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G-CSF Receptor Blockade Ameliorates Arthritic Pain and Disease

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Abstract

G-CSF or CSF-3, originally defined as a regulator of granulocyte lineage development via its cell surface receptor (G-CSFR), can play a role in inflammation, and hence in many pathologies, due to its effects on mature lineage populations. Given this, and because pain is an extremely important arthritis symptom, the efficacy of an anti-G-CSFR mAb for arthritic pain and disease was compared with that of a neutrophil-depleting mAb, anti-Ly6G, in both adaptive and innate immune-mediated murine models. Pain and disease were ameliorated in Ag-induced arthritis, zymosan-induced arthritis, and methylated BSA/IL-1 arthritis by both prophylactic and therapeutic anti-G-CSFR mAb treatment, whereas only prophylactic anti-Ly6G mAb treatment

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was effective. Efficacy for pain and disease correlated with reduced joint neutrophil numbers and, importantly, benefits were noted without necessarily the concomitant reduction in circulating neutrophils. Anti-G-CSFR mAb also suppressed zymosan-induced inflammatory pain. A new G-CSF-driven (methylated BSA/G-CSF) arthritis model was established enabling us to demonstrate that pain was blocked by a cyclooxygenase-2 inhibitor, suggesting an indirect effect on neurons. Correspondingly, dorsal root ganglion neurons cultured in G-CSF failed to respond to G-CSF in vitro, and *Csf3r* gene expression could not be detected in dorsal root ganglion neurons by single-cell RT-PCR. These data suggest that G-CSFR/G-CSF targeting may be a safe therapeutic strategy for arthritis and other inflammatory conditions, particularly those in which pain is important, as well as for inflammatory pain per se.

Of all the cells implicated in rheumatoid arthritis (RA) pathogenesis, neutrophils possess the greatest cytotoxic potential, due to their ability to release products such as degradative enzymes and reactive oxygen species (1). G-CSF is a key regulator of granulocyte production from progenitor cells (2–4). Its receptor (G-CSFR), as well as being expressed on myeloid progenitors, is highly expressed on neutrophils and eosinophils, but also at a lower level on other cell types, such as monocytes/macrophages (2–4). It therefore potentially has other functions due to its actions on mature myeloid populations (2, 3, 5, 6). G-CSF administration is widely used clinically to treat neutropenia associated with chemotherapy and to mobilize hemopoietic stem cells for transplantation (7). However, the most relevant and acutely harmful side effect of G-CSF administration to cancer patients is bone or musculoskeletal pain (8). G-CSF has been reported to induce pain by directly acting on neurons (9, 10).

G-CSF can be produced in vitro by many cell types, such as macrophages, endothelial cells, fibroblasts, and chondrocytes, in response to inflammatory stimuli, such as IL-1 and TNF (7, 11, 12); its formation is also regulated by IL-17 released (13). These data suggest that G-CSF may have a role in inflammation. In this connection, elevated levels of G-CSF have been found in RA patients and correlate with disease activity and severity (14). Numerous reports have documented arthritis flares with G-CSF treatment for Felty syndrome (15). We have demonstrated that G-CSF can exacerbate murine collagen-induced arthritis (16), and endogenous G-CSF has been implicated in arthritis models (17–20). Neutrophil number and function were also implicated in these inflammatory arthritis models. However, anti-inflammatory effects of administered G-CSF have been observed in some inflammatory conditions, including adjuvant arthritis in rats (21).

Because pain is an extremely important arthritis symptom, we tested whether targeting G-CSFR with a neutralizing mAb would suppress it, as well as disease, in various arthritis models. We report in this study that it is the case even with a therapeutic mAb protocol and even under conditions where a neutrophil-depleting mAb failed. We also established a G-CSF-driven arthritis model enabling downstream pathways to be potentially elucidated. We were unable to stimulate dorsal root ganglion (DRG) neuronal activity with G-CSF in vitro.

Materials and Methods

Mice

C57BL/6 mice (8–12 wk old) were from the Walter and Eliza Hall Institute (Parkville, Australia) or the South Australian Health and Medical Research Institute (Adelaide, Australia). All experiments were approved by the University of Melbourne or South Australian Health and Medical Research Institute Animal Ethics Committees.

Ag-induced arthritis

As before (22, 23), mice were immunized with methylated BSA (mBSA, 200 µg; Sigma-Aldrich), emulsified in CFA, intradermally in the base of the tail. Arthritis was induced 7 d later by an intra-articular (i.a.) injection of mBSA into the left knee, with the right knee being injected with saline.

Zymosan-induced arthritis model

For the induction of zymosan-induced arthritis (ZIA) (22), mice were injected with 300 µg of sonicated zymosan in a 10-µl vol into the left knee joint, whereas the contralateral knee received saline as a control.

Inflammatory pain model

Inflammatory pain was induced by intraplantar injection of zymosan (100 µg) with saline as the control (22, 24). Paw swelling was measured using spring calipers (Mitutoyo, Tokyo, Japan).

mBSA-induced arthritis models

For the induction of mBSA arthritis models (23), mice were injected with 200 µg of mBSA in 10 µl into the left knee, and saline was injected into the contralateral knee, followed by s.c. injection on days 0–2 of either murine IL-1β (250 ng; R&D Systems), human G-CSF (250 ng; R&D Systems), or saline. In some experiments, indomethacin (1 mg/kg) or the cyclooxygenase (COX)-2 selective inhibitor, SC581258 (1 mg/kg), was given i.p. once pain was evident and daily thereafter.

mAb treatment

For mAb administration, mice were given i.p. anti-G-CSFR (100 µg, clone 5E2-VR81; CSL) (20), anti-Ly6G (100 µg, clone 1A8) (19, 25), or both isotype IgGs [IgG1 (20) and IgG2a (anti-β-galactosidase) (26), respectively], except where indicated.

Pain readings

As an indicator of pain, the differential distribution of weight between the inflamed limb or paw relative to the noninflamed limb or paw was measured using an incapitance meter (IITC Life Science). This technique has been validated for measurement of both arthritic knee and footpad pain (22, 23, 27). Three measurements were taken for each time point and averaged.

Quantitative PCR

Quantitative PCR (qPCR) was performed as previously described (22). Briefly, total RNA was extracted from joint cells or skin from the hind paw using an ISOLATE II RNA mini kit (Bioline, Taunton, MA) and reverse transcribed using Tetro reverse transcriptase (Bioline). qPCR was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA) and predeveloped TaqMan probe/primer combinations for murine *Csf3r*, *Tnf*, *Il1b*, and *Ubc* (Life Technologies). All samples were assayed in duplicate. Threshold cycle numbers were transformed to threshold cycle values, and the results were expressed relative to the reference gene, *Ubc*.

Cell population analysis

Cell populations from blood and joints were analyzed by flow cytometry (28). Briefly, Fc receptors on cells were blocked with normal mouse serum (1:4 dilution) and stained with fluorochrome-conjugated mAbs specific for mouse CD45-FITC (clone OX-1), CD11b-allophycocyanin-Cy7 (clone M1/70), Ly6G-allophycocyanin-Cy7 (clone 1A8), Ly6C-PerCP (clone AL-21), F4/80-PE (clone BM8), MHC class II (MHC-II)-V500 (clone M5/114.15.2), CD11c-PE-Cy7 (clone HL3), CD62L-PE (clone MEL-14), and the corresponding isotype controls, either from BD Biosciences or BioLegend. Cells were analyzed using a CyAn ADP analyzer (Beckman Coulter).

Histology

At termination, the knee joints were removed, fixed, decalcified, and paraffin embedded. Frontal sections (7 μ m) were stained with H&E (22, 23, 27). For the mBSA models, cellular infiltration, synovitis, pannus formation, cartilage damage, and bone erosions were scored separately from 0 (normal) to 5 (severe) as described previously (22, 23) and plotted as a total histological score. For ZIA and Ag-induced arthritis (AIA), cell infiltration, synovial hyperplasia, and bone erosion were scored separately from 0 (normal) to 3 (severe) (22, 23).

Neuron isolation and responses

As before (29), whole DRGs from all spinal levels were collected, digested, and mechanically triturated. Cell suspensions were washed twice in supplemented DMEM (containing 10% heat-inactivated FBS and 1% penicillin/streptomycin) (Thermo Fisher Scientific), resuspended in supplemented DMEM containing 1% N1 neuronal supplement (Sigma-Aldrich), plated onto coverslips coated with poly-L-lysine (Sigma-Aldrich) and laminin (100 μ g/ml), and cultured for 24 or 48 h at 37°C (95% O₂, 5% CO₂).

Measurement of intracellular Ca²⁺ concentration in DRG neurons.—Neuronal cells were loaded with Fura-2/AM ester (5 μ M, 45 min, 37°C) in calcium assay buffer (30) and incubated for 30 min before imaging. Fluorescence was measured at 37°C using a Leica DMI-6000B imaging system (Leica Microsystems, Mannheim, Germany) with a \times 10 objective. Images were collected at 1-s intervals (excitation, 340 nm/380 nm; emission, 530 nm). Neurons were challenged with vehicle, G-CSF (200 ng/ml), and capsaicin (0.5 μ M; TRPV1 agonist). KCl was applied at the end of the experiment to identify neurons (30).

Data are presented as $F/F_0 - 1$, where F is the measured fluorescence intensity and F_0 is the basal fluorescence.

G-CSF stimulation and immunofluorescence.—Prior to stimulation, neuronal cells were serum starved overnight in supplemented DMEM with 1% (v/v) N1 (30) at 37°C. Neurons were then stimulated for 15 min with PBS, G-CSF (200 ng/ml), or PMA (2 μM; Sigma-Aldrich). Cells were washed with ice-cold PBS, fixed (4% paraformaldehyde) for 20 min, incubated in methanol (100%, -20°C, 20 min), blocked, and permeabilized (0.01% Triton X-100, 5% FBS, and 5% goat serum in PBS, 60 min). Neurons were then stained with mouse anti-mouse NeuN (clone A60; Millipore) and rabbit anti-mouse phospho-p44/42 MAPK (Erk1/2, clone 197G2; Cell Signaling Technology) mAbs followed by Alexa Fluor 568-conjugated anti-mouse IgG (Thermo Fisher Scientific) and Alexa Fluor 488-conjugated anti-rabbit IgG, F(ab')₂ fragment (Thermo Fisher Scientific). Cells were stained with DAPI (1 μg/ml, 5 min; EMD Millipore) and coverslipped. Slides were examined using a Zeiss Axioskop 2 at ×10 magnification, and images were captured by a Zeiss AxioCam MRm. The percentage of neurons positive for phospho-ERK1/2 was calculated relative to the total NeuN-positive neuronal population.

Single-cell RT-PCR

Thirty-two single dissociated L5 DRG neurons from one mouse (C57BL/6; male; 16 wk old) were picked using a micromanipulator at ×40 magnification (31). Cells were under a continuous slow flow of sterile and RNA/DNase-free PBS to reduce contamination. After a cell was picked, the glass capillary was broken into a tube containing 10 μl of lysis buffer and DNase (TaqMan Gene Expression Cells-to-CT Kit; Thermo Fisher Scientific). The whole content was used for cDNA synthesis (SuperScript VILO cDNA synthesis kit; Thermo Fisher Scientific), and *Csf3r* expression was measured using TaqMan RT-PCR for 50 cycles. For every coverslip a bath control was taken and analyzed together with samples. *Tubb3* expression served as positive control. Three cells were excluded because no *tubb3* expression was present. Twenty-nine cells were used to calculate *Csf3r* frequency.

Statistical analysis

For mRNA expression and joint cell analysis, a Student *t* test or a one-way ANOVA was used. For pain readings, a Student *t* test or two-way ANOVA was used, and for histologic scores, a Kruskal–Wallis test was used (version 5.04; GraphPad Software, San Diego, CA). A Bonferroni post hoc test was also used when appropriate. Data were plotted as mean ± SEM with significance indicated. A *p* value <0.05 was regarded as significant.

Results

Therapeutic G-CSFR neutralization ameliorates AIA pain and disease

AIA is a widely used adaptive immune-mediated monoarticular arthritis model (32). Neutrophils, as well as TNF, IL-1β, and PGs, have been implicated in the articular hypernociception in AIA (33, 34). First, anti-G-CSFR mAb was given therapeutically to determine the optimal dose at which it might ameliorate AIA pain and disease. Pain was measured by differences in hind limb weight distribution using the validated incapitance

meter method (22, 23, 27, 35). An i.p. dose of 100 μ g of anti-G-CSFR mAb, given at days 1, 3, and 5 after i.a. Ag (mBSA) challenge, optimally and rapidly reversed pain (Fig. 1A) and inhibited arthritis progression (histology, day 7) (Fig. 1B). It also led to some reduction at day 6 in the percentage of blood neutrophils (Fig. 1C), identified as in Supplemental Fig. 1, to around the steady-state value (8–10%) (data not shown). Ten micrograms of anti-G-CSFR mAb also effectively inhibited pain and arthritis (Fig. 1A, 1B), without lowering the percentage of circulating neutrophils (Fig. 1C), whereas mice treated with 1 μ g of anti-G-CSFR mAb had similar pain and disease as mice given 100 μ g of isotype IgG (Fig. 1A, 1B). It is interesting that the degree of reduction by anti-G-CSFR mAb in the cellular infiltration into the AIA joints did not correlate with the relative blood neutrophil percentages (see below and Discussion). A dose of 100 μ g of anti-G-CSFR mAb was therefore used in the subsequent experiments.

We next assessed whether therapeutic treatment with the neutrophil-depleting anti-Ly6G mAb (19, 25) would be similarly efficacious. Following mAb administration beginning on day 1 after AIA induction, G-CSFR blockade was again found to be effective at reversing pain rapidly (Fig. 1D) and inhibiting disease severity (day 7) (Fig. 1E), whereas anti-Ly6G mAb and the isotype controls were ineffective. Interestingly, the anti-Ly6G mAb reduced the percentage of circulating neutrophils more than the anti-G-CSFR mAb (Fig. 1F), even though only G-CSFR blockade reduced arthritis and pain.

Notwithstanding the challenges faced in defining categorically macrophages and dendritic cells (DCs) by surface marker expression (26, 36), the joint cell populations at day 7 were subsequently analyzed. Among the CD45⁺ cells, there were F4/80⁻CD11b⁺ neutrophils, which were also Ly6G⁺, as well as three F4/80⁺CD11b⁺ populations, which were designated as follows: a MHC-II⁻CD11c⁻ macrophage subset (R1), a MHC-II⁺CD11c⁻ macrophage subset (R2), and a MHC-II⁺CD11c⁺ subset (R3), designated as monocyte-derived DCs (Mo-DCs) (26) (Supplemental Fig. 2A). Following therapeutic G-CSFR neutralization in AIA mice, the numbers of all of these myeloid populations were reduced as compared with the isotype-treated mice (Fig. 1G), consistent with the cellular infiltration score (Fig. 1E). Paralleling the findings for pain and disease, such reduction was not seen following therapeutic anti-Ly6G mAb administration (Fig. 1G). We also examined the surface expression of CD62L and CD11b on the joint neutrophils and macrophages, because they are modulated following G-CSF addition to murine neutrophils in vitro (18, 37); however, following either mAb administration, we could not detect any differences in surface CD62L and CD11b expression among the neutrophil and macrophage populations, suggesting that G-CSF is not modulating these surface markers in this model (data not shown).

We also monitored changes in some gene expression in the AIA joints. By qPCR, expression of the G-CSFR (*Csf3r*) gene, which is highly expressed in neutrophils and also to a lesser extent in macrophages (38), and expression of the inflammatory mediator genes TNF (*Tnf*) and IL-1 β (*Il1b*) were all upregulated in the day 7 AIA (i.a. mBSA) joint (Supplemental Fig. 2B), and were all lowered upon G-CSFR neutralization but not by anti-Ly6G mAb (Fig. 1H). This decrease in total joint *Csf3r* expression was not observed in sorted (CD11b⁺Ly6G⁺F4/80⁻) neutrophils (data not shown), indicating that the decrease

reflected their numbers rather than a G-CSF–dependent regulation as part of its mechanism of action.

Thus, therapeutic neutralization with anti–G-CSFR mAb, but not with anti-Ly6G mAb, effectively ameliorated AIA pain and disease, which was associated with reduced joint myeloid cell numbers and the expression of certain genes associated with inflammation (see Discussion).

G-CSFR neutralization suppresses the onset of AIA pain and disease

The dissociation noted above in the relative effectiveness of therapeutic anti–G-CSFR mAb versus anti-Ly6G mAb administration in the AIA model prompted us to evaluate whether both mAbs would suppress AIA pain and disease onset when given prophylactically. Mice were pretreated i.p. with mAbs at days –3 and –1, with day 0 being the time of i.a. Ag challenge. When assessed at day 1, both anti–G-CSFR mAb- and anti-Ly6G mAb-treated mice had significantly less pain (Fig. 2A); arthritis, which at this time point was predominantly cellular infiltration, was abrogated in mice pretreated with either mAb (Fig. 2B). The anti-Ly6G mAb again lowered the percentage of circulating neutrophils more than did the anti–G-CSFR mAb, with the latter again to around the steady-state value (Fig. 2C).

Joint cells were also analyzed on day 1 after AIA induction. Among the CD45⁺ cells, there were F4/80[–]CD11b⁺Ly6G⁺ neutrophils and F4/80⁺CD11b⁺ macrophages, which were either MHC-II[–] (R1) or MHC-II⁺ (R2), and CD11b[–] cells (26) (Supplemental Fig. 2C). Following both G-CSFR and Ly6G neutralization, there was a reduction in the numbers of all these populations, with the two mAbs having similar effects this time (Fig. 2D). Following either mAb administration, we again could not detect any differences in surface CD62L and CD11b expression among the neutrophil and macrophage populations (data not shown).

There was again increased expression in AIA (i.a. mBSA) joints of the genes noted above (*Csf3r*, *Tnf*, and *Il1b*) (Supplemental Fig. 2D); expression of all of these genes was lowered by prophylactic anti–G-CSFR mAb or anti-Ly6G mAb treatment (Fig. 2E).

These data indicate that G-CSFR signaling and neutrophils are required for the onset of AIA pain and disease.

Therapeutic G-CSFR neutralization ameliorates ZIA pain and disease

Intra-articular zymosan induces an acute, innate immune-driven monoarticular arthritis, with neutrophils being implicated (24). We therefore tested whether therapeutic treatment with anti–G-CSFR mAb would also be efficacious for ZIA pain and disease, and again compared it with anti-Ly6G mAb administration. mAbs were administered therapeutically (days 1, 3, and 5) after i.a. zymosan injection. Neutralizing anti–G-CSFR mAb effectively and rapidly reversed arthritic pain, whereas anti-Ly6G–treated mice still displayed similar pain to isotype-treated mice (Fig. 3A). Also, arthritis development in anti–G-CSFR mAb-treated mice was inhibited unlike in both anti-Ly6G– and isotype-treated mice (Fig. 3B). Consistent with our observations above, the percentage of blood neutrophils was reduced more by the anti-Ly6G mAb (day 6), with the value for the anti–G-CSFR mAb group again being similar to that for the steady-state (Fig. 3C).

To examine changes in joint cellular composition at day 7, a similar gating strategy as for the AIA model at day 1 (Supplemental Fig. 2C) was used, as no Mo-DCs were seen (data not shown). At day 7 following ZIA induction, there was a significant reduction in joint CD45⁺ cells, including Ly6G⁺ neutrophils and both subsets of F4/80⁺ macrophages in the anti-G-CSFR-treated mice (Fig. 3D), but not in the anti-Ly6G-treated mice, as in the AIA model using a therapeutic protocol (Fig. 1G). Once again, lower expression for the *Csf3r*, *Tnf*, and *Il1b* genes was seen in joints from anti-G-CSFR-treated mice, but not from anti-Ly6G-treated mice (Fig. 3E).

Thus, therapeutic neutralization with anti-G-CSFR mAb, but not with anti-Ly6G mAb, also ameliorated ZIA pain and disease, which was associated with reduced joint myeloid cell numbers and the expression of certain genes associated with inflammation (see Discussion).

G-CSFR neutralization suppresses the onset of ZIA pain and disease

Given once more the dissociation between the relative effectiveness of therapeutic anti-G-CSFR mAb versus anti-Ly6G mAb administration, we therefore again evaluated whether for ZIA both anti-G-CSFR mAb and anti-Ly6G mAb would reduce pain and disease when given prophylactically. Abs were administered on days -1, 1, and 4, with day 0 being the time of ZIA induction.

Treatment with anti-G-CSFR or anti-Ly6G mAb blocked the pain (Fig. 4A) and suppressed arthritis severity (day 7) (Fig. 4B). Once again, the anti-Ly6G mAb, unlike the anti-G-CSFR mAb, reduced the percentage of the blood neutrophils to below the steady-state value (Fig. 4C).

As regards joint cellular composition, anti-G-CSFR or anti-Ly6G mAb blockade led to a reduction in the numbers of infiltrating CD45⁺ cells compared with isotype-treated mice, with lower numbers of neutrophils and MHC-II⁻ and MHC-II⁺ macrophages being present (Fig. 4D). Additionally, there was lower joint *Csf3r*, *Tnf*, and *Il1b* expression following G-CSFR and Ly6G neutralization (Fig. 4E).

These data indicate that G-CSFR signaling and neutrophils are required for the onset of ZIA pain and disease.

G-CSFR neutralization suppresses the onset of inflammatory pain

Zymosan can also induce significant inflammatory pain and footpad swelling when given intraplantarly, with neutrophils being implicated. We tested whether anti-G-CSFR mAb could also inhibit these responses and compared it again with anti-Ly6G mAb. Prior to zymosan injection (day 0), the mAbs and their respective isotype controls were administered i.p. on days -3 and -1. Both mAbs significantly inhibited the inflammatory pain (Supplemental Fig. 3A) and swelling (Supplemental Fig. 3B). Furthermore, qPCR analysis of the plantar tissue showed that both mAbs effectively reduced zymosan-induced *Csf3r*, *Tnf*, and *Il1b* expression (Supplemental Fig. 3C).

mBSA/IL-1 arthritis pain is G-CSF–dependent

The so-called monoarticular mBSA/IL-1 arthritis model, induced by i.a. mBSA followed by s.c. IL-1 β , is T lymphocyte–dependent and its severity is less in G-CSF^{-/-} mice (17). As shown in Fig. 5A, in this model the onset of pain was also prevented by anti-G-CSFR and anti-Ly6G mAbs, and the arthritis was suppressed (Fig. 5B).

G-CSF drives arthritic pain and disease

We showed above that G-CSFR signaling is required for mBSA/IL-1–induced arthritic pain and disease. This type of model, which is driven by systemic cytokines, enables cytokine-dependent downstream mechanisms to be explored directly (23, 39), as done for IL-1 β above. Because we wanted to examine further how G-CSF is involved in regulating arthritic pain and disease, we determined whether systemic G-CSF might be able to replace IL-1 β in the mBSA/IL-1 model.

We therefore modified the mBSA/IL-1 arthritis model with s.c. G-CSF administered systemically (days 0–2) to mice given i.a. mBSA at day 0. mBSA/G-CSF–injected mice developed pain in the mBSA-injected joint relative to the contralateral joint, which was similar in magnitude and kinetics to what was observed in mBSA/IL-1 β –injected mice (Fig. 6A). As above (Fig. 5) and before (23), mBSA s.c. saline-injected mice did not develop any signs of arthritic pain (Fig. 6A). Nonsteroidal anti-inflammatory drugs represent the first level of approach in patients to treat G-CSF–induced pain (8), and G-CSF–induced mechanical hyperalgesia is inhibited by the broad COX inhibitor indomethacin (40). We found that indomethacin (Fig. 6B) and the more specific COX-2 inhibitor, SC58125 (Fig. 6C), when given once pain was evident (day 3), both reversed it rapidly, indicating eicosanoid involvement in this new mBSA/G-CSF model.

mBSA/G-CSF–injected mice developed arthritis to a similar extent as mBSA/IL-1 β –injected mice compared with mBSA/saline-injected mice, indicating that exogenous G-CSF can also drive arthritic disease as a coarthritogenic stimulus. For the same experiment in which the COX inhibitors could rapidly reverse G-CSF–driven arthritic pain (Fig. 6B, 6C), arthritic disease was not reduced (day 7) (Fig. 6D) (see Discussion).

Our data showing eicosanoid involvement in mBSA/G-CSF arthritic pain suggest perhaps an indirect effect of G-CSF on neurons in this model. However, direct effects of G-CSF have been reported, including as the mechanism for G-CSF–induced mechanical hyperplasia (9). In cultures of DRG neurons we were unable to demonstrate either G-CSF–stimulated elevation in intracellular Ca²⁺ concentration (Supplemental Fig. 4A, 4B) or ERK-1/2 phosphorylation (Supplemental Fig. 4C) unlike the positive controls capsaicin and PMA, respectively. Also, we could not detect STAT3 phosphorylation following G-CSF stimulation in these neurons (data not shown). Additionally, by single-cell RT-PCR, we could not detect *Csf3r* mRNA expression in *Tubb3*⁺ L5 DRG neurons (0 of 29) (data not shown). Taken together, these data do not support a direct action of G-CSF on neurons (see Discussion).

We investigated the systemic effects of G-CSF on circulating and mBSA-injected joint myeloid cell populations in the mBSA/G-CSF arthritis model. In day 7 mBSA-injected joints, the number of CD45⁺ joint cells and neutrophils, but not macrophages (gated as in

Supplemental Fig. 2C), were increased in s.c. G-CSF– versus s.c. saline-injected mice (Fig. 6E). Following G-CSF administration, by day 3 there was an increase in the percentage of blood neutrophils that continued to at least day 7 (Fig. 6F).

TNF and IL-1 β have also been considered to be involved in G-CSF–induced bone and musculoskeletal pain in cancer patients (8). By qPCR, joint expression for *Csf3r*, *Tnf*, and *Il1b* were increased following G-CSF administration (Fig. 6G), all paralleling the increase in joint neutrophil number.

These data show that exogenous G-CSF can drive arthritic pain and disease, with the pain being dependent on a COX-2 product.

Discussion

To our knowledge, this is the first study showing that a neutralizing anti-G-CSFR mAb can prevent and rapidly reverse arthritic pain, thereby implicating G-CSF in such pain. This approach was effective in both adaptive (lymphocyte-dependent) and innate (lymphocyte-independent) immune arthritis models; arthritic disease was also ameliorated and the onset suppressed, thus extending the number of arthritis models in which G-CSF signaling is implicated in the pathogenesis (3, 16–20). This strategy was also able to block inflammatory pain in an intraplantar model.

Our neutralizing mAb approach, in addition to incorporating both prophylactic and therapeutic protocols in a range of arthritis models, included a comparison with a well-established neutrophil depletion method using anti-Ly6G mAb. This allowed us to find protocols in which only anti-G-CSFR mAb was effective in suppressing pain and arthritis, and to show that a reduction in joint myeloid cell number, particularly of neutrophils, but not in circulating neutrophils, correlated with the benefit. In this connection, joint *Csf3r* mRNA expression correlated with joint neutrophil number and the degree of pain and disease, and perhaps could be used more broadly as a surrogate readout for neutrophil numbers. In support of this suggestion, the maintenance of *Csf3r* mRNA expression in sorted neutrophils from AIA joints upon therapeutic anti-G-CSFR administration is consistent with this parameter correlating with joint neutrophil numbers.

The dissociation between the efficacies of the anti-G-CSFR and anti-Ly6G mAbs found with therapeutic administration in the AIA and ZIA models was perhaps surprising, but also interesting—these data suggest that, once neutrophils are present in the inflamed joints, anti-Ly6G mAb is not readily effective at depleting them there but G-CSFR signaling is still required locally for arthritis pain and disease development. Consistent with these data for a role in local G-CSFR signaling in joints, a benefit for AIA pain and disease could be obtained under conditions, that is, at a low dose of anti-G-CSFR mAb, where circulating neutrophils were not significantly reduced when compared with control levels (Fig. 1C); this finding, along with the success of the therapeutic protocols, has obvious implications for potential clinical studies (see below).

We also established a new G-CSF–driven arthritis model, which we have termed mBSA/G-CSF arthritis. Thus, we have now shown that G-CSF can exacerbate both systemic (16)

and monoarticular arthritis. This model also allowed us to show COX-2 dependence of G-CSF-driven arthritic pain and, at least under the conditions employed in this particular model, to dissociate arthritis development from pain. It is possible that the COX inhibitors may suppress arthritic disease when administered differently, for example, during longer periods. The availability of this model will enable such a question and additional G-CSF-dependent downstream mechanisms to be further explored.

The COX-2 dependence of mBSA/G-CSF-induced joint pain and the COX dependence of G-CSF-induced mechanical hyperalgesia (40) indicate perhaps an indirect effect at the level of the neuron via an eicosanoid in this model. Consistent with this notion, we were unable to detect *Csf3r* mRNA in DRG neurons, in agreement with studies using single-cell (41) and genome-wide RNA sequencing (42), and could not demonstrate enhanced intracellular Ca^{2+} levels, as well as ERK-1/2 and STAT3 phosphorylation, in these neurons in response to G-CSF in vitro. These data are at odds with those from another group who reported that G-CSF can induce pain by direct action on sensory nerves (9), identified a G-CSFR-dependent neuronal transcriptome for DRG neurons (10), and showed stimulation by G-CSF of ERK-1/2 and STAT3 in DRG neurons in vitro (9, 10). Also, even though exogenous G-CSF can exacerbate the behavioral hypersensitivity in a murine chronic neuropathic pain model (43), it can also counteract acute neuronal degeneration and drive neurogenesis reportedly via its receptors on neurons (44). The different findings on whether G-CSF acts directly on DRG neurons could depend on variation in the isolation and culture methods and need to be interpreted cautiously, as G-CSFR expression and function could vary. A thorough analysis of G-CSFR expression by different neuronal populations would also seem to be warranted.

Also, note that TNF and IL-1 β have been implicated in G-CSF-induced mechanical hyperalgesia in the murine paw and that TNF-induced hyperalgesia is dependent on neutrophil migration (40). The dependence noted above of arthritic joint *Tnf* and *Il1b* mRNA expression on G-CSFR signaling (Figs. 1H, 2E, 3E, 4E) is consistent with these murine paw data. It can be seen that the reduction in *Tnf* and *Il1b* mRNA expression by anti-G-CSFR mAb paralleled the reduction in G-CSFR mRNA, as well as the number of joint neutrophils and macrophages (Figs. 1G, 2D, 3D, 4D). In this context, the contribution of infiltrating neutrophil numbers to *Tnf* and *Il1b* expression and its significance for arthritis has been mentioned (20, 45). The reduction in joint *Il1b* mRNA expression upon anti-G-CSFR administration is in line with what was reported recently for the protein levels of IL-1 β and a number of cytokines/chemokines (20). Interestingly, both TNF and IL-1 β can upregulate G-CSF expression in vitro in a number of tissue-resident cell types, including human synovial fibroblasts and chondrocytes (11, 12), indicating a mutual interdependence and a possible positive feedback loop among these cytokines. Further studies are required to explore the significance and mechanism of this dependence. Our data on cytokine expression are also in line with the observation that G-CSF serum levels in RA patients correlate with those of TNF and IL-1 β (14).

Another CSF, namely, GM-CSF, which can also affect neutrophil and monocyte/macrophage functions, including in inflammation (2), can be regulated concomitantly with G-CSF in vitro (11, 12). Also GM-CSF blockade/depletion is effective in treating AIA, ZIA, and

mBSA/IL-1 pain and arthritis (22, 23), as well as zymosan-induced inflammatory pain (22); blockade/depletion of either CSF is beneficial for collagen-induced arthritis (17, 46, 47), K/BxN-serum transfer arthritis (19, 47), and experimental autoimmune encephalomyelitis (48, 49). All of these data suggest that there may be a functional link between these two CSFs in inflammation.

Given the efficacy also shown above for arthritic pain and disease following prophylactic neutrophil depletion by the anti-Ly6G mAb, neutrophils are likely to be a key cell target for G-CSFR-mediated signaling in the AIA and ZIA models, although other cell types, such as monocytes/macrophages (50), may also be targets. Neutrophils have previously been implicated in AIA (45) and in zymosan-induced hyperalgesia (24), as well as in arthritis in general (6, 51). G-CSF is a prosurvival factor for granulocytes, which could contribute to the G-CSFR dependence of their numbers in the arthritic joints noted above, perhaps by delaying apoptosis and resulting in further inflammatory responses (52); however, control over neutrophil trafficking, possibly via chemokine formation, is also another potential mechanism that increases such numbers (7, 17, 19, 20, 37, 45). G-CSF can also have other proinflammatory activities, such as modulating neutrophil adhesion molecules and enhancing angiogenesis, in addition to the elevation of degradative enzymes and reactive oxygen species (2, 20, 51).

As mentioned, we found above that in the AIA and ZIA models the relative reduction in joint macrophage numbers was similar to that observed for joint neutrophils. This could be of functional significance, as synovial macrophage numbers in RA correlate with disease severity and efficacy of a number of disease-modifying antirheumatic drugs (53). These findings appear to be different to what was reported in a recent study using the same anti-G-CSFR mAb in type II collagen Ab-induced arthritis where only minimal effects on monocyte/macrophage numbers in the joints were noted (20). There is evidence that extravasation of inflammatory monocytes is markedly reduced after neutrophil depletion (54).

With regard to potential clinical relevance, pain is the dominant symptom in arthritis with the highest impact on disease burden. Also, a possible adverse side effect of G-CSFR and G-CSF targeting, which will need to be monitored, is neutropenia and thus susceptibility to infections; however, the data above suggest that therapeutic benefit can be achieved with anti-G-CSFR mAb treatment without a dramatic reduction in circulating neutrophils, as has been noted recently (20). These observations, along with the success of the therapeutic protocols noted above, are encouraging for the clinical safety trial that has just commenced using an anti-G-CSFR mAb in healthy volunteers (Australian and New Zealand Clinical Trials Registry, ACTRN 12616000846426). We suggest that such an approach may be beneficial for other inflammatory/autoimmune conditions, including those in which pain is an important feature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article:

AIA	Ag-induced arthritis
COX	cyclooxygenase
DC	dendritic cell
DRG	dorsal root ganglion
i.a.	intra-articular(ly)
mBSA	methylated BSA
MHC-II	MHC class II
Mo-DC	monocyte-derived DC
qPCR	quantitative PCR
RA	rheumatoid arthritis
ZIA	zymosan-induced arthritis

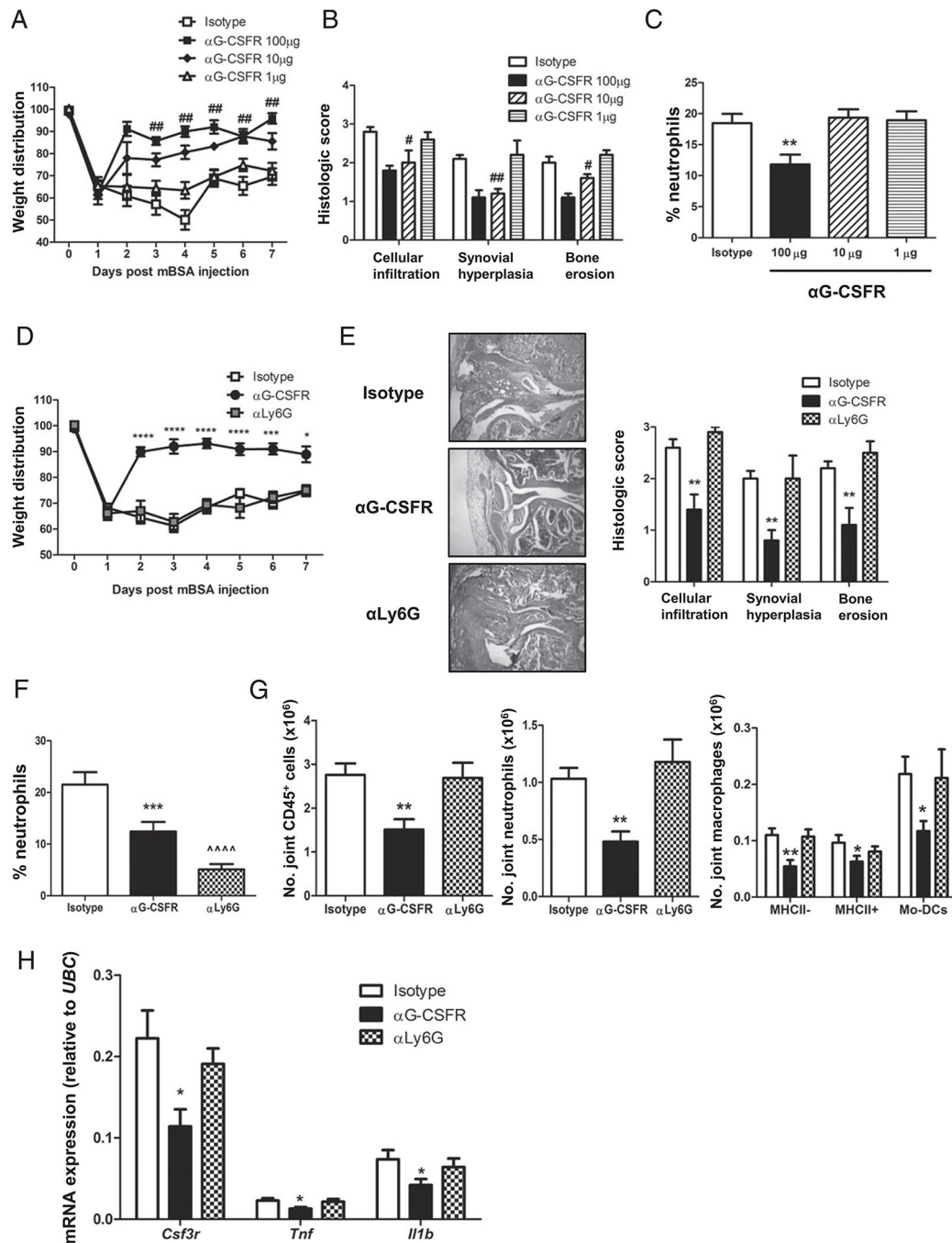
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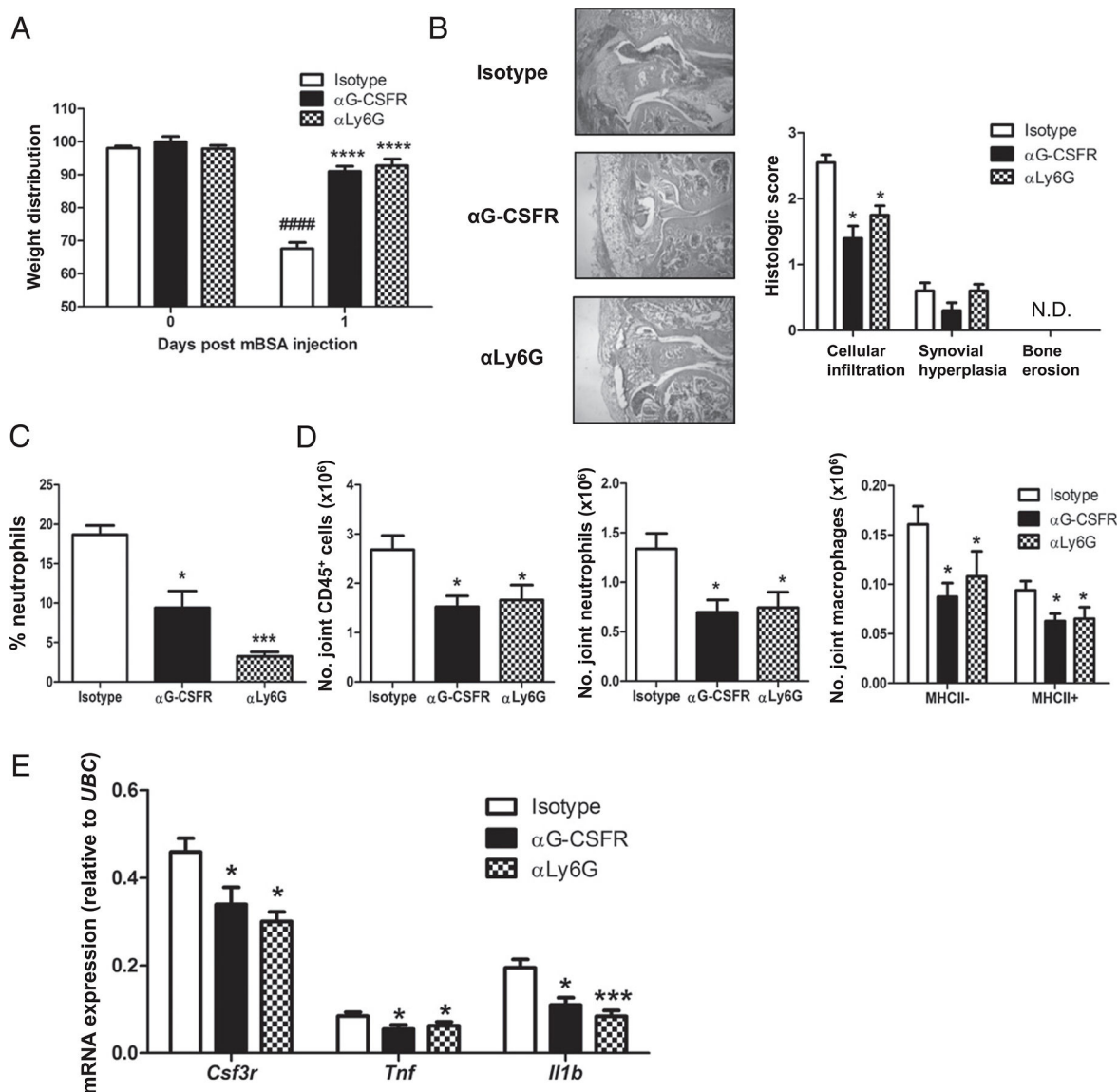
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**FIGURE 1.**

Therapeutic G-CSFR neutralization ameliorates AIA pain and disease. C57BL/6 mice were induced with AIA (day 0). For (A)–(C), 100, 10, or 1 μ g of anti-G-CSFR mAb or 100 μ g of isotype IgG1 control was administered i.p. on days 1, 3, and 5. (A) Pain (incapacitance meter) and (B) arthritis severity (histology) were also evaluated (day 7, $n = 10$ per group). (C) The percentage of blood neutrophils was determined (day 6, $n = 10$ per group). (D–H) Anti-G-CSFR, anti-Ly6G, and isotype control mAbs (100 μ g) were administered i.p. on days 1, 3, and 5. (D) Pain (incapacitance meter) and (E) arthritis (histology, day 7) were

evaluated ($n = 10\text{--}15$ per group). Histology original magnification $\times 60$. (F) Blood was collected on day 6 and the percentage of neutrophils was determined ($n = 10$ per group). (G) Day 7 mBSA-injected joints were collected for cellular analysis. Numbers of infiltrating CD45⁺ leukocytes (neutrophils, MHC-II^{-/+} macrophages, Mo-DCs) ($n = 5\text{--}10$ per group) are shown. (H) Joint mRNA (qPCR) expression was measured (day 7, $n = 9\text{--}10$ per group). Results are mean \pm 6SEM. The p values were obtained using a two-way ANOVA test (A and D) for pain readings, a Kruskal–Wallis test (B and E) for histology, and a one-way ANOVA (C, F, G, and H) for blood cells, joint cells, and gene expression. $\dagger\dagger p < 0.01$, $\dagger\dagger\dagger p < 0.001$, IgG1 versus anti-G-CSFR (100 μg); $\# p < 0.05$, $\#\# p < 0.01$, IgG1 versus anti-G-CSFR (10 μg); $* p < 0.05$, $** p < 0.01$, $*** p < 0.001$, $**** p < 0.0001$, isotype versus anti-G-CSFR; $^^^^ p < 0.0001$, isotype versus anti-Ly6G.

**FIGURE 2.**

G-CSFR neutralization suppresses the onset of AIA pain and disease. C57BL/6 mice were induced with AIA (day 0), and anti-G-CSFR mAb (100 μg), anti-Ly6G mAb (100 μg), or isotype control mAbs were administered i.p. on days -3 and -1. (A) Pain (incapacitance meter) and (B) arthritis (histology, day 1) development were assessed ($n = 10$ per group). Histology original magnification $\times 60$. (C) Blood was collected on day 0 prior to i.a. mBSA injection and the percentage of blood neutrophils was determined (flow cytometry) ($n = 10$ per group). (D) Day 1 mBSA-injected joints were collected for cellular analysis (flow cytometry). Numbers of infiltrating CD45⁺ leukocytes (neutrophils and MHC-II^{-/+} macrophages) ($n = 5-10$ per group) are shown. (E) AIA joint mRNA expression (qPCR) was measured (day 1, $n = 6-10$ per group). Results are mean \pm SEM. The p values were obtained using a two-way ANOVA test (A) for pain readings, a Kruskal-Wallis test (B) for histology, and a one-way ANOVA (C, D, and E) for blood cells, joint cells, and gene expression. * p

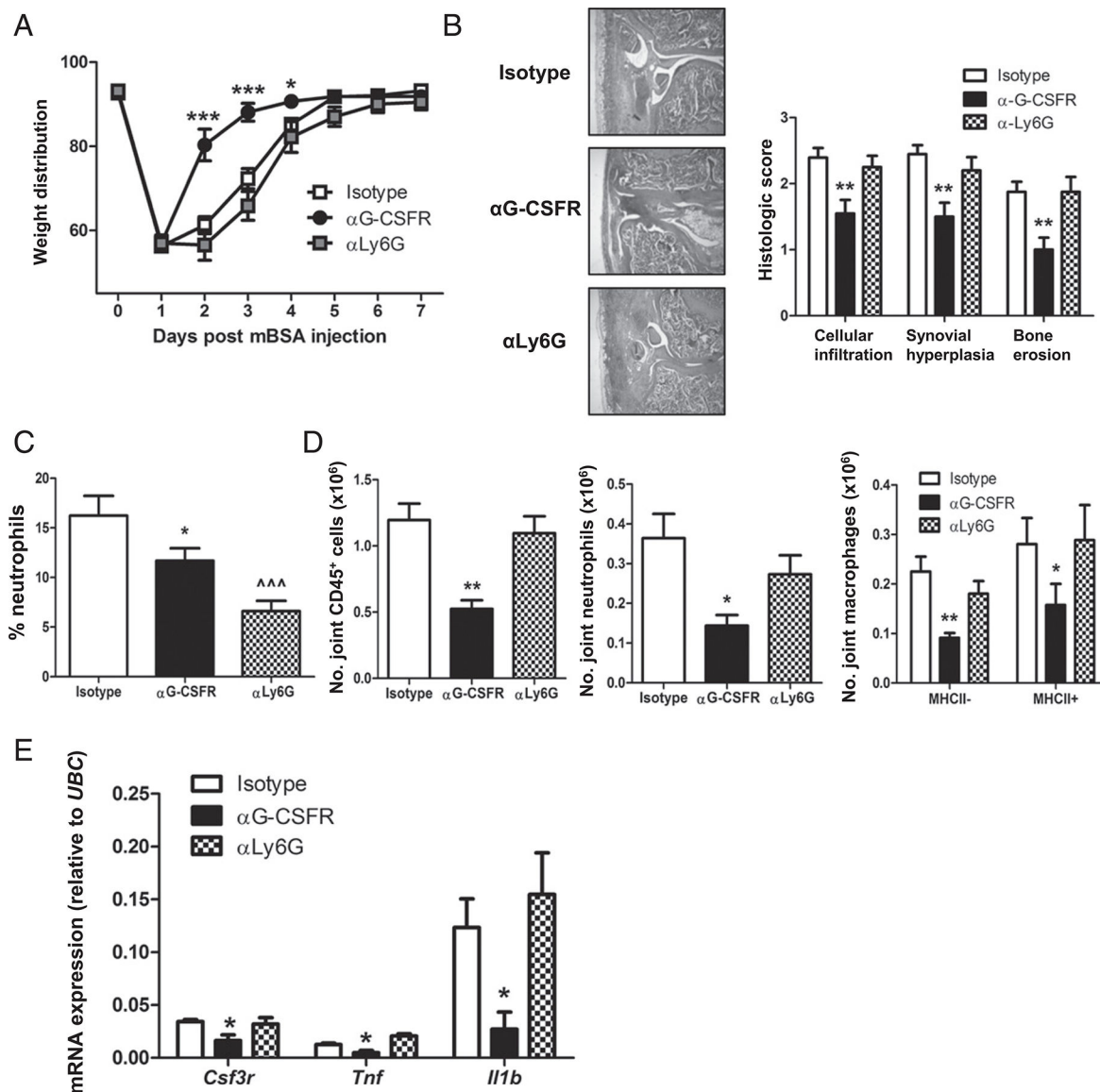
< 0.05, *** p < 0.001, **** p < 0.0001, isotype versus anti-G-CSFR or anti-Ly6G; #### p < 0.0001, day 0 versus day 1 in isotype-treated AIA mice. N.D., not detected.

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**FIGURE 3.**

Therapeutic G-CSFR neutralization ameliorates ZIA pain and disease. C57BL/6 mice received an i.a. injection of zymosan (day 0), with anti-G-CSFR, anti-Ly6G, and isotype control mAbs being administered i.p. on days 1, 3, and 5. (A) Pain (incapacitance meter) and (B) arthritis (histology, day 7) were assessed ($n = 10$ per group) are shown. Histology original magnification $\times 60$. (C) Blood was collected on day 6 and the percentage of blood neutrophils was determined ($n = 10$ per group). (D) Day 7 zymosan-injected joints were collected for cellular analysis, and numbers of infiltrating CD45⁺ leukocytes (neutrophils and MHC-II⁺ macrophages) were measured ($n = 10$ –15 per group). (E) Joint mRNA expression (qPCR) was measured on day 7 ($n = 10$ per group). Results are mean \pm SEM. The p values were obtained using a two-way ANOVA test (A) for pain readings, a Kruskal–Wallis test (B) for histology, and a one-way ANOVA (C–E) for blood cells, joint cells, and

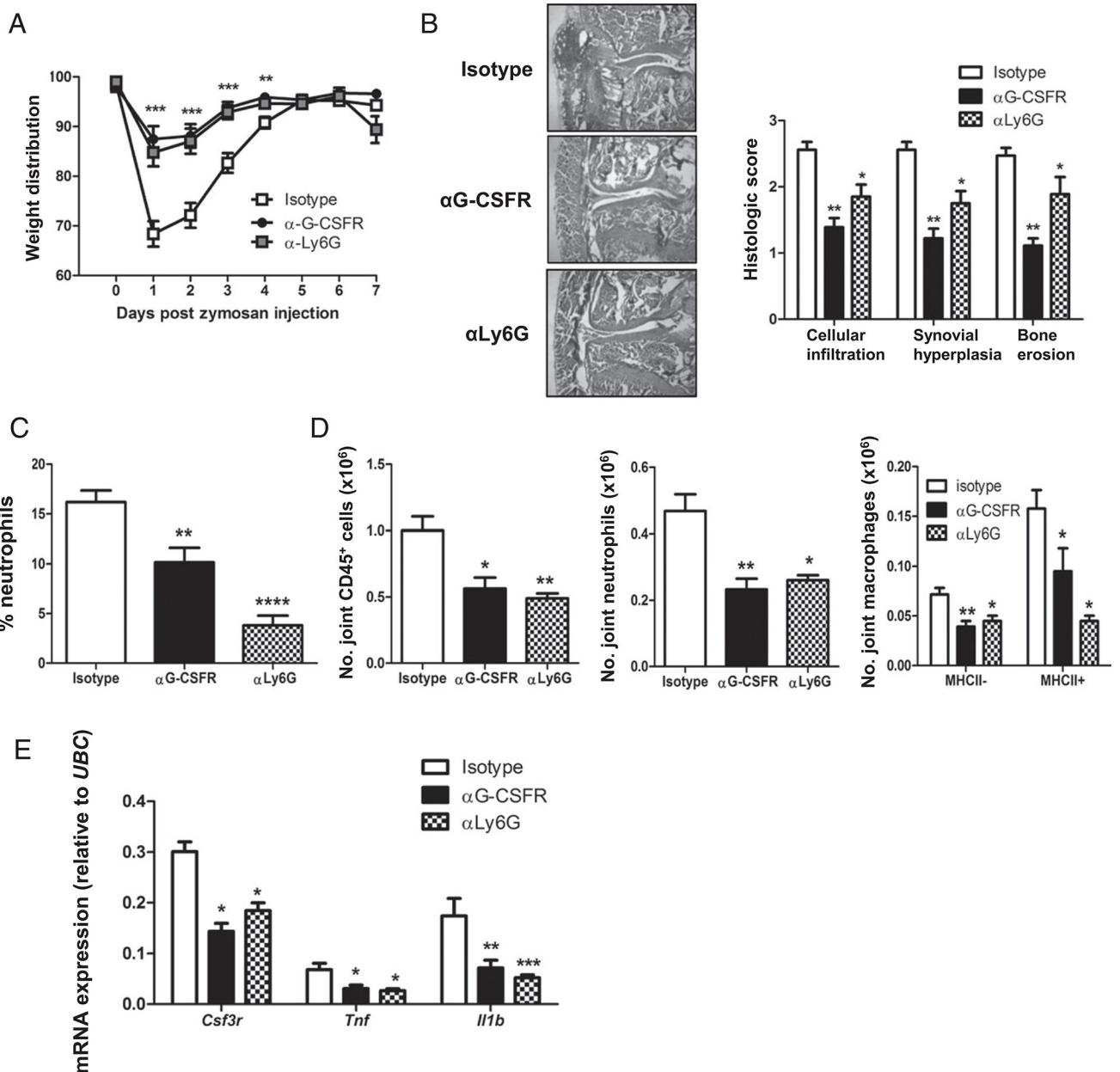
gene expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, isotype versus anti-G-CSFR; ^^ $p < 0.001$, isotype versus anti-Ly6G.

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**FIGURE 4.**

G-CSFR neutralization suppresses the onset of ZIA pain and disease. C57BL/6 mice received an i.a. injection of zymosan (day 0), with anti-G-CSFR, anti-Ly6G, and isotype control mAbs being administered i.p. on days -1, 1, and 4. (A) Pain (incapacitance meter) and (B) arthritis (histology, day 7) were assessed ($n = 10$ per group) are shown. Histology original magnification $\times 60$. (C) Blood was collected on day 5 and the percentage of blood neutrophils was determined ($n = 10$ per group). (D) Day 7 zymosan-injected joints were collected for cellular analysis, and numbers of infiltrating CD45⁺ leukocytes (neutrophils and MHC-II^{-/+} macrophages) measured ($n = 10$ –15 per group). (E) Joint mRNA expression (qPCR) was measured on day 7 ($n = 10$ per group). Results are mean \pm SEM. The p values were obtained using a two-way ANOVA test (A) for pain readings, a Kruskal-Wallis test (B)

for histology, and a one-way ANOVA (C–E) for blood cells, joint cells, and gene expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, isotype versus anti–G-CSFR or anti-Ly6G.

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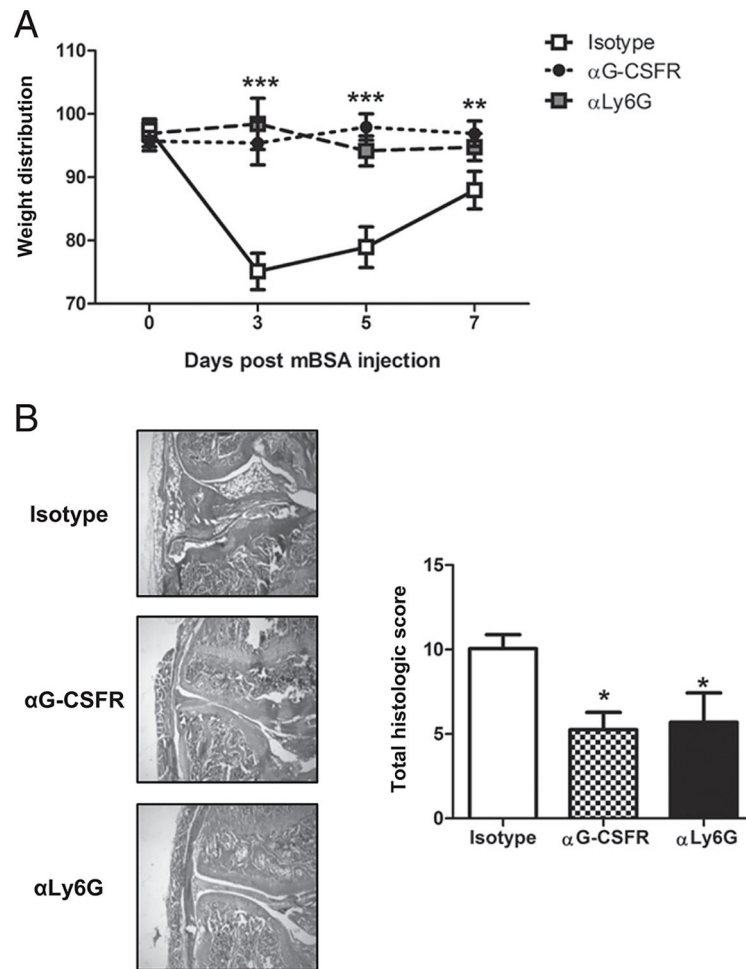


FIGURE 5. mBSA/IL-1 arthritis pain is G-CSF-dependent. mBSA/IL-1 arthritis (mBSA i.a. [day 0] and IL-1 s.c. [days 0–2]) was induced in C57BL/6 mice, with anti-G-CSFR, anti-Ly6G, and isotype control mAbs being injected on days –1, 1, and 4. **(A)** Pain (incapacitance meter) and **(B)** arthritis (histology, day 7) development were evaluated ($n = 10$ per group). Histology original magnification $\times 60$. Results are mean \pm SEM. The p values were obtained using a two-way ANOVA test (A) for pain readings and a Kruskal–Wallis test (B) for histology. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, isotype versus anti-G-CSFR or anti-Ly6G.

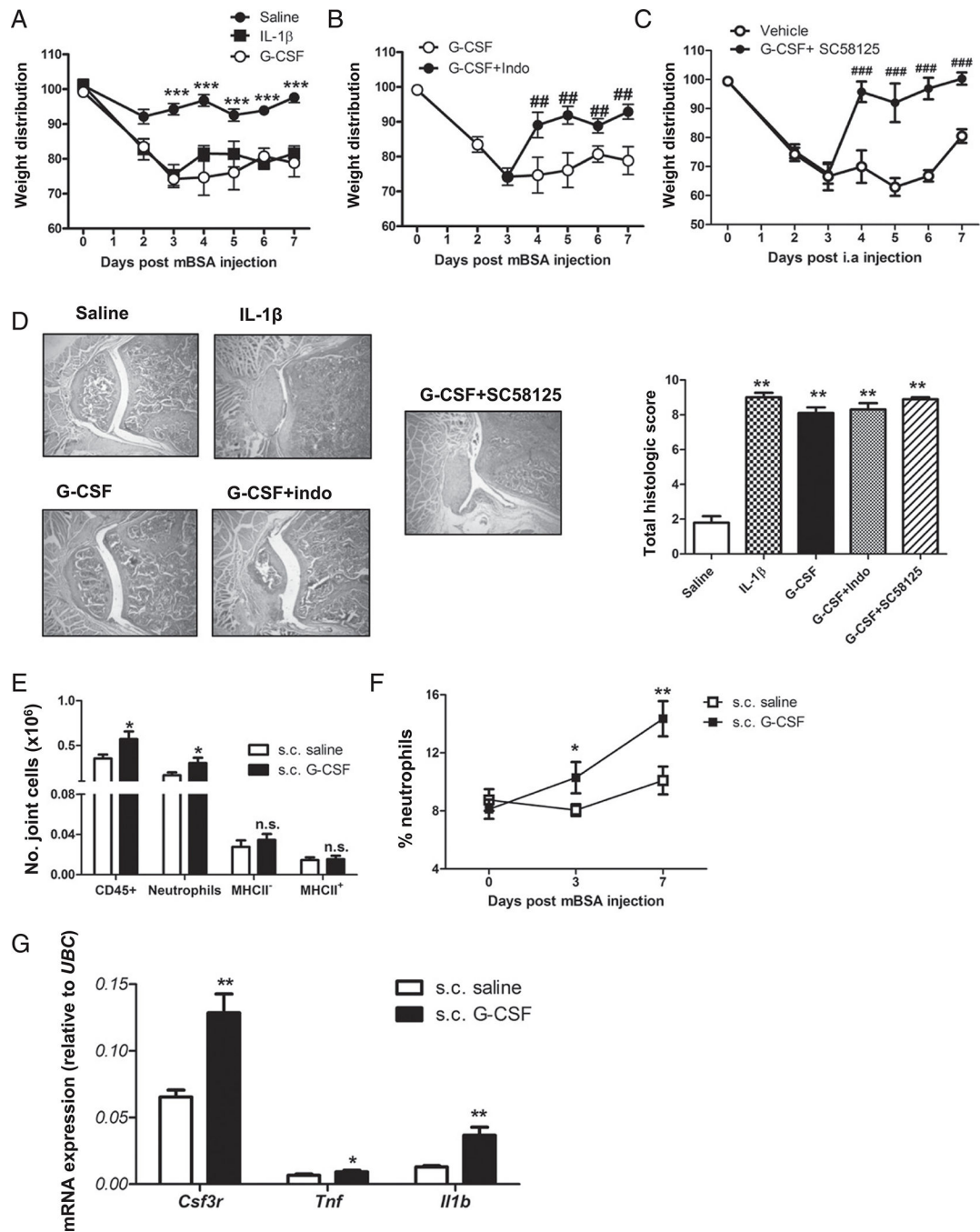


FIGURE 6.

G-CSF drives arthritic pain and disease. (A) mBSA/IL-1 or mBSA/G-CSF arthritis (i.a. mBSA [day 0], s.c. saline, IL-1 [250 ng], or G-CSF [250 ng], respectively [days 0–2]) was induced in C57BL/6 mice. mBSA/G-CSF mice were administered i.p. with (B) indomethacin (1 mg/kg; Indo) or (C) the COX-2 selective inhibitor, SC58128 (5 mg/kg), once G-CSF-driven pain was evident (day 3). (A–C) Pain (incapacitance meter) and (D) arthritis (histology, day 7) were measured ($n = 10$ per group). Histology original magnification $\times 60$. (E) Infiltrating cells (CD45⁺, neutrophils, MHC-II⁻ and MHC-II⁺

macrophages) in mBSA-injected joints were quantified (day 7, $n = 10$ per group). (F) The percentage of blood neutrophils was determined on days 0, 3, and 7 ($n = 10$ per group). (G) mBSA-injected joint gene expression (qPCR) was measured on day 7 ($n = 15$ per group). Results are mean \pm SEM. The p values were obtained using a two-way ANOVA test (A–C) for pain readings, a Kruskal–Wallis test (D) for histology, and an unpaired t test (E–G) for cell counts and gene expression. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, saline versus G-CSF or IL-1 β ; $##p < 0.01$, $###p < 0.001$, G-CSF versus G-CSF plus Indo or G-CSF plus SC58125; $^^p < 0.01$, saline versus G-CSF plus Indo or G-CSF plus SC58125.