G-CSF rescue of FOLFIRINOX-induced neutropenia leads to systemic immune suppression in mice and humans

Victoire Cardot-Ruffino1,2, Naima Bollenrucher,1 Luisa Delius,1 S Jennifer Wang,1,2 Laureen K Brais,3 Joshua Remland,1 C Elizabeth Keheler,4 Keri M Sullivan,3 Thomas A Abrams,3,5 Leah H Biller,3,5 Peter C Enzinger,3,5 Nadine J McCleary,3,5 Anuj K Patel,3,5 Douglas A Rubinson,3,5 Benjamin Schlechter,3,5 Sarah Slater,3,5 Matthew B Yurgelun,3,5 James M Cleary,3,5 Kimberly Perez,3,5 Michael Dougan,4,5 Kimmie Ng,3,5 Brian M Wolpin,3,5 Harshabad Singh,3,5 Stephanie K Dougan1,2

ABSTRACT
Background Recombinant granulocyte colony-stimulating factor (G-CSF) is routinely administered for prophylaxis or treatment of chemotherapy-induced neutropenia. Chronic myelopoiesis and granulopoiesis in patients with cancer has been shown to induce immature monocytes and neutrophils that contribute to both systemic and local immunosuppression in the tumor microenvironment. The effect of recombinant G-CSF (pegfilgrastim or filgrastim) on the production of myeloid-derived suppressive cells is unknown. Here we examined patients with pancreatic cancer, a disease known to induce myeloid-derived suppressor cells (MDSCs), and for which pegfilgrastim is routinely administered concurrently with FOLFIRINOX but not with gemcitabine-based chemotherapy regimens.

Methods Serial blood was collected from patients with pancreatic ductal adenocarcinoma newly starting on FOLFIRINOX or gemcitabine/ nabpaclitaxel combination chemotherapy regimens. Neutrophil and monocyte frequencies were determined by flow cytometry from whole blood and peripheral blood mononuclear cell fractions. Serum cytokines were evaluated pretreatment and on-treatment. Patient serum was used in vitro to differentiate healthy donor monocytes to MDSCs as measured by downregulation of major histocompatibility complex II (HLA-DR) and the ability to suppress T-cell proliferation in vitro. C57BL/6 female mice with pancreatic tumors were treated with FOLFIRINOX with or without recombinant G-CSF to directly assess the role of G-CSF on induction of immunosuppressive neutrophils.

Results Patients receiving FOLFIRINOX with pegfilgrastim had increased serum G-CSF that correlated with an induction of granulocytic MDSCs. This increase was not observed in patients receiving gemcitabine/nabpaclitaxel without pegfilgrastim. Interleukin-1β also significantly increased in serum on FOLFIRINOX treatment. Patient serum could induce MDSCs as determined by in vitro functional assays, and this suppressive effect increased with on-treatment serum. Induction of MDSCs in vitro could be recapitulated by addition of recombinant G-CSF to healthy serum, indicating that G-CSF is sufficient for MDSC differentiation. In mice, neutrophils isolated from spleen of G-CSF-treated mice were significantly more capable of suppressing T-cell proliferation.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ Pegfilgrastim was known to induce neutrophil production from the bone marrow to remedy chemotherapy-induced neutropenia. Neutrophils from patients with cancer include a subset of immature neutrophils capable of suppressing T-cell proliferation that contribute to the immunosuppressive tumor microenvironment.

WHAT THIS STUDY ADDS
⇒ Neutrophils newly produced in response to pegfilgrastim include a larger fraction of immunosuppressive granulocytic myeloid-derived suppressor cells (Gr-MDSCs) than were present at baseline. This study demonstrates induction of MDSCs in humans with pancreatic cancer receiving FOLFIRINOX and pegfilgrastim versus gemcitabine-based regimens that do not require granulocyte colony-stimulating factor (G-CSF) supportive care. Similar results were observed in a mouse model of chemotherapy-induced neutropenia and mechanistically implicate G-CSF in the enhanced degree of immunosuppression.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ We do not advocate withholding pegfilgrastim or filgrastim for chemotherapy-induced neutropenia. For patients where antitumor T-cell responses are expected to contribute to clinical benefit, we recommend considering selecting a chemotherapy regimen that is unlikely to induce neutropenia requiring G-CSF administration. Further clinical trials should investigate this consideration prospectively.

Conclusions Pegfilgrastim use contributes to immune suppression in both humans and mice with pancreatic cancer. These results suggest that use of recombinant G-CSF as supportive care, while critically important for mitigating neutropenia, may complicate efforts to induce antitumor immunity.
The acute inflammatory response involves rapid influx of neutrophils and monocytes into damaged tissue to clear potential pathogenic threats and initiate wound healing. Damaged epithelial cells and activated fibroblasts at the site of injury produce hematopoietic growth factors and chemokines for long range communication with the bone marrow to increase production of myeloid lineage cells. This emergency myelopoiesis is evolutionarily important for protection against ischemic damage and a variety of other acute threats. However, in the context of chronic inflammation, the constant strain on the hematopoietic system to produce neutrophils and monocytes can cause these cells to exit the bone marrow in an immature state. These immature monocytes and neutrophils have been loosely called myeloid-derived suppressor cells (MDSCs), a broad term encompassing a variety of different subtypes and lineages capable of inhibiting adaptive immunity. In the setting of chronic inflammation, the ability to suppress T-cell responses and thus dampen further tissue destruction may be advantageous.

Cancer grows slowly over time and induces chronic inflammation driven by the presence of microbes or factors associated with necrotic cell death such as oxidized DNA, adenosine triphosphate (ATP), or high mobility group box 1 protein (HMGB1) activate innate pattern recognition receptors in immune cells. Both cancer cells and associated fibroblasts can secrete chemokines and growth factors, with granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), C-C motif chemokine ligand 2 (CCL2), and ligands for C-C motif chemokine receptor 2 (CXCR2) being important for production and recruitment of monocyte and neutrophil lineage MDSCs capable of inducing both local and systemic immune suppression. Tumor-associated macrophages can also secrete interleukin (IL)-1β, which further activates MDSCs and correlates with metastasis. In mice, MDSCs are identified as Gr-1+CD11b+ and express a transcriptional program consistent with their ability to sequester metabolites and suppress T-cell responses. Immature myeloid cells have been shown to accumulate in the spleen, which serves as a reservoir for replenishment of MDSCs in the tumor microenvironment. Granulocytic MDSCs (Gr-MDSCs) are derived from immature neutrophils and are recruited into the tumor by C-X-C motif chemokines CXCL1, CXCL2, and CXCL3 acting on CXCR2. Monocytic MDSCs (Mo-MDSCs) express Ly6C and, like monocytic-derived macrophages, are recruited into the tumor by CCL2 acting on C-C motif chemokine receptor 2 (CCR2). In preclinical models, blockade of CCR2, CXCR2 or both leads to decreased accumulation of MDSCs in the tumor and overall improved antitumor immunity.

Human MDSCs have been more difficult to quantify due to lack of consensus as to which surface markers best identify granulocytic and monocytic MDSC lineages. MDSCs are functionally defined by their ability to suppress T-cell responses; however, functional assays are difficult to perform with intrinsically short-lived cells that survive for only a few hours after isolation. Nevertheless, most groups agree that CD15+ cells that survive density gradient centrifugation to appear in the mononuclear cell fraction are Gr-MDSCs. Other surface properties of Gr-MDSCs include CD33+HLA-DR CD16+CXCR2+. Surface low-density lipoprotein receptor LOX-1 or CD84 may further distinguish MDSCs of the granulocytic lineages in humans. Similar to mice, human Gr-MDSCs express CXCR2 and can be recruited to tissues by CXCL1, CXCL2, and IL-8. Mo-MDSCs and healthy monocytes cannot be easily separated based on density. Mo-MDSCs in humans are typically identified as CD14+CD33+HLA-DRlow.

Pancreatic ductal adenocarcinoma (PDAC) induces high rates of MDSC formation in both mice and humans. Pancreatic tumor cells themselves secrete both GM-CSF and CXCL1, which orchestrate an immunosuppressive microenvironment. CXCL1 was identified as the dominant tumor cell-secreted factor responsible for conferring a T cell poor, immunotherapy refractory state in mouse models of pancreatic cancer. Furthermore, cancer-associated fibroblasts in PDAC secrete high levels of G-CSF, GM-CSF and myeloid recruiting chemokines. In mouse models of pancreatic cancer, depletion of Gr-MDSCs or Mo-MDSCs leads to reduced tumor growth. Compensatory increases in one myeloid cell population can offset reductions in another, although CD11b agonism appears to target multiple myeloid lineages simultaneously, reducing myeloid cell accumulation in PDAC mouse models and synergizing with checkpoint blockade. Reprogramming of myeloid cells away from an MDSC phenotype using agonistic anti-CD40 or cIAP1/2 antagonism has also shown success in preclinical models. In patients with PDAC, MDSCs have been identified in both blood and tumor, and their presence correlates with poor prognosis. Cytokines such as IL-6 secreted by malignant cells induce signal transducer and activator of transcription 3 (STAT3) signaling in Mo-MDSCs which confers a suppressive program and can increase the frequency of circulating tumor cells in patients with PDAC as well as contribute to cachexia. Overall, these findings have led to multiple clinical trials for PDAC aimed at reducing MDSCs.

In a phase Ib/II study, the CCR2 small molecule inhibitor PF-04136309 was evaluated in patients with locally advanced PDAC in combination with FOLFIRINOX chemotherapy versus FOLFIRINOX alone. The authors collected bone marrow biopsies and peripheral blood to show a reduction in both circulating monocytes and tumor Mo-MDSCs in patients receiving the CCR2 inhibitor. Monocyte frequencies in the bone marrow increased, suggesting that CCR2 is required for egress from the bone marrow. Tumor burden also decreased, with more than one-third of CCR2 inhibitor-treated patients becoming eligible for surgery versus none in the FOLFIRINOX control arm. Unfortunately, a study of CCR2 inhibitor with gemcitabine/ab/paclitaxel in patients with advanced PDAC failed to meet its endpoints for safety or efficacy. Why CCR2 inhibitor performed so well in locally advanced disease but not in the metastatic setting...
is unclear. One possibility is that inhibition of monocyte trafficking cannot be sustained long-term due to risk of infection and that these agents may be best used as either a short-term bridge to surgery or in conjunction with therapies that stimulate antitumor T cells. Another possible explanation could involve differences between the chemotherapy backbones used for these two trials. The effects of FOLFIRINOX versus gemcitabine/n(ab) paclitaxel chemotherapy and associated supportive care regimens on hematopoietic cell survival and MDSC differentiation have not been reported.

Current treatments for both localized and advanced PDAC include combination chemotherapy regimens. FOLFIRINOX extends median overall survival in patients with advanced PDAC compared with gemcitabine (11.1 vs 6.8 months). Addition of albumin-conjugated paclitaxel (n(ab)paclitaxel, brand name Abraxane) to gemcitabine extends median overall survival by 1.8 months compared with gemcitabine alone (8.5 vs 6.7 months). FOLFIRINOX and gemcitabine/n(ab)paclitaxel have not been compared head-to-head in a randomized trial for advanced PDAC, and an ongoing trial is addressing this question in the context of identifying molecular predictors favoring one regimen over the other (NCT04469556). In the neoadjuvant setting, a parallel phase 2 trial using FOLFIRINOX or gemcitabine/n(ab)paclitaxel showed similar median overall survival post surgery. FOLFIRINOX has a slightly less favorable safety profile with overall higher rates of grade 3/4 toxicities including fatigue, diarrhea and need for growth factor use compared with gemcitabine/n(ab)paclitaxel. Nevertheless, both chemotherapy regimens are used in standard practice.

Clinical trials for PDAC typically include a chemotherapy backbone combined with a novel agent. Given the success of immunotherapy in other cancer types, many immunotherapy agents are being tested in PDAC, with thus far limited success outside of the 1% of PDAC tumors that are microsatellite stable and can be treated with checkpoint inhibitor therapy. Which chemotherapy backbone would best combine with immunotherapy agents has been a source of much debate. FOLFIRINOX has a perceived slightly higher efficacy, potentially due to responsiveness of the approximately 5% of patients with breast cancer type 1/2 susceptibility protein (BRCA1/2) mutations to platinum-based agents, but the side effect profile limits clinical enrollment and has resulted in gemcitabine/n(ab)paclitaxel being more commonly combined with immunotherapy in clinical trials. To date, there is not a strong scientific rationale for which chemotherapy regimen would best synergize with antitumor immunity in humans. FOLFIRINOX has been reported to increase effector T-cell responsiveness in peripheral blood, although it should be noted that T-cell restimulation assays were performed from frozen peripheral blood mononuclear cells (PBMCs), a condition that does not support survival of Gr-MDSCs during the freeze-thaw process. Resected tumors treated with FOLFIRINOX show a marked influx of myeloid cells into the tumor center, although whether these myeloid cells are contributing to vs a consequence of tumor cell death is unclear.

Here we evaluated patients with PDAC newly starting on FOLFIRINOX or gemcitabine/n(ab)paclitaxel. We found that FOLFIRINOX, but not gemcitabine/n(ab) paclitaxel, was associated with an increase in Gr-MDSCs as determined by flow cytometry and functional assessment of patient serum. This increase in Gr-MDSCs was caused by pegfilgrastim administered as supportive care for FOLFIRINOX-induced neutropenia. We showed that G-CSF added to healthy serum was sufficient to recapitulate the immunosuppressive effects of post-FOLFIRINOX patient serum. We further developed a mouse model of pegfilgrastim rescue of neutropenia to formally demonstrate that neutrophil-lineage cells arising from G-CSF treatment were more immunosuppressive than similarly isolated cells from cancer-bearing mice alone.

**METHODS**

**Ethics approval**

All animal protocols were approved by the Dana-Farber Cancer Institute Committee on Animal Care (protocol #14–019, 14–037) and are in compliance with the National Institutes of Health and National Cancer Institute ethical guidelines for tumor-bearing animals.

**Human samples**

Whole blood and serum were collected from patients with PDAC receiving FOLFIRINOX or gemcitabine/n(ab)-paclitaxel at Dana-Farber Cancer Institute under protocol #03–189 (see table 1). Blood and serum were used for immune cells and cytokine analysis.

Healthy donor serum was obtained from consenting patients at Massachusetts General Hospital under protocol #21–590. Participants were women between 30 and 75 years of age (inclusive) and had no symptoms of acute illness, no history of immune-mediated disease, no use of immunomodulating medications within 1 month prior to enrollment, received no vaccines within 5 weeks prior to enrollment, and had never received an organ transplant. Blood was collected at two study visits based 4 weeks apart. During each visit, physical well-being and eligibility were verified by the study team and a detailed medical history (including relevant comorbidities, current and recent medications, and vaccination history) was reviewed. Serum from healthy donors were used for cytokine analysis controls.

Healthy donor monocytes and T cells were obtained from de-identified leukapheresis cones from the Kraft Blood Donor Center.

**Human blood processing**

Whole blood was processed within 24 hours of collection. From the fresh whole blood, 200 µl were used for flow cytometry staining. The remaining blood was used for PBMC isolation by gradient centrifugation using
PBMCs were washed once in phosphate-buffered saline, 2 mMol/l l-glutamine, 1% penicillin/streptomycin, 1% minimal essential media nonessential amino acids, 1 mmol/l sodium pyruvate, and 0.1 mmol/l 2-mercaptoethanol with DNase I (Sigma-Aldrich). PBMCs were washed once in phosphate-buffered saline (PBS) with ethylenediaminetetraacetic acid (EDTA) 2 mM and stained with Zombie NIR fixable viability kit (BioLegend) prior to staining for flow cytometry. Stained cells were fixed with 1% formalin (Sigma) and analyzed with an SP6800 Spectral Cell Analyzer (SONY).

The following antibodies were used for human cells. From BioLegend: aCD3-PE clone OKT3, aCD19-PE clone HIB19, aCD33-PE-Cy7 clone WM53, aCD15-PB clone MMA, aCD14- FITC clone M5E2, aCD16- BV785 clone 3G8, aHLA-DR-BV570 clone L243, aPD1-PE clone EH12.2H7, aCD137-PE-Cy7 clone 4B4-1, aCD8-PE clone HIT8a, CX3CR1-BV421 clone 2A9-1, ACCR3-PE clone 5E8, aCD14-BV605 clone M5E2, aCD4-PB clone OKT4, aCD25-BV421 clone BC96, CCR2-BV510 clone K036C2, CXCR2-PerCP-Cy5.5 clone 5E8, aCD33-FITC clone P67.6. From BD Biosciences: aCD3-PE-CF594 clone UCHT1, aCD45-PE-CF594 clone H130, aCD4-BV510 clone SK3.

**Human CD14+ MDSC differentiation**

Monocytes were isolated from healthy donor leukopaks using a human CD14 isolation kit (Miltenyi) as per the manufacturer’s protocol. Monocytes were resuspended in RPMI complete without FBS and plated into a thermosensitive 6-well plate (Thermo Scientific 174901). 20 µl of human serum (either from healthy donors or patients) was added for a final concentration of 20% serum. 75 pg/mL of M-CSF (Peprotech) was added to each condition. Some monocytes were also cultured with 10 ng/mL of human IL-6 (Peprotech), 10 ng/mL GM-CSF (Peprotech), G-CSF (Miltenyi), IL-20 (Peprotech), or IL-33 (Peprotech). On days 3–5, the medium was changed, 400 µl of fresh RPMI complete without FBS was added and replenishment of serum and/or recombinant cytokines as indicated. On day 7, the cells were harvested using cold PBS aided by a cell scraper.

**Human T-cell proliferation assay**

PBMCs were isolated from healthy donor fresh blood using Ficol-Paque PLUS (Cytiva). The PBMCs were then washed, and T cells isolated using the human Pan T-cell isolation kit (Miltenyi) as per the manufacturer’s protocol. After isolation, the T cells were stained with CFSE (Invitrogen), washed in FBS and then cultured in RPMI complete with CD3/CD28 activation beads (Gibco) in a U-bottom 96-well plate. 3 hours later, 50,000 MDSCs were added to each well containing pre-activated T cells. After 3 days of incubation at 37°C, the cells were analyzed by flow cytometry.

**Human serum cytokine analysis**

On the day of collection, patient serum was centrifuged at 450 g for 15 min, aliquoted and stored at −80°C. Then cytokines were analyzed by cytokine bead array (Eve Technologies, Canada).

**Mouse tumor graft and treatment**

Female C57BL/6j mice aged 6–8 weeks were purchased from Jackson Labs (stock #000664) and used for experiments after acclimatizing for at least 1 week to being housed at the Dana-Farber Cancer Institute Redstone Facility. Tumor inoculations were performed as described. Briefly, female C57BL/6 mice were subcutaneously injected with
250,000 6694c2 cells. Tumor size was measured twice weekly, and tumor volume calculated. Mice were treated with FOLFIRINOX (5-fluorouracil 60 mg/kg, leucovorin 75 mg/kg, irinotecan 50 mg/kg and oxaliplatin 5 mg/kg) or PBS with or without G-CSF (5 µg/mouse, Neupogen) resuspended in PBS. Five groups of n=5 mice per group were used for a total of 25 mice per experiment. Group 1=no tumor, no treatment; Group 2=tumor, PBS; Group 3=tumor, FOLFIRINOX; Group 4=tumor, G-CSF; Group 5=tumor, FOLFIRINOX+G CSF. All values were compared with those obtained for the control group of tumor-bearing mice treated with PBS. Mice were euthanized on day 12 post implantation. Tumors were weighed at the time of euthanasia. Investigators were not blinded. Humane endpoints include body condition score of 2 or less, 20% or more weight loss, tumor ulceration, tumor size >2000 mm3, or other signs of morbidity. No mice in the study were euthanized for humane endpoints or excluded.

Mouse flow cytometry
Blood was collected by retro-orbital bleeding and erythrocytes lysed with red cell lysis buffer (8.26 g NH4Cl, 1 g KHCO3, 37 mg EDTA, 1 L water). Spleens were crushed through a 40 micron cell strainer, washed and lysed with ACK buffer. Bone marrow was isolated from femurs by flushing with PBS using a 27-gage syringe. Cells were resuspended in flow cytometry buffer (2% FBS in PBS) and stained with Zombie NIR viability dye and other flow cytometry antibodies prior to fixation with 1% formalin. Samples were analyzed using an LSR Fortessa X-20 (BD).

The following antibodies were used from BioLegend: Ly6G-PE-Cy7 clone 1A8, Ly6C-FITC clone HK1.4, CD45-PE-Dazzle clone 30-F11, Siglec-F-BV421 clone S17007L, CD11b-APC clone M1/70, CD4-BV421 clone GK1.5, CD8a-APC clone 53–6.7.

Mouse MDSC suppression assay
T cells were isolated from pooled spleen and lymph nodes of C57BL/6J mice using Mouse T Cell Isolation Kit (Invitrogen). T cells were washed twice with PBS, counted, and labeled with CFSE (Life Technologies) as per the manufacturer’s protocol. 50,000 CFSE-labeled T cells were plated into a U-bottom 96 well plate with CD3/CD28 activation beads (Life Technologies). The plate was placed in a 37°C incubator while preparing the MDSCs. Spleen cells from two to three tumor-bearing mice per group were pooled at 12 days after inoculation. Neutrophil isolation was performed using the Mouse Neutrophil Enrichment Kit (Invitrogen). Isolated cells were washed with RPMI complete, counted, and 25,000 cells added to the stimulated T cells. MDSCs and T cells were cocultured for 3 days prior to analysis by flow cytometry.

Tumor immunofluorescence staining
Subcutaneous tumors were fixed with Z-fix (Fisher Scientific) and frozen in OCT (Fisher Scientific). Samples were cut into 8 µm sections using a cryostat (Leica) and analyzed by immunofluorescence using antibodies against Arginase 1 (Life Technologies) and Gr-1 (clone RB6-8C5, BioLegend). Sections were imaged using Leica Thunder Imager Live cell and 3D Assay microscope.

Statistics
Pairwise, group comparisons and correlation were performed using Wilcoxon matched-pairs signed-rank test, one-way analysis of variance test, and Pearson coefficient calculation. GraphPad Prism software was used for statistical analysis.

Data accessibility
All data are presented in the manuscript. Any additional data may be obtained from the investigators on request.

RESULTS

FOLFIRINOX is associated with an increase in circulating Gr-MSDCs
Peripheral blood was collected from patients with PDAC newly starting either FOLFIRINOX or gemcitabine/n(ab) paclitaxel chemotherapy regimens (figure 1A and table 1). Whole blood was analyzed by flow cytometry to quantify neutrophils and monocytes according to the gating strategy in online supplemental file 2. No significant changes in neutrophils as a percentage of CD45+ cells were observed for either chemotherapy regimen (figure 1B). FOLFIRINOX treatment induced an early decrease in monocytes, although this population recovered in subsequent cycles (figure 1C). Absolute neutrophil counts increased significantly in patients receiving FOLFIRINOX, as expected from concurrent administration of pegfilgrastim in this population (figure 1D). To quantify MDSCs in whole blood, we analyzed CD15+CD16− immature neutrophils and found that this population increased during treatment with FOLFIRINOX but not with gemcitabine/n(ab)paclitaxel (online supplemental file 2). No significant changes were observed in any of the major lymphocyte populations (online supplemental file 2).

PBMC were isolated by density gradient centrifugation, a process that retains lymphocytes, monocytes, Mo-MSDCs and Gr-MSDCs but not healthy granulocytes. From the PBMC fraction, we defined Mo-MSDCs as CD45+CD33+CD15+HLA-DRlow/neg and Gr-MSDCs as CD45+CD33+CD15+ (figure 1E and online supplemental file 2). We observed a significant increase in the percentage of Gr-MSDCs on treatment with FOLFIRINOX but not with gemcitabine/n(ab)paclitaxel (figure 1F). This difference was also apparent when comparing absolute abundance of Gr-MSDCs (figure 1G). Mo-MSDCs were less abundant overall, and their frequency as a percentage of total CD45+ cells or total monocytes was not significantly affected by either chemotherapy regimen (figure 1H,I). However, absolute abundance of Mo-MSDCs was significantly increased in patients after receiving FOLFIRINOX, consistent with higher overall white blood cell counts (figure 1J).

Figure 1  Patients receiving FOLFIRINOX show an increase in circulating Gr-MDSCs. (A) Timing of treatments received by the FOLFIRINOX group and the gemcitabine/n(ab)paclitaxel group of patients with metastatic pancreatic cancer. (B,C) Whole blood collected at cycle 1 day 1 (C1D1) of treatment and either cycle 2 day 1 or cycle 3 day 1 (C2/3D1) of FOLFIRINOX treatment or C1D15 of gemcitabine/n(ab)paclitaxel treatment was stained with the antibodies indicated in online supplemental file 2 and analyzed by flow cytometry. N=9 FOLFIRINOX; N=7 gem/n(ab)paclitaxel. Percentage of CD15+ neutrophils (B) and CD14+ monocytes (C) out of total CD45+ immune cells. (D) Absolute neutrophil counts (ANC) values were obtained from patient records corresponding to the same time points analyzed in B and C. Patients were included even if no corresponding flow cytometry was performed on whole blood. N=18 FOLFIRINOX; N=8 gem/n(ab)paclitaxel. (E) Representative flow cytometry plot showing the Gr-MDSCs gating strategy. (F–J) Peripheral blood mononuclear cells were collected by density gradient centrifugation using Ficoll and analyzed by flow cytometry according to the gating scheme shown in online supplemental file 2. (F) Percentage of Gr-MDSCs (CD45+CD33+ CD15+) N=19 FOLFIRINOX; N=8 gem/n(ab)paclitaxel. (G) Absolute counts of Gr-MDSCs from PBMC fraction were calculated from patient samples where whole blood neutrophil frequencies and ANC values were known. N=9 FOLFIRINOX; N=6 gem/n(ab)paclitaxel. (H) Mo-MDSCs (CD45+CD33+ CD15 CD14+) out of total CD45+ immune cells. N=19 FOLFIRINOX; N=8 gem/n(ab)paclitaxel. (I) Mo-MDSCs out of total CD14+ cells. N=19 FOLFIRINOX; N=8 gem/n(ab)paclitaxel. (J) Absolute counts of Gr-MDSCs from PBMC fraction were calculated from patient samples where whole blood neutrophil frequencies and ANC values were known. N=9 FOLFIRINOX; N=6 gem/n(ab)paclitaxel. Wilcoxon matched-pairs signed-rank test was used throughout. Gr-MDSCs, granulocytic MDSCs; MDSCs, myeloid-derived suppressor cells; Mo-MDSCs, monocytic MDSCs; PBMC, peripheral blood mononuclear cell.
FOLFIRINOX-treated patient serum contains increased G-CSF
To determine whether systemic growth factors and chemokines associated with emergency myelopoiesis were affected by chemotherapy treatment, we measured a panel of cytokines and chemokines from patient serum by cytokine bead array. For this panel, we also included serum from five healthy age-matched people who donated two blood samples 28 days apart. The mean of healthy controls is indicated by a dashed line in each panel. Not surprisingly, all patients treated with FOLFIRINOX showed a marked increase in serum G-CSF, consistent with administration of pegfilgrastim (recombinant pegylated G-CSF) on day 3 of each FOLFIRINOX cycle as prophylaxis for chemotherapy-induced neutropenia (p=0.002) (figure 2A). After treatment, G-CSF levels were positively correlated (p=0.0309) with the percentage of Gr-MDSCs (figure 2B). Given that total neutrophil frequencies were not significantly altered in patients receiving FOLFIRINOX (figure 1B) and that rescue of neutrophil production was successfully achieved by pegfilgrastim treatment (figure 1D), we hypothesize that a higher fraction of these newly-produced neutrophils were Gr-MDSCs.

We examined other circulating markers of inflammation and noted a significant increase in the IL-1 family member IL-18 (p<0.0001) on treatment with FOLFIRINOX but not gemcitabine/nab-paclitaxel (figure 2C). The innate inflammatory cytokines IL-1β, IL-6 and tumor necrosis factor-α were elevated in all patients with PDAC above levels found in similarly aged healthy controls but were not significantly affected by either chemotherapy regimen (figure 2D–F). The IL-1R antagonist (IL-1RA) was significantly increased in the FOLFIRINOX cohort (figure 2G). Gr-MDSC and neutrophil recruitment into tissues is mainly driven by CXCR2 binding CXCL1/2/5 or IL-8. The chemokines CXCL1 and CXCL5 were significantly decreased in the circulation of FOLFIRINOX-treated patients and reached levels similar to that of healthy controls whereas IL-8 was not significantly affected by either chemotherapy regimen (figure 2H). Of the 71 cytokines and chemokines analyzed, 3 other factors were significantly increased by FOLFIRINOX treatment (online supplemental table 1). These were FLT3 ligand, TRAIL, and CTACK (CCL27), all of which increased on FOLFIRINOX administration although concentrations remained within the normal range of healthy control blood donors and were not investigated further (online supplemental file 2). CCL8, a monocyte recruiting chemokine and PDGFα/bβ were significantly decreased with FOLFIRINOX treatment but remained within the range of healthy control values (online supplemental file 2).

FOLFIRINOX-treated patient serum increases suppressive capacity of Mo-MDSCs in vitro
Mo-MDSCs can be induced in vitro by differentiating healthy donor monocytes with recombinant GM-CSF and IL-6 (figure 3A). These induced Mo-MDSCs (iMDSCs) adopt a large spreading shape that is morphologically distinct from control monocytes cultured for a week with healthy serum (figure 3B). To develop a functional assay to measure systemic immune suppression in patients with PDAC, we adapted a method previously used to test systemic immune suppression in patients with sepsis or COVID-19. On coculture at a ratio of 1:1 with CFSE-labeled T cells activated with anti-CD3/CD28, iMDSCs suppress T-cell proliferation whereas monocytes differentiated with healthy serum and M-CSF are not suppressive (figure 3C). Healthy donor serum with maintenance levels of M-CSF (75 pg/mL) does not induce Mo-MDSCs due to low concentrations of growth factors. However, in patients with acute or chronic inflammation, high levels of myeloid-differentiation factors in serum can induce formation of Mo-MDSCs in vitro (figure 3C). To test whether serum from FOLFIRINOX-treated patients could induce the differentiation of Mo-MDSCs in vitro, we isolated CD14+ cells from a healthy donor and cultured them for 1 week in medium containing maintenance levels of M-CSF with 20% patient serum from pretreatment or post-treatment time points and performed a T-cell suppression assay. Post-treatment serum induced significantly more suppressive Mo-MDSCs than baseline serum, suggesting that treatment induced changes in serum factors capable of increasing the suppressive capacity of Mo-MDSCs (figure 3C,D).

G-CSF is sufficient to induce human Mo-MDSCs in vitro
Neither GM-CSF nor IL-6, the two recombinant cytokines used as positive controls in our Mo-MDSC differentiation assay, were affected by FOLFIRINOX treatment (figure 2E and online supplemental table 1). GM-CSF was undetectable across most of the patients in our cohort. We therefore hypothesized that other serum growth factors, such as G-CSF, could be responsible for the increased differentiation capacity of Mo-MDSCs observed in serum from FOLFIRINOX-treated patients. Human monocytes express the G-CSF receptor. To test for a direct effect of G-CSF on Mo-MDSC formation, we cultured magnetic bead-purified CD14+ cells obtained from healthy donor PBMCs with 200 pg/mL of G-CSF, which is similar to G-CSF concentrations measured in post-FOLFIRINOX patient serum. After 7 days of differentiation, we cocultured our putative Mo-MDSCs with CFSE-labeled T cells and measured proliferation on activation with anti-CD3/CD28. G-CSF alone was sufficient to induce Mo-MDSCs that suppressed T-cell proliferation similarly to the positive control of GM-CSF/IL-6 (figure 4A). This confirms the ability of G-CSF to directly induce Mo-MDSCs formation in vitro. We further analyzed these induced Mo-MDSCs by flow cytometry and found that in vitro differentiated Mo-MDSCs expressed CD33 and lower levels of HLA-DR compared with monocytes cultured with healthy serum (figure 4B). G-CSF reduced HLA-DR expression in vitro differentiated Mo-MDSCs in a dose-dependent fashion (figure 4C), again suggesting that G-CSF is sufficient to induce Mo-MDSCs.
Figure 2  G-CSF and IL-18 are increased in patients on FOLFIRINOX treatment. Serum from patients receiving FOLFIRINOX, gemcitabine/nab-paclitaxel, or healthy donor serum were analyzed for the indicated cytokine and chemokines by cytokine bead array. Samples were collected at cycle 1 day 1 (C1D1) of treatment and either cycle 2 day 1 or cycle 3 day 1 (C2/3D1) of FOLFIRINOX treatment or C1D15 of gemcitabine/nab-paclitaxel treatment. (A) Circulating levels of G-CSF. N=17 FOLFIRINOX and N=6 gemcitabine/nab-paclitaxel. (B) Correlation between serum G-CSF from FOLFIRINOX-treated patients and the percentage of Gr-MDSCs at C2D1 or C3D1. N=12 patients had paired data for analysis. Pearson’s correlation was used for statistical analysis. (C–J) Circulating levels of IL-18, IL1β, IL-6, TNFα, IL-1RA, IL-8, CXCL1 and CXCL5. Mean values of the healthy control samples are indicated with a dashed line. N=17 FOLFIRINOX and N=6 gemcitabine/nab-paclitaxel. Wilcoxon matched-pairs signed-rank test was used throughout. CXCL, C-X-C motif chemokine; G-CSF, granulocyte colony-stimulating factor; Gr-MDSCs, granulocytic MDSCs; IL, interleukin; IL-1RA, IL-1R antagonist; TNF, tumor necrosis factor.
G-CSF rescue of chemotherapy-induced neutropenia in mice induces Gr-MDSCs that are more suppressive than ones induced by the cancer alone

Although we demonstrated that FOLFIRINOX-treated patient serum or recombinant G-CSF are sufficient to induce Mo-MDSCs in vitro, we could not formally demonstrate that pegfilgrastim is responsible for the increase in Gr-MDSCs in vivo as all patients receiving pegfilgrastim also received FOLFIRINOX. We therefore developed a mouse model of chemotherapy-induced neutropenia and G-CSF rescue. Immune-competent C57BL/6 mice were inoculated subcutaneously with the poorly immunogenic pancreatic cancer cell line 6694c2, originally derived from a spontaneous tumor from a mouse expressing oncogenic KrasG12D and monoallelic loss of p53 in pancreatic acinar cells.20 Human G-CSF binds to murine G-CSF receptor; therefore, we treated mice with clinical-grade pegfilgrastim (Neulasta) and/or FOLFIRINOX according to the schedule shown (figure 5A). Mice were euthanized on day 12, at which time point tumor size was not significantly different among the treatment groups (figure 5B). Examination of the bone marrow showed an increase in production of CD11b+ cells in tumor-bearing mice compared with mice with no tumors (figure 5C). This increase was further augmented by G-CSF administration, indicating that our model recapitulated enhanced myelopoiesis observed in the setting of cancer and growth factor supplementation. Similar to our finding in patients with PDAC receiving FOLFIRINOX, frequencies of Mo-MDSCs in blood (defined in mice as CD11b+Ly6C+) were not affected by any of the treatments (figure 5D,E and online supplemental file 2). However, circulating neutrophils, defined as CD11b+Ly6G+, were dramatically affected (figure 5D,E). G-CSF treated mice showed a striking increase in peripheral blood neutrophils. Conversely, neutrophils were nearly absent in blood from mice receiving FOLFIRINOX alone but were restored to normal levels by combination treatment of FOLFIRINOX.
The mean fluorescence intensity of Ly6G also shifted with treatment, consistent with rapidly formed neutrophils expressing lower levels of this neutrophil differentiation marker (Figure 5D). FOLFIRINOX treatment reduced eosinophil and neutrophil populations significantly in blood and spleen as previously reported (Figure 5E and online supplemental file 2). Neutrophils, but not eosinophils, were restored with G-CSF treatment, similar to the effects of G-CSF in humans. To determine whether G-CSF-induced neutrophils were qualitatively different from those present in tumor-bearing mice, we isolated CD11b+ cells from the spleens of mice from each treatment group and examined their immunosuppressive potential ex vivo by coculturing CD11b+ cells with CFSE-labeled T cells and a 1:1 ratio with anti-CD3/CD8 stimulation (Figure 5F). Neutrophils from mice treated with G-CSF were more capable of suppressing T-cell proliferation than neutrophils from tumor-bearing mice or mice treated with FOLFIRINOX alone (Figure 5F). Thus, G-CSF administration in mice is sufficient to induce an increase in systemic Gr-MDSCs with immunosuppressive capacity. To determine whether the systemic increase in Gr-MDSCs

---

**Figure 4** Recombinant G-CSF is sufficient to induce suppressive, MHC class II-low Mo-MDSCs in vitro. (A) Monocytes were obtained from healthy donor blood using positive selection on CD14+ magnetic beads. Monocyte were then cultured in vitro with 75 pg/mL M-CSF and the indicated concentrations of G-CSF or GM-CSF/IL-6 for 7 days. Induced Mo-MDSCs were then cultured with CFSE-labeled T cells for 3 days coculture at a 1:1 ratio, and T-cell proliferation was assessed by flow cytometry. Representative of three independent experiments. (B) Representative flow cytometry plots showing the gating strategy for MDSCs-induced in vitro. MHC-II (HLA-DR) expression on induced MDSCs is typically low. (C) Flow plot and quantification of HLA-DR mean fluorescence intensity of CD33+ SSCA high cells after monocytes were cultured for days with 75 pg/mL M-CSF with the addition of G-CSF (200 pg/mL or 10 ng/mL) or 10 ng/mL each of GM-CSF and IL-6. Representative of four independent experiments. GM-CSF, granulocyte macrophage CSF; IL, interleukin; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; Mo-MDSCs, monocytic MDSCs.
**Figure 5**  G-CSF-induced neutrophils in mice are more immunosuppressive than neutrophils induced by the cancer alone. (A) Diagram of mouse inoculation with 6694C2 pancreatic tumor cells different treatments received. FOLFIRINOX was dosed intravenously (oxaliplatin: 5 mg/kg, 5-FU: 15 mg/kg, irinotecan: 50 mg/kg, leucovorin: 75 mg/kg) and G-CSF was dosed intraperitoneally (clinical grade Neulasta, 20 µg/mouse per day). (B) Tumor weight on day 12 did not significantly differ among the treatment groups. n=4–5 mice per group. Representative of three independent experiments. (C) Bone marrow was harvested from mice treated as in A, as well as non-tumor bearing mice. Single cell suspensions were stained for CD11b and analyzed by flow cytometry. n=4–5 mice per group. Representative of three independent experiments. (D) Representative flow plots of Ly6G and Ly6C expression gated on CD11b+ live cells from blood of mice treated with PBS, G-CSF, FOLFIRINOX or FOLFIRINOX+G-CSF. Representative of three independent experiments. (E) Bar graph representing the percent CD11b+ cells out of total CD45+ cells and the percentage of eosinophils, Mo-MDSCs and neutrophils out of CD11b+ cells in blood from non-tumor bearing mice (teal bars) or tumor bearing mice treated with PBS, G-CSF, FOLFIRINOX or FOLFIRINOX+G-CSF. n=3–5 mice per group. Representative of three independent experiments. (F) Neutrophils were enriched using a negative magnetic bead enrichment strategy for isolating live neutrophils from spleens of naïve mice or tumor-bearing mice from each treatment group (PBS, G-CSF, FOLFIRINOX or FOLFIRINOX+G-CSF). T cells from healthy mice were labeled with CFSE, stimulated with anti-CD3/CD28 beads and cocultured with freshly isolated CD11b+ cells at a 1:1 ratio. CD8 T-cell proliferation was measured by flow cytometry for CFSE dye-dilution 72 hours later and proliferation indexes were calculated. n=2 pooled samples from two to three mice each. Representative of two independent experiments. (G) Immunofluorescence images and quantification of Gr-MDSCs (Arg1+Gr1+) in frozen tumor slices from PBS, G-CSF, FOLFIRINOX or FOLFIRINOX+G-CSF treated mice. Representative of n=3–5 mice per group and one experimental replicate. One-way analysis of variance with multiple hypothesis testing was used for statistical analysis. Arg1, arginase; GM-CSF, granulocyte macrophage colony-stimulating factor; Gr-MDSCs, granulocytic myeloid-derived suppressor cells; Mo-MDSCs, monocytic MDSCs; PBS, phosphate-buffered saline.
resulted in more Gr-MDSCs in the tumor, we performed immunofluorescence staining for the neutrophil marker Gr-1 and arginase (Arg1) which is highly expressed in both Gr-MDSCs and Mo-MDSCs. We observed a significant increase in Gr-MDSCs (Gr1+Arg1+) inside the tumor in the FOLFIRINOX+G-CSF treated group compared with all other treatment regimens (figure 5G). Collectively these data suggest that G-CSF is sufficient in mice to induce systemic neutrophilia containing a higher fraction of Gr-MDSCs than would be achieved by either normal hematopoiesis or tumor-induced emergency hematopoiesis. These systemic Gr-MDSCs can accumulate in the tumor microenvironment thereby potentially contributing to both local and systemic immune suppression.

**DISCUSSION**

Febrile neutropenia is a potentially fatal complication of cancer care. Use of recombinant G-CSF (pegfilgrastim, filgrastim and lenograstim) and to a lesser extent GM-CSF (sargramostim and molgramostim) has improved outcomes for patients with cancer and prevented dose reductions or delays in life-saving chemotherapy agents.\(^{50,52}\) Pegylated G-CSF provides longer lasting growth factor support and can be self-administered at home after completing FOLFIRINOX infusion.\(^{39,50}\) Nevertheless, these agents come at a cost. Here we report a negative impact of G-CSF on induction of systemic immune suppression in both humans and mice. Current treatment for PDAC does not rely on invoking antitumor immunity, and thus the generation of MDSCs may be unimportant for patients receiving standard of care FOLFIRINOX. However, we are hopeful that future treatments for PDAC will invoke antitumor T-cell responses. While we do not advocate withholding G-CSF from patients with neutropenia, we do suggest being aware of the negative impact of G-CSF when designing clinical trials aimed at inducing T cell-based antitumor immunity. We further suggest that gemcitabine/n(ab)paclitaxel, which less frequently requires G-CSF supportive care, may be a more preferred treatment for patients with PDAC who are also receiving checkpoint inhibitor therapy for microsatellite instable or high tumor mutational burden disease.

Although we conducted this study in humans with PDAC and mouse PDAC models, our results are likely applicable across cancer types. Most telling, we show that addition of G-CSF to healthy human serum is capable of inducing functional Mo-MDSCs that suppress T-cell responses in vitro. These results indicate that supraphysiologic G-CSF, independent of cancer status, can induce systemic immune suppression. We were only able to functionally assess the suppressive capacity of Mo-MDSCs in vitro due to technical limitations in culturing of human neutrophils. However, we note that the frequency of Gr-MDSCs increases significantly with FOLFIRINOX treatment and is positively correlated with serum levels of G-CSF. G-CSF is widely used for neutropenia in patients with cancer and is given across multiple hematologic and solid tumor types.\(^{56}\) In a similar study of patients with breast cancer, peripheral blood MDSCs were found to increase on treatment with doxorubicin, cyclophosphamide and pegfilgrastim (G-CSF).\(^{45}\) G-CSF suppression of T-cell responses is occasionally desirable. Human CD4 T cells from G-CSF mobilized blood are less capable of allogeneic responses in mixed lymphocyte cultures,\(^{74}\) and similarly G-CSF administration in a mouse model can reduce graft versus host disease symptoms.\(^{55}\) Intriguingly, both serum G-CSF and CD14+CD15+ Gr-MDSCs were increased in the normal course of pregnancy, suggesting that chronic G-CSF production may have evolved for maternal-fetal tolerance.\(^{56}\)

There are several limitations to our study. First, all patients receiving G-CSF also received FOLFIRINOX whereas all patients not receiving G-CSF were treated with gemcitabine/n(ab)paclitaxel. The choice of treatment regimen may have been selected for older or sicker patients in the group receiving gemcitabine/n(ab) paclitaxel. Although we demonstrated that G-CSF use correlated with abundance of Gr-MDSCs and was sufficient on its own to induce Mo-MDSCs, we cannot conclude that it is the only factor contributing to the observed difference in MDSCs between patients receiving two different chemotherapy regimens. Human neutrophils are not amenable to differentiation after they exit the bone marrow, thus we were unable to directly assess the effects of G-CSF on human Gr-MDSC formation in vitro as we did for Mo-MDSCs. Given the high rate of neutropenia observed with FOLFIRINOX, there are no patients at our center who receive FOLFIRINOX without G-CSF. Retrospective analysis from other centers indicates that prophylactic G-CSF is associated with fewer FOLFIRINOX dose reductions and a trending benefit in progression-free survival.\(^{57}\) Ideally, we would have tested paired blood samples from patients receiving gemcitabine/n(ab)paclitaxel with G-CSF compared with patients receiving gemcitabine/n(ab)paclitaxel without growth factor support; however, G-CSF is not commonly given with gemcitabine/n(ab)paclitaxel at our center and thus would require a much larger clinical cohort. Second, all patients in our study received pegfilgrastim, a version of G-CSF with polyethylene glycol additions for half-life extension. It is possible that filgrastim, which has a shorter half-life, would have different effects on production of Gr-MDSCs. Finally, we do not show a clear link between G-CSF usage and antitumor T-cell responses. T-cell recognition of PDAC has been difficult to demonstrate outside of the 1% of patients with MSI-high tumors.\(^{41}\) We therefore conclude that while G-CSF does induce systemic immune suppression, this is still a theoretical concern for patients with PDAC. Our results may be more relevant to patients with immunotherapy-responsive tumor types.

Human MDSCs have been difficult to study. Here we demonstrate that pegfilgrastim induces MDSCs in humans and present a novel mouse model of chemotherapy-induced neutropenia with G-CSF rescue. In humans we show that even though PDAC induces Gr-MDSCs in treatment-naïve patients, the absolute
counts of Gr-MDSCs and Mo-MDSCs further increase after FOLFIRINOX with G-CSF in a manner not observed with gemcitabine/\(n(ab)\)paclitaxel. In mice we showed that myeloid cells from the spleen were more suppressive on a per-cell basis after FOLFIRINOX treatment with G-CSF than with FOLFIRINOX alone or from untreated cancer-bearing mice. Collectively, these results strongly implicate G-CSF as a causative factor in MDSC induction. We caution against use of G-CSF where not clinically necessary and encourage research into alternative means of chemotherapy and/or growth factor support that may be less prone to MDSC induction.

Supplemental material This content has been supplied by the author(s), it has not been vetted by BMJ Publishing Group Limited (BMJ) and may not have been peer-reviewed. Any opinions or recommendations discussed are solely those of the author(s) and are not endorsed by BMJ. BMJ disclaims all liability and responsibility arising from any reliance placed on the content. Where the content includes any translated material, BMJ does not warrant the accuracy and reliability of the translations (including but not limited to local regulations, clinical guidelines, terminology, drug names and drug dosages), and is not responsible for any error and/or omissions arising from translation and adaptation or otherwise.

Open access This is an open access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited, appropriate credit is given, any changes made indicated, and the use is non-commercial. See http://creativecommons.org/licenses/by-nc/4.0/.

REFERENCES


ORCID iDs

Victoire Cardot-Ruffino http://orcid.org/0000-0002-5595-187X
Douglas A Rubinson http://orcid.org/0000-0002-5337-5231
Stephanie K Dougan http://orcid.org/0000-0002-2263-363X

Ethics approval This study involves human participants and was approved by DF/HCC studies 03-189, 02-103 and 21-590 were approved by the DFCI IRB (IRB #000035, FWA #0001121) prior to the commencement of study activities. Written informed consent in accordance with federal regulations at 45 CRF 46.117 / 21 CFR 50.27 was obtained from all participants prospectively enrolled in study #21-590 and 03-189. A waiver of consent was granted under 45 CFR 46.116(d) for the retrospective use of previously collected samples from participants enrolled in study #12-013. These studies were conducted in accordance with the US Common Rule.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

Twitter Douglas A Rubinson @labra3

Contributors VC-R, NB, LD and SJW conducted experiments and analyzed data. LKB, JR, CEK, KMS, MD and SJW coordinated sample collection and analyzed data. TAA, LHB, PCE, NJM, AKP, DAR, BS, SS, MBY, JMC, KP and HS were involved in sample annotation, data interpretation and understanding translational relevance of findings. MD, KN, and BMW supervised biobanking efforts. SKD, HS, VC-R, NB and LD designed the study and interpreted the data. SKD and VC-R wrote the manuscript with input from all of the authors. SKD and HS supervised the project and are the guarantors.

Competing interests SKD received research funding unrelated to this project from Eli Lilly and Company, Novartis Pharmaceuticals, Genocea, and Bristol-Myers Squibb and is a founder, science advisory board member and equity holder in Kojin. MD has research funding from Eli Lilly; he has received consulting fees from Genentech, ORIC Pharmaceuticals, Partner Therapeutics, SU2 Biotech, AzurRx, Eli Lilly, Mallinkrodt Pharmaceuticals, Adtrim, Faghour Therapeutics, Palleon, and Moderna; and he is a member of the Scientific Advisory Board for Neodendro Therapeutics, Veravas and Cerberus Therapeutics. HS receives research funding from AstraZeneca and consulting fees from Merck and Devpoint Therapeutics. DAR is on the scientific advisory board of AxialTX and a consultant for Boston Scientific and Instylla. MBY receives research funding from Janssen Oncology and fees for peer review services from UpToDate. NJM receives research funding from Bristol-Myers Squibb. PCE has received consulting fees from ALX Oncology, Arcus Bioscience, Astellas, AstraZeneca, Blueprint Medicines, Celgene, Coherus, Daltichi-Sankyo, Five Prime, Ideaya, Istari, Legend, Lilly, Luxo, Merck, Novartis, Ono, Servier, Taiho, Takeda, Turning Point, Therapeutics, Xencor, and Zymeworks. KN received research funding from Pharmavite, Evergrande Group, Janssen, Revolution Medicines and is on the SAB or received consulting fees from Bayer, GlaxoSmithKline, and Pfizer. BMW receives consulting fees from Celgene, GRAIL, and Mirati and research support from Celgene, Eli Lilly, Novartis, and Revolution Medicines.

Patient consent for publication Not applicable.


