Supplemental Information

CXCR2 Inhibition Profoundly Suppresses Metastases and Augments Immunotherapy in Pancreatic Ductal Adenocarcinoma

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Figure S1, related to Figure 1

The effects of CXCR2 signaling in pancreatic tumors are dependent on location (A) IHC for CXCR2 on a human pancreatic cancer TMA. The left image is representative of tumor epithelium scored as 'high'. The right image is representative of tumor stroma scored as 'high'. (B) Kaplan-Meier analyses of patient survival stratified on high and low tumor cell CXCR2 expression and high and low stromal expression, p value, Log Rank.
Figure S2, related to Figure 2

**Stromal characterization of KPC PDAC**

(A) H&E, (B) Picrosirius red staining (collagen) (C-I) IHC for (C) F4/80 (macrophages), (D) MPO (neutrophils and precursors), (E) S100A9 (neutrophil precursors, BMDCs), (F) CD3 (T cells), (G) αSMA (activated fibroblasts), (H) Tenascin C, and (I) Ki67. Scale bars = 200 μM.
Figure S3, related to Figure 3

Effects of Cxcr2 deletion on stromal markers within the primary tumor

(A-D) Boxplots showing quantification of (A) MPO IHC, (B) F4/80 IHC, (C) CD3 IHC, and (D) Tenascin C IHC in tumors from KPC and KPC Cxcr2−/− mice treated as indicated. p values, Mann Whitney, n ≥ 3. Note that there is no box, and only a median line for MPO+ cells in KPC Cxcr2−/− mice because all the tumors assessed had the same score. (E-G) IHC for (E) Ki67, (F) cleaved caspase 3, and (G) picrosirius red, in tumors from KPC or KPC Cxcr2−/− mice as indicated.
Figure S4, related to Figure 5

Effects on stromal markers within the primary tumor and liver in response to CXCR2-inhibiting pepducin

(A-D) Boxplots showing quantification of (A) MPO IHC, (B) F4/80 IHC, (C) CD3 IHC, and (D) Tenascin C IHC in tumors from KPC mice treated with scrambled pepducin, CXCR2-inhibiting pepducin, gemcitabine or CXCR2-pepducin combined with gemcitabine as indicated. P values, Mann Whitney, n ≥ 3. (E-G) IHC for (E) Ki67, (F) cleaved caspase 3, and (G) picrosirius red, in tumors from KPC mice treated with scrambled pepducin or CXCR2-inhibiting pepducin. (H-K) IHC for CD3, S100A9, NIMP1 (neutrophils), F4/80 and CXCR2, as indicated, in (H) pre-metastatic livers of KPC mice, (I) KPC liver metastases, (J) the livers of KPC mice treated with CXCR2 pepducin and gemcitabine from 10 weeks of age, and (K) the livers of KPC mice treated with CXCR2 pepducin and gemcitabine once symptoms are apparent. Scale bars = 200 μM.
Figure S5, related to Figure 6

Validation of AZ13381758 (CXCR2 SM) and its effects on stromal markers in primary tumors (A-C) MIP2 ligand-stimulated CD11b expression in blood from C57Bl/6 mice (A) 2 hours, (B) 6 hours, or (C) 24 hours following a single dose of CXCR2 antagonist AZ13381758 at 50 mg/kg or 100 mg/kg. (D) Mean number of circulating neutrophils following a single bolus dose of AZ13381758 in C57Bl/6 mice treated as indicated, n=3. (E) Quantification of MIP2–CXCR2-induced stimulation of CD11b expression on neutrophils ex vivo (performed 15 minutes after AZ13381758 was spiked into whole blood pooled from 10 mice). F) Blood neutrophil depletion in pooled whole blood from CXCR2 SM treated KPC mice. Recombinant MIP2 was added followed by anti-CD11b antibody and the number of CD11b-expressing cells determined by flow cytometry. (G-J) Boxplots showing quantification of (G) MPO IHC, (H) F4/80 IHC, (I) CD3 IHC, and (J) Tenascin C IHC in tumors from KPC mice treated with vehicle, CXCR2 SM, or CXCR2 SM in combination with gemcitabine as indicated. P values, Mann Whitney, n ≥ 3. (K-M) IHC for (L) Ki67, (L) cleaved caspase 3, and (M) picrosirius red, in tumors from KPC mice treated with vehicle, CXCR2 SM, or CXCR2 SM in combination with gemcitabine as indicated.
Table S1, related to Figure 6

In vitro potency of AZ13381758 versus mCXCR2 and hCXCR2

| Species | Compounds                          | Values                  |
|---------|------------------------------------|-------------------------|
|         | hCXCR2 antibody (HY29) (µg/ml)     | mCXCR2 antibody (18-74-5) (µg/ml) | AZ13381758 (µM) |
| mCXCR2  | >10 ± 0                            | 0.44 ± 0                | 0.026 ± 0       |
| hCXCR2  | 0.74 ± 0.129                       | >10 ± 0                 | 0.03 ± 0.001    |

Values = mean IC50 ± std dev (n = 2)
Supplemental Experimental Procedures

Human Pancreatic Cancer Microarray

Tissue microarrays containing at least 3 cores of resected PDAC from each of 184 patients with full clinicopathological data were obtained from Glasgow Biorepository. Tissue was collected prospectively with local ethical approval and fully informed consent. Following immunohistochemistry, expression was scored using a weighted histoscore (\(= \Sigma (0 \times \% \text{ unstained}) + (1 \times \% \text{ low}) + (2 \times \% \text{ medium}) + (3 \times \% \text{ high})\)).

Human Gene Expression Analysis

Fresh frozen PDAC tissue samples were obtained from 47 patients undergoing pancreaticoduodenectomy at the West of Scotland Pancreatic Unit and macrodissected to acquire samples from tumor border and adjacent normal tissue. Tissue was collected prospectively with local ethical approval and fully informed consent. Only histologically proven PDACs were included. Complete clinicopathological, follow-up and recurrence data were available. RNA was isolated from frozen tissue by TRIzol (Invitrogen) extraction according to the manufacturer's instructions. Samples with a RNA integrity number (RIN) above 7.0 were selected for downstream analysis. Transcript profiling was performed as described previously (GEO GSE55643). Kaplan-Meier survival analysis was used to analyze overall survival from time of surgery. A Log Rank test was used to compare length of survival between curves. Statistical significance was set at a \(p\) value of < 0.05. All statistical analyses were performed using SPSS version 19.0 (Version 19.0. Armonk, NY: IBM Corp.)
**Immunohistochemistry**

IHC was performed on formalin fixed paraffin embedded pancreatic tissue using standard protocols. Quantification was performed using visual counts at x20 or x40 fields of view using an Olympus BX53 microscope (where possible, >30 fields per animal). To quantify stromal stains, pictures were taken of >30 fields of view at x40 magnification, and Adobe Photoshop 5.1 used to pixel count the positive staining.

**Immunohistochemistry Antibodies:**

| Protein               | Clone | Conc. | Supplier                  |
|-----------------------|-------|-------|---------------------------|
| MPO                   | 1/200 | Dako A0398 |
| F4/80                 | A3-1  | 1/400 | Abcam ab6640              |
| CD3                   | SP7   | 1/75  | Vector VP-RM01            |
| Ki67                  | SP6   | 1/200 | Thermo RM-9106            |
| Cleaved Caspase 3     | ASP175| 1/50  | Cell Signaling 9661       |
| CXCR2                 | 19    | 1/200 | Human: Invitrogen AHR1532X |
| CXCL2                 | 40605 | 1/200 | R&D systems MAB452        |
| CXCL1                 | 1/100 | AbCam ab86436 |
| αSMA                  | 1A4   | 1/20000 | Sigma-Aldrich A2547   |
| CK19                  | A53-B | 1/500 | AbCam ab194399           |
| 1A8                   | 1A8   | 1/500 | Biolegend 127602         |
| Tenascin C            | M-Tn12| 1/1000 | Sigma-Aldrich T3413     |
| Nimp1                 | NIMP-R14| 1/50  | Abcam ab2557            |
| S100A9/Calgranulin    | M-19  | 1/1000 | Santa Cruz sc-8115      |
Animal Experiments

All animal experiments were performed under UK Home Office licence and approved by the local ethics committee. Mice were maintained in conventional cages and given access to standard diet and water ad libitum. Mice were genotyped by Transnetyx (Cordoba, TN, USA). Cxcr2−/− mice on a BALB/c background were obtained from The Jackson Laboratory (Cacalano et al., 1994). KPC mice were first described by (Hingorani et al., 2005) and were bred in house on a mixed background. Mice were monitored at least 3 times weekly and culled when exhibiting symptoms of PDAC. For short term drug studies pancreatic malignancy was confirmed by abdominal palpation.

Laser Capture Microdissection

Primary pancreatic tumors were dissected from mice, snap frozen, and mounted in optimal cutting temperature (OCT) cryostat compound. 35 µM sections were cut from these tumors and stained with crystal violet. Using Leica microdissection microscope LMD 6500 areas of tumor and stroma were dissected and tissue immediately snap frozen and RNA extracted.

RNA Extraction

Portions of murine pancreatic tumors were frozen in RNALater (Qiagen) solution until required. LCM RNA was snap frozen. RNA extraction was performed using Qiagen RNeasy Plus Mini Kit (Qiagen) and homogenized in a Precellys with ceramic beads (Stretton scientific). DNA was removed with Turbo DNA-free Kit (Applied
Biosystems). RNA integrity was assessed using Agilent 2100 Bioanalyzer in conjunction with RNA 6000 Nano LabChip kits (Agilent).

**qPCR**

SYBR Green qPCR kits (Thermo Scientific) were utilized to perform real time PCR for Cxcr2 and its ligands Cxcl1, Cxcl2, and Cxcl5. cDNA was prepared from mouse total RNA using standard protocols. qPCR was performed using these primers:

- **CXCL1 F:** 5’ CTGGGATTCACTCAAGAACATC
- **CXCL1 R:** 5’ CAGGGTCAAGGCAAGCCTC
- **CXCL2 F:** 5’ CCAACCACCAGGCTACAGG
- **CXCL2 R:** 5’ GCGTCACACTCAAGCTCTG
- **CXCL5 F:** 5’ GTTCCATCTCGCCATTCATGC
- **CXCL5 R:** 5’ GCGGCTATGACTGAGGAAGG
- **CXCR2 F:** 5’ ATGCCCTCTATTCTGCCAGAT
- **CXCR2 R:** 5’ GTGCTCCGGTGTTATGAGATGAC

Opticon monitor software (Bio-Rad) was used for data collection and analysis. All PCR products were analyzed in the exponential phase of amplification. Quantification of the relative expression of Cxcr2 and its ligands transcripts was performed using standard curves normalized to Gapdh transcripts.

**mRNASeq analysis**

Sequencing was done by Source BioScience using Illumina TruSeq RNA library preparation, and Illumina HiSeq sequencing and HiSeq2000 analysis. Reads were
analysed using the bcbio-nextgen framework (https://bcbio-nextgen.readthedocs.org/en/latest/). After quality control and adaptor trimming, reads were aligned to the UCSC mouse mm10 genome build using STAR Counts for known genes were generated using the function featureCounts in the R/Bioconductor package “Rsubread”. The R/Bioconductor package “edgeR” was used to identify differentially expressed genes.

**Cxcr2+/− signature PDAC subtype enrichment**

To determine the enrichment of Cxcr2+/− differentially expressed mouse genes in PDAC subtypes, mouse identifiers were first mapped to their corresponding human HGCN Symbol using the R/Bioconductor package “biomaRt”. After mapping, gene set enrichment in PDAC was performed using the R package ‘GSVA’ (function gsva - arguments: method=”gsva”, mx.diff=TRUE). GSVA implements a non-parametric unsupervised method of gene set enrichment that allows an assessment of the relative enrichment of a selected pathway across the sample space. The output of GSVA is a gene-set by sample matrix of GSVA enrichment scores. GSVA enrichment scores were generated exclusively for both upregulated and downregulated genes using transformed count data and stratified on the basis of subtype. A Kruskal-Wallis test was applied to the stratified scores to determine whether the distributions were significantly different.

**Efficacy testing of CXCR2 small molecule inhibitor (AZ13381758)**

**In vitro:** HEK 293s Gqi5 cells over-expressing murine or human CXCR2 receptor were cultured in DMEM + Glutamax (Invitrogen), 10% FCS, 1000 µg/ml G418, 300 µg
Hygromycin B. 24 hours before the assay, cells were plated in black 384 well poly-D-lysine coated plates at 12000 cells per well in 70 µl growth media/well and grown overnight at 37 °C in a CO₂ incubator. The following day the cells were loaded with Calcium 3 dye (Molecular devices) and Fluo-4 AM stocks (Invitrogen) for 30 minutes at 37 °C at room temperature, treated with AZ13381758 and then stimulated with MIP2 (R&D Systems) at 0.5 mg/ml. Species selectivity of each assay was demonstrated using therapeutic antibodies HY29 and 18-74-5 that block human and murine receptor respectively.

**In vivo**: Non tumour bearing female C57BL/6J mice (Harlan UK) were dosed with a single oral bolus of AZ13381758, n = 3 mice per treatment group. Animals were terminated using rising levels of CO₂, and terminal blood samples were slowly taken via vena cava into lithium-heparin tubes. Whole blood (80 µl) was placed into polypropylene tubes. A range of concentrations of 10 µl recombinant MIP2 (R&D Systems) was added followed by 10 µl of PE-labeled rat anti-mouse CD11b antibody (Serotec). Following 40 minutes incubation in the dark at room temperature the tubes were placed on ice. The cells were processed using Immunoprep (Beckman Coulter) to lyse red blood cells and fix leukocytes. The level of CD11b expression was determined using a Becton Dickinson Facs Caliber. Neutrophils were gated based on their forward scatter / side scatter profile.

**Ex vivo**: AZ13381758 was spiked into whole murine blood pooled from 10 mice and incubated at room temperature for 15 minutes before the CD11b assay was performed.
Flow Cytometry

Tumours were minced and incubated at 37 °C for 20-30 minutes in an enzymatic cocktail (Dnase (0.5 mg/ml, Sigma) and collagenase type V (2 mg/ml, Sigma) in RPMI 1640 (Sigma) to make a single cell suspension. This was passed through a 70 µM filter (BD Biosciences), washed in PBS supplemented with 2% foetal bovine serum and 2 mM EDTA, counted and used immediately for flow cytometry. Flow cytometry was performed with the antibodies listed below. All surface staining were performed in in PBS supplemented with 2% foetal bovine serum and 2 mM EDTA. Fixable viability dye (eBioscience) was used to discriminate between live and dead cells. Acquisition and analyses were performed on a BD LSRII system using BD FACSDIVA software (BD Biosciences). Collected data were analysed using FlowJo software (version 10.0.7r2 tree Star). Cells were gated based on CD45 positivity, followed by CD3, CD4/8 and CD62L/CD44.

| Antibody | Fluorochrome | Clone | Vendor |
|----------|--------------|-------|--------|
| CD45     | BV (Brilliant Violet) 570 | 30-F11 | Biolegend |
| CD3      | PE-Cyanine 7 | 145-2C11 | Biolegend |
| CD4      | APC (allophycocyanin) | RM4-5 | eBioscience |
| CD8      | PE (phycoerytrin) | 53-6.7 | eBioscience |
| CD44     | BV650        | IM7   | Biolegend |
| CD62L    | BV605        | MEL-14| Biolegend |
| PD1      | eFluor450    | RMP1-30 | eBioscience |