified in (D). There were no significant differences between genotypes. Data are presented as mean ± SEM. **P < 0.01; ***P < 0.001 vs contralateral, one-way ANOVA with Tukey’s multiple comparisons post hoc, n = 3 to 6. Scale bar = 200 μm. ANOVA, analysis of variance; DH, dorsal horn; GFAP, glial fibrillary acidic protein; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; Iba1, ionized calcium binding adapter molecule 1; KO, knockout; PNL, partial sciatic nerve ligation; WT, wild-type.
4. Discussion

Given the well-described roles of GM-CSF in peripheral inflammation and inflammation-induced pain, we hypothesized that it may also play a role in neuroinflammation associated with neuropathic pain. To date, there are very few studies examining GM-CSF in neuropathy, and to the best of our knowledge, we are the first to show that central inhibition of GM-CSFR signalling in the PNL model is analgesic, thus supporting a role of GM-CSF in neuropathic pain. In agreement with reports of mRNA and protein expression of GM-CSFR in the CNS,

we confirmed transcript expression in glial cells and showed that nerve injured mice treated with a centrally delivered antagonizing mAb had attenuated mechanical hyperalgesia. By contrast, peripheral delivery was found to have no effect, thus implicating a centrally driven mechanism in line with successful demonstration of enhanced analgesic efficacy with a BBB penetrative construct that facilitates CNS delivery, as we have previously shown. In conjunction with these data, nerve injured KO mice had attenuated mechanical hyperalgesia compared with WT controls, equivalent to the magnitude of alleviation generated by CAM3003. Given that we observed functional expression of both GM-CSFRα- and β-chain subunits in microglia and astrocytes, we profiled the immunophenotypic response of these cells to nerve injury in the presence and absence of the receptor. Interestingly, we did not detect differences in spinal gliosis between genotypes but observed enhanced proinflammatory

Figure 4. Granulocyte-macrophage colony-stimulating factor (GM-CSF) induces p-ERK signalling in microglia, which is inhibited by antibody neutralisation. Neonatal mouse microglia (A) and astrocytes (B) were stimulated with GM-CSF for 5 to 60 minutes, and p-ERK activation was quantified. Granulocyte-macrophage colony-stimulating factor–induced (0.6 ng/mL) p-ERK activation in microglia could be inhibited by preincubation (30 minutes) with the anti-GM-CSFR mAb CAM3003 (C). Phorbol 12-myristate 13-acetate (100 ng/mL) was used as a positive control. Data are presented as mean ± SEM, n = 3.

Figure 5. Granulocyte-macrophage colony-stimulating factor potentiates LPS-induced cytokine release from neonatal microglia and astrocytes. Neonatal microglia (A) and astrocytes (B) were stimulated for 24 hours with increasing concentrations of LPS (0.06-1 μg/mL) +/− GM-CSF (5 ng/mL), and the protein expression of IL-1β, TNF-α, IL-6 and IL-10 released into the supernatant was measured by ELISA. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs control (−GM-CSF); 2-way ANOVA with Sidak’s multiple comparisons test, n = 3 to 4. ANOVA, analysis of variance; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.
cytokine release from glial cell cultures after GM-CSF stimulation, thus implicating a GM-CSF–mediated neuroinflammatory mechanism.

Although GM-CSFR–positive macrophages may be driving peripheral sensitization in inflammation-induced pain, an analgesic effect in nerve injured mice was only exhibited by intrathecal administration of the inhibitory mAb, CAM3003, thus supporting a centrally mediated mechanism. A significant body of literature exists around a role of the receptor in the CNS, and studies to date have focused on models of MS (eg, EAE; reviewed in19) and stroke (reviewed in20). In EAE, GM-CSF has been widely described as pathogenic through actions on myeloid cells resulting in proinflammatory cytokine production. By contrast, GM-CSF has also been associated with beneficial neuroprotective effects in rodent models of stroke as a result of direct action on neurons.43 In the CNS, GM-CSF expression has been described on microglia, astrocytes, neurons,42,33,42,43 and oligodendrocytes,4 but we only observed immunoreactivity that predominantly colocalised with glial–specific markers in the mouse spinal cord, and we saw no evidence for neuronal expression (data not shown). In vitro data confirmed functional expression of the receptor in microglia and astrocytes, so we focused our study on these cell types.

The role of glial cells in chronic pain mechanisms has been well characterised in several neuropathic and inflammatory pain paradigms, and many of the initial findings were based on studies using general glial inhibitors. A striking observation from our study was the significant gliosis response in the KO, despite the reduced pain behaviours compared to the WT. Although dissociation between gliosis and chronic pain pathways has been documented before,11,29,40 we acknowledge the fact that our studies were limited to gross immunophenotypic differences, and further work examining markers of activation may elucidate the contribution of these cells. Interestingly, it has been shown that direct administration of exogenous GM-CSF into the brain induces a rapid gliosis response,22 but it seems that in this model, GM-CSF was not the sole driver of gliosis. Nonetheless, the absence of overt change in the gliosis response does not preclude a glial-mediated effect of GM-CSF in this model.

The presence of proinflammatory mediators, particularly IL-1β, TNF, and IL-6, within the joints of patients with RA and osteoarthritis6,19 corroborates animal studies that have shown that inhibition of GM-CSF signalling reduces cytokine release in joint tissue.1,2,13 As we found no IHC evidence of an altered glial response in association with the KO behavioural phenotype, we sought to examine whether GM-CSFR deletion impairs the ability of microglia and astrocytes to launch an inflammatory response based on evidence that GM-CSF contributes to proinflammatory cytokine networks,25,26 and that we had shown glial expression of both the α- and β-chain receptor subunits. Here, we used a simplified in vitro model to measure protein expression changes in a selection of putative mediators subsequent to LPS ± GM-CSF stimulation. The rationale for this experimental paradigm is based on published and in-house data that in response to nerve injury, glial cells contribute to pain pathways through releasing a range of mediators such as TNF-α, IL-1β, and IL-6 that promote a proinflammatory environment. Lipopolysaccharide stimulation increases cytokine synthesis in a toll-like receptor-4 (TLR4) dependent manner, which is a key molecule involved in generating responses to pathogen–associated molecular patterns and endogenous molecules released from damaged tissue.34 Activation of TLR4 subsequently activates nuclear factor-κB and the transcription of proinflammatory mediators.34 Whilst the application of LPS to spinal cord induces pain behaviour, TLR4 inhibition attenuates pain-associated behaviours in models of nerve injury.47 Therefore, as we are proposing a glial-mediated mechanism in the GM-CSF signalling pathway, LPS-induced priming of these cells is a relevant in vitro paradigm to enable us to further elucidate the KO behavioural phenotype. Although GM-CSF did not seem to induce cytokine release in its own right, preincubation with GM-CSF significantly potentiated LPS-induced IL-1β, TNFα, IL-6, and IL-10 release from microglia as previously reported39 and IL-6 release from astrocytes.

A number of groups have demonstrated algesic effects of proinflammatory cytokines and have shown that whilst cytokine administration elicits the onset of mechanical and thermal hyperalgesia in rodents, this can be reversed using neutralising antibodies or inhibitors.4 Accordingly, in experimental models of neuropathic and inflammatory pain, cytokines and their cognate receptors are upregulated and the administration of neutralising antibodies or inhibitors alleviates pain-associated behaviours.2,3 Dysregulation of cytokines has also been reported in patients with painful neuropathies. For example, compared with healthy controls, cytokines such as IL-1β and TNFα were found to be elevated in both blood and CSF patient samples.3,49 Despite there being fewer studies showing reversal of pain with neutralising or inhibitory treatments, there is evidence of beneficial effects in some subsets of patients.51 In the spinal cord, proinflammatory cytokines secreted by glial cells have been shown to directly enhance neuronal excitability and synaptic transmission while simultaneously reducing inhibitory currents.18,31,50,54 This suggests that GM-CSF may not affect glia but instead elicit its pronociceptive effects indirectly through glial cell–mediated cytokine release, a response that may be impaired in the KO. Evidently, further study would be required to confirm this link in vivo.

Functional analysis revealed that GM-CSF–induced dose-dependent p-ERK responses in neonatal microglia could be inhibited by treatment with CAM3003. Unexpectedly, we did not detect p-ERK responses in astrocytes for reasons that remain unclear but could be related to high p-ERK basal levels. We did, however, observe significant potentiation of LPS-induced IL-6 release after GM-CSF pretreatment, which is indicative of a functional GM-CSFR signalling response in these cells despite being less marked than those seen in microglia. Literature reports of GM-CSF expression and signalling in astrocytes are few,24 with the focus tending to be on GM-CSF secretion by astrocytes rather than their response to this factor. Nonetheless, astrocytes have been implicated in the maintenance of neuropathic pain,21 and our data suggests that they may indirectly contribute to the behavioural phenotype we observed.

In summary, we have identified for the first time, a centrally mediated mechanism for GM-CSF signalling in the context of neuropathic pain that is driven primarily by glial cell mediator release. In light of these rodent data and the availability of a human anti–GM-CSFR antibody, mavrilimumab with demonstrated safety in the clinic,7 it is interesting to consider the potential of this molecule as a pain therapeutic. Moreover, reports showing increased GM-CSF in intervertebral disc associated with pain55 and increased serum GM-CSF in patients with disc herniation and pain,51 identify a potential population of patients who may benefit from anti–GM-CSFR antibody treatment. Finally, we have also demonstrated efficacy with a novel construct exemplifying an engineered solution to overcome traversing the endothelial cell–derived BBB and deliver proteins to the CNS.52 As previously shown, greater penetration of the CNS results in greater analgesia and prolonged exposure in the central
compartment results in a longer duration of the analgesia.\textsuperscript{52} Certainly, this approach provides a promising avenue for centrally targeted entities not only for the treatment of neuropathic pain but also for other CNS diseases.

Conflict of interest statement
Authors who were employees of MedImmune or Astrazeneca at the time of the work have a theoretical conflict of interest through being employed by the organisation that both funded the work and has a potential commercial interest in the findings.

Acknowledgements
The authors thank all the staff of MedImmune’s Biological Services unit for their invaluable help and technical assistance in performing the surgeries. All work described in this article was funded by MedImmune.

Appendix A. Supplemental digital content
Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A516.

Article history:
Received 5 July 2017
Received in revised form 15 November 2017
Accepted 1 December 2017
Available online 12 December 2017

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