ApoE-TREM2 axis induces pathogenic senescent-like myeloid cells in prostate cancer

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Abstract

Tumour cells promote the expansion and intra-tumoural recruitment of Myeloid-derived suppressor cells (MDSCs), a subset of immature myeloid cells, that support tumour cell proliferation and confer treatment resistance. While immature myeloid cells have a very short lifespan, whether pathogenic MDSCs can persist in the tumour microenvironment remains unknown. Here, we report the identification of a subset of long-lasting MDSCs that upregulate markers of cellular senescence and the TREM2 receptor. Senescent-like MDSCs possess higher pro-inflammatory capabilities compared to canonical MDSCs. Genetic and pharmacological elimination of senescent-like MDSCs decreases tumour progression in different mouse models of prostate cancer. Mechanistically, we find that Apolipoprotein E (ApoE) secreted by prostate tumour cells binds TREM2 in senescent-like MDSCs, thereby regulating the survival of these cells. ApoE and TREM2 mRNA levels are upregulated in prostate cancers and correlate with poor patients’ prognosis. Taken together, these results reveal a novel mechanism by which the tumour microenvironment shapes the intra-tumoural immune response. Pathogenic senescent-like MDSCs persist longer in the tumour microenvironment and can be eliminated by histone deacetylase inhibitors enhancing the efficacy of standard therapy in prostate cancer.

Main Text

Increased numbers of circulating and tumour infiltrating myeloid-derived suppressor cells (MDSCs) have been observed in patients affected by different types of tumour including prostate cancers. MDSCs are known to support prostate tumourigenesis by suppressing the T cells response, promoting angiogenesis, senescence evasion, and therapy resistance. Compounds that block the recruitment of MDSCs (e.g. anti-CXCR2) have been extensively tested as anti-cancer compounds in pre-clinical trials in different cancers and are currently in clinical phase of investigation either alone or in combination with different immunotherapies.

MDSCs are a population of terminally differentiated and heterogeneous immature myeloid cells that are produced by the bone marrow and recruited in the tumour microenvironment by the tumour secretome. In healthy subjects and in patients affected by different inflammatory conditions, MDSCs possess a short circulating half-life. However, if MDSCs can persist in the tumour microenvironment remains enigmatic. This is the reason why inhibition of the intra tumoral recruitment of MDSCs, rather than elimination, has been proposed as a potential anti-cancer strategy.

To characterise their intra-tumoural recruitment and vitality of MDSCs, we infused bone marrow (BM)-derived MDSCs obtained from CD45.1 mouse, Y.1+ cells, i.v., in CD45.2 Pten null prostate conditional (Ptenpc/-) mice (Figure 1a-c). Ptenpc/- mice develop prostate tumours that recruit MDSCs through CXCL2, CXCL3 and CXCL5. While the percentage of alive CD45.1 MDSCs in the tumours decreased over time as detected by Annexin V, a small percentage of infused cells persisted in the tumour microenvironment for several weeks remaining Annexin V negative (Figure 1b-c). Note that CD45.1
MDSCs were not detected alive in the spleen, bone marrow and blood of tumour-bearing mice (Extended Data Figure 1a-c). The long-lasting presence of donor MDSCs in the tumour of *Pten*<sup>pc/-</sup> mice was validated by the infusion of MDSCs differentiated from bone marrow precursors (BMPs) collected from mCherry mice. In line with the previous experiment, mCherry<sup>+</sup> donor MDSCs were still present in the prostate tumours of *Pten*<sup>pc/-</sup> mice several weeks after their infusion (Extended Data Figures 1d–e).

Intrigued by this finding, we next checked whether persistent MDSCs could upregulate markers of cellular senescence. Senescent cells have the capability to indefinitely survive *in vivo* in the tissue microenvironment and resist to different apoptotic stimuli by remaining metabolically active<sup>15</sup>. In line with this hypothesis, multi-parametric flow cytometry analysis showed that CD45.1 persistent MDSCs stained positive for C<sub>12</sub>FDG which detects increased SA-Beta-Galactosidase (b-Gal) activity at acidic pH<sup>16</sup>, a hallmark of cellular senescence<sup>15</sup> (Figure 1b-c). Additionally, persistent MDSCs stained positive for both p16 and p21, two key markers of cellular senescence<sup>15</sup>, as detected by Immunofluorescent (IF) staining (Figure 1d; Extended Data Figure 1f-g). Interestingly, the percentage of tumour-infiltrating C<sub>12</sub>FDG<sup>+</sup> PMN-MDSCs increased with disease progression, being particularly enriched in Pten null castration-resistant (CR) prostate tumours reaching 50% of the total MDSCs (Figure 1e).

In order to validate this finding, C<sub>12</sub>FDG positive and negative PMN-MDSCs were sorted from *Pten*<sup>pc/-</sup> prostate tumours and tested for markers of cellular senescence by qPCR (Figure 1f). Tumour-infiltrating C<sub>12</sub>FDG<sup>+</sup> PMN-MDSCs expressed higher mRNA levels of *p16ink4a* (*p16*), *p21Cip* (*p21*) and *PAI-1* (Figure 1g) and stained positive for SA-b-Galactosidase when analysed *ex vivo*, thereby validating the C<sub>12</sub>FDG marker analysed by flow cytometry (Figure 1h).

Whole gene expression profile analysis identified a signature enriched in C<sub>12</sub>FDG positive PMN-MDSCs (Senescent-like PMN-MDSCs signature). Differential expression analyses between C<sub>12</sub>FDG<sup>+</sup> and C<sub>12</sub>FDG<sup>-</sup> PMN-MDSCs showed that C<sub>12</sub>FDG<sup>+</sup> MDSCs upregulate genes involved in mitochondrial respiration and oxidative phosphorylation (Figure 1i) and possess a peculiar senescence-associated secretory phenotype (SASP), enriched in factors regulating inflammation, angiogenesis and granulocyte chemotaxis (Figure 1k; Extended Data Figures 1h). Of note, senescent-like MDSCs were also detected in human prostate tumour biopsies by using the C<sub>12</sub>FDG staining (Figure 1l). These data were further validated using available single-cell RNA sequencing (scRNA-seq) of human prostate tumours<sup>17</sup>. For this analysis, PMN-MDSCs were identified using a published gene signature<sup>18</sup>, whereas senescent like-PMN-MDSCs were spotted by using the Senescent-like PMN-MDSCs signature (Extended Data Figure 1i-k). In sum, these results demonstrate that tumour-infiltrated PMN-MDSCs can acquire features of senescent cells and that the C<sub>12</sub>FDG staining can be used to identify this cell population in prostate tumours.

To assess whether factors secreted by tumour cells impact on MDSCs vitality and induce the upregulation of markers of senescence, we co-cultured BM-derived MDSCs in presence or absence of conditioned medium (C.M.) derived from TRAMP-C1 cells, a murine prostate cancer cell line<sup>19</sup>. The C.M.
of prostate cancer cells increases the survival of MDSCs and triggers the upregulation of senescent markers as detected by the C\textsubscript{12}FDG staining and western blot analysis for p21 and additional markers of cellular senescence (Extended Data Figure 2a-c). As observed in vivo (Figure 1i), BM-derived senescent-like MDSCs possess increased mitochondrial respiration and spare respiratory capacity when compared to canonical MDSCs (Extended Figure 2d-e). Additionally, these cells also produced more ROS, Arginase I, immunosuppressive cytokines (Extended Figure 2f-h) and possessed a higher immunosuppressive activity than canonical MDSCs (Extended Figure 2i). The secretome of senescent-like MDSCs also showed an increased chemotaxis for canonical MDSCs and promoted the proliferation of murine prostate tumour cells to a higher extend that the secretome of canonical PMN-MDSCs (Extended Data Figure 2j, k).

Taken together, these data suggest that factors secreted by tumour cells control, in a paracrine manner, the vitality of PMN-MDSCs in prostate tumours and promote the upregulation of senescence markers. Senescent-like PMN-MDSCs show increased immunosuppressive activity and pro-tumourigenic functions than canonical PMN-MDSCs.

To identify factors secreted by tumour cells that induce a senescence-like state in PMN-MDSCs, we performed a bioinformatic analysis by comparing receptors found upregulated in senescent-like PMN-MDSCs with secreted factors increased in \textit{Pten}\textsuperscript{pc/-} prostate tumours. TREM2 was one of the most upregulated receptors expressed in senescent-like MDSCs and ApoE-TREM2 was the most significant ligand-receptor pair identified in the network analysis (Figure 2a-c; Extended Data Figure 2i-m). ApoE is an apolipoprotein involved in lipid metabolism and trafficking that binds to different receptors\textsuperscript{20}. However, in its lipidated form ApoE binds with high affinity to TREM2 activating the downstream Dap12/Syk pathway\textsuperscript{21,22}. Of note, ApoE/TREM2 was the most significantly correlated ligand/receptor couple in human prostate cancers when compared to a list of additional upregulated factors and known ApoE interactors (Figure 2d; Extended Data Figure 2n). Recent evidence demonstrates that TREM2 upregulation in tumour-associated macrophages leads to tumour immune evasion and treatment resistant\textsuperscript{23}. However, the role of TREM2 in the biology of MDSCs remains unknown.

We, therefore, assessed the levels of TREM2 expression on tumour-infiltrating PMN-MDSCs in \textit{Pten}\textsuperscript{pc/-} mice. Flow cytometry analyses confirmed that senescent-like PMN-MDSCs expressed high TREM-2 levels (Figure 2e). Accordingly, CD45.1\textsuperscript{+} donor MDSCs infused in \textit{Pten}\textsuperscript{pc/-} mice and analysed 4 weeks after the infusion expressed both C\textsubscript{12}FDG and high level of TREM2 (Figure 2f). BM-MDSCs co-cultured \textit{in vitro} with C.M. from TRAMP-C1 cells also co-expressed high levels of both C\textsubscript{12}FDG and TREM2 whereas canonical BM-MDSCs culture in absence of C.M. did not (Figure 2g). Finally, senescent-like BM-MDSCs upregulated the downstream signalling pathway of TREM2 as detected by increased phosphorylation of Syk and Erk1/2 (Figure 2h).

We next performed a protein profile analysis using different fractions of C.M. obtained from TRAMP-C1 prostate tumour cells. C.M. was concentrated on 100 kDa centrifuge filters. Then, proteins were denatured by heat inactivation in order to obtain different fractions of C.M. containing macromolecules above or
under 100kDa threshold. ApoE was detected in the total C.M. fraction before and after heat inactivation and in the fraction containing high molecular weight protein complexes (>100kDa) (Figure 2i). Co-culture experiments with different fractions showed that only the fractions containing ApoE were capable to prolong the survival of BM-MDSCs and trigger the senescence-like state (Figure 2j). To functionally validate the ApoE-TREM2 axis for the induction of senescence in MDSCs, we cultured BM-MDSCs in the presence of C.M. of TRAMP-C1 infected with shApoE (C.M. TRAMP-C1<sup>shAPOE</sup>) or shEV cells (C.M. TRAMP-C1<sup>ShEV</sup>). While the C.M. of TRAMP-C1<sup>ShEV</sup> promoted the survival and increased C<sub>12</sub>FDG positivity in BM-MDSCs, the C.M. of TRAMP-C1<sup>shAPOE</sup> was ineffective (Figure 2k). Mechanistically, we found that the C.M of ApoE deficient prostate tumour cells did not increase the levels of pSyk and of its downstream regulators in BM-MDSCs (Figure 2l). These data were further validated in vitro and in vivo by using Trem2 wt (Trem2<sup>wt</sup>) and Trem2 mutant BM-MDSCs (BM-MDSCs<sup>Trem2mut</sup>) that lack the capability to fully activate the downstream TREM2 signalling<sup>24</sup> (Figure 2m-n).<sup>2</sup> Pten<sup>pct-/-</sup> mice were reconstituted with these BMPs to generate the Pten<sup>pct-/-; Trem2<sup>wt</sup></sup> and Pten<sup>pct-/-; Trem2<sup>mut</sup></sup> mice (Figure 2o). Pten<sup>pct-/-</sup>; Trem2<sup>mut</sup> mice were less infiltrated by C<sub>12</sub>FDG<sup>+</sup> PMN-MDSCs and developed prostate tumours of smaller size than control mice thereby demonstrating that TREM2 is required for the survival of senescent-like MDSCs (Figure 2 o-q).

Intriguingly, single cell analysis in human prostate tumour samples showed that the majority of senescent-like MDSCs were also positive for TREM2 and that the senescence-like signature strongly correlated with TREM2 expression (Figure 2r, s). This was also validated in different human prostate cancer datasets using bulk RNA-seq data (Extended data Figure 3a, b).

In human prostate tumour biopsies, we found a cluster of PMN-MDSCs that express high TREM2 levels as detected by both multi-parametric flow cytometry analysis and multiplex immune fluorescence (Extended Data Figure 3c-e). Finally, bioinformatic analysis showed that ApoE and TREM2 mRNA levels increased with disease progression in prostate cancers and that TREM2 strongly correlated with both ApoE and the PMN-MDSCs signature<sup>18</sup> in both primary and metastatic tumours (Extended Data Figure 3f-m). Of note, increased ApoE mRNA levels were also associated with poor disease-free and overall survival in patients affected by prostate cancer (3/5 datasets analysed; Extended Data Figure 3n-p).

To determine the contribution of senescent-like PMN-MDSCs to tumour progression in vivo, we reconstituted lethally irradiated untouched, sham-operated or castrated-<sup>Pten</sup>pct-/ mice with BMPs from C57 or 3MRp16<sup>25</sup> mice that were deprived of T, B and natural killer (NK) cells<sup>1</sup> (yielding Pten<sup>pct-/-; C57</sup> mice and Pten<sup>pct-/; 3MRp16</sup> mice; Figure 3a, Extended Data Figure 4a). In the 3MRp16 model, treatment with Ganciclovir promotes the selective elimination of senescent cells over-expressing p16ink<sup>25</sup>, thus making it a suitable model to study senescent-like MDSCs where this gene is upregulated (Figure 1g). Treatment with Ganciclovir resulted in the effective killing of senescent-like PMN-MDSCs in the tumour microenvironment and led to a reduction in prostate cancer tumours volume, normalization of glands affected by prostate cancer and reduction of Ki-67 positivity in both castration-sensitive and castration-resistant Pten<sup>pct-/</sup> mice (Figure 3 a-g, Extended Data Figure 4 a-f).
Since TREM2+ senescent-like PMN-MDSCs persist in the tumour microenvironment, we next attempted to identify compounds capable to eliminate these pathogenic cells. To identify small molecules that selectively kill senescent-like PMN-MDSCs, we screened compounds from the NCI drug repository team of NIH (DTP program) (Figure 4a; Extended Data Figure 5a). All the drugs included in these libraries are FDA approved and used in the clinic for different diseases. Within the compounds tested only five compounds showed activity against these cells significantly affecting their viability (>50% at 10μM concentration). These five hits included Carfilzomib (proteasome inhibitor), Ceritinib (ALK inhibitor), Valrubicin (Anthracycline), Vorinostat and Romidepsin (HDAC inhibitors) (Figure 4b; Extended Data Figure 5b).

Between these drugs, we found that Vorinostat and Romidepsin, two HDAC inhibitors\(^{26}\), were capable to eliminate senescent-like MDSCs at nanomolar concentrations as assessed in a dose-response assay (Figure 4b). Pathway analysis showed that HDAC inhibition in senescent-like MDSCs impacted the TREM2 pathways decreasing the mRNA expression of TREM2, Dap12 and Syk already 6 hours after the administration of the compound (Figure 4 c-e). Accordingly, western blot analysis of senescent-like BM-MDSCs treated with Romidepsin showed increased acetylation of H3K9 and a downregulation of the TREM2 signalling as assessed by decreased total and phosphorylated-Syk and Erk levels (Figure 4f).

Given that senescent-like MDSCs were enriched in CR prostate cancers (Figure 1e), we next assessed whether Romidepsin could enhance the efficacy of enzalutamide (ENZA) by promoting the elimination of senescent-like PMN-MDSCs \textit{in vivo}. ENZA, a small molecule that binds the AR and suppresses the androgen receptor-signalling axis, is a standard of care for patients insensitive to first-line ADT\(^{27,28}\). CR \textit{Pten}pc-/- mice were treated with Romidepsin in combination with ENZA for four weeks at the indicated dosages. Co-treatment of CR prostate tumours with ENZA and Romidepsin strongly decreased the percentage of senescent-like MDSCs and lead to a reduction in tumour cells proliferation as detected by decreased Ki67 staining, and percentage of glands affected by invasive prostate cancer (Fig. 4g-j). Next, we assessed whether this combination of compounds could be further improved by the addition of a CXCR2 inhibitor, a compound under clinical evaluation in prostate cancer. We and others have previously shown that CXCR2 inhibitors partially decrease, without abolishing, the recruitment of MDSCs in prostate tumours blocking tumour progression\(^{5,1,7}\). Triple combination of ENZA, Romidepsin and anti-CXCR2 (aCXCR2) lead to the strongest inhibition of prostate cancer when compared to mice treated with the double combination (Figure 4g-j and Extended Data Figure 5c). These changes were associated with a robust reduction of both senescent-like and canonical PMN-MDSCs infiltration in these tumours (Figure 4g). These data were also validated in RM1 allograft\(^{29}\), a model of aggressive prostate cancer driven by Ras and Myc overexpression (Figure 4k-o). Taken together, these data demonstrate that Romidepsin kills senescent-like PMN-MDSCs and when administered in combination with a CXCR2 inhibitor further impacts prostate cancer progression enhancing the efficacy of ENZA.

In conclusion, we have identified a novel mechanism driven by the tumour microenvironment to reinforce its pool of tumour-infiltrating PMN-MDSCs adding novel insights on the mechanism by which ApoE produced by prostate tumour cells promotes immunosuppression in the tumour microenvironment\(^{30}\). These data also add novel knowledge on the role played by MDSCs in cancer, describing a new
unexpected feature for this immune subset. Previous evidence demonstrates that MDSCs can support tumourigenesis in a number of tumours through different mechanisms\textsuperscript{3,7,10}. However, the discovery that MDSCs can persist into prostate cancers, express markers of senescence, live longer and remain metabolically active was unexplored. This discovery also opens a novel scenario for the therapeutic targeting of MDSCs proving through preclinical studies that Romidepsin can target this persisting pro-tumourigenic immune population.

**Methods**

**Animals**

All mice were maintained under specific pathogen-free conditions in the IRB facility and experiments were performed according to state guidelines and approved by the local ethics committee. Male C57BL/6 or NSG mice 6–8 weeks of age were purchased from Jackson Laboratories (Envigo) and acclimated for at least a week before use. C57BL/6 3MRp16 (3MRp16) mice\textsuperscript{31} were kindly provided by Prof. Demaria (Groningen, Netherlands). C57BL/6 Trem2 mutant (Trem2\textsuperscript{mut}) mice\textsuperscript{32} were kindly provided by Prof. Mike Sasner (The Jackson Laboratory). \textit{Pten}\textsuperscript{pc-/-} mice were generated and genotyped as previously described\textsuperscript{17}. Female \textit{Pten}\textsuperscript{loxP/loxP} mice were crossed with male \textit{PB-Cre4} transgenic mice and genotyped for Cre using following primers: primer 1 (5’-AAAAGTTCCCCTGCTGATGATTTGT-3’) and primer 2 (5’-TGTTTTTGACCAATTAAGTGGCTGTG-3’) for \textit{PTEN}\textsuperscript{loxP/loxP}, primer1 (5’ TGATGGACATGTTCAGGGATC 3’) and primer2 (5’CAGCCACCAGCTTGCATGA 3’) for \textit{Probasin}-CRE. Surgical castration was performed under anesthesia with isoflurane. Mice were monitored postoperatively for recovery from anesthesia and checked daily for 2 days postoperatively. Surgical skin clips were removed on postoperative day 5. Mice undergoing treatment were administered control vehicle or therapeutic doses of the appropriate agents. Any mouse suffering distress or greater than 15% weight loss during treatment was euthanized by CO\textsubscript{2} asphyxiation. At the completion of study, mice were euthanized by CO\textsubscript{2} asphyxiation and tissue was collected for histology, mRNA analysis, protein analysis, and single cell suspensions for flow cytometry. For allograft experiments, 2,5x10\textsuperscript{6} TRAMP-C1 cells or 2x10\textsuperscript{5} RM1 cells were injected subcutaneously into the flank of male respectively NSG or C57BL/6 mice. For TRAMP-C1 allograft when tumours were approximately 100 mm\textsuperscript{3}, mice were randomized to the treatment groups. For RM1 allograft, castration was performed 3 days after injection and mice were randomized to the treatment groups. Tumour growth was monitored daily by measuring the tumour size with caliper. The tumour volume was estimated by calculating \(R1*R2*R3*4/3\pi\), where R1 and R2 are the longitudinal and lateral radii, and R3 is the thickness of tumour protruding from the surface of normal skin. Animals were sacrificed when the tumour reached approximately 600 mm\textsuperscript{3}.

**Treatments**

\(\alpha\text{CXCR2}\) (AZD5069; Astrazeneca) was administered with daily intraperitoneal injections at a final concentration of 100mg/kg on a Monday through Friday schedule. Control animals received vehicle.
Enzalutamide (APExBio) was administered daily by oral gavage with a dose of 30mg/kg/day on a Monday through Friday schedule. Romidepsin (0.03mg/kg per mouse; MedChemExpress) was administered twice per week via intraperitoneal injection.

**Bone marrow reconstitution**

Bone marrow was flushed from the femurs of male C57BL/6 or 3MRp16 mice under sterile conditions with RPMI 1640 using a 21-gauge needle. Mononuclear cells were filtered, collected and checked for viability using trypan blue. Recipient Pten\(^{pc/-}\) mice were lethally irradiated (900 rad) and transplanted i.v. two hours after with 1 \(\times 10^7\) viable bone marrow cells from either C57/BL6 or 3MRp16 mice.

**TRAMP-C1 conditioned media collection**

TRAMP-C1 cells were cultured and expanded in the appropriate medium. When the cells were at about 40–50% confluence, the medium was replenished with fresh complete medium. After 3 days the conditioned medium was centrifuged at 500 \(g\) for 5 min, 2,000 \(g\) for 10 min and 4,600 \(g\) for 20 min at 4 °C to remove dead cells and debris. The medium was then aliquot in single use tubes and store at -80°C or fractionated as followed. Fractions of conditioned media were collected using Centrifugal Filter Unit 100 KDa cutoff (Amicon® Ultra, merckmillipore). The fractions were reconstituted to initial volume with PBS. Heat inactivation was performed heating the samples at 65°C for 30 min.

**Viral infection and establishment of stable cell lines**

TRAMP-C1 cells were infected with lentivirus encoding mouse ApoE shRNA (in TRC1.5 Vector: pLKO.1-puro). Infected cells were selected in 3 \(\mu\)g/ml puromycin-containing medium for 7 days, and the selected cells were collected and used for further experiments.

**Differentiation of senescent-like BM-MDSCs in vitro**

Murine MDSCs were differentiated in vitro as previously described\(^{33}\). Briefly, bone marrow precursors were flushed from the femurs of C57/BL6 or TREM2\(^{mut}\) mice with RPMI 1640 medium. The cell pellet was resuspended in RPMI 1640 containing 10% heat-inactivated FBS, and the cells were cultured in vitro in the presence of 40 ng/ml GM-CSF and 40 ng/ml IL-6. On day 4, the cells were washed and resuspended with TRAMP-C1 conditioned medium or TRAMP-C1 ShApoE. After 3 days, the cells were analysed by flow cytometry or used for in vitro experiments.

**Senescence associated β-galactosidase (SA-β-gal) Assay**

For in vitro experiment, SA-β-gal staining was performed using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, Cat. No 9860) according to the manufacturer's instructions.

**Measurement of SA-β-Gal activity by flow cytometry**

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SA-β-Gal activity was measured according to the method described previously by Debacq-Chainiaux et al. Cells were pre-treated with 100 nM bafilomycin A1 (Bafilomycin A1 from Streptomyces griseus, Calbiochem cat.196000) in fresh cell culture medium at 37 °C for 1 h to induce lysosomal alkalinization. Thereafter, the fluorogenic substrate 5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside (C₁₂FDG Thermo Fisher Scientific, cat. D2893) was added to the cell culture medium to yield a final concentration of 25 μM. After incubation, the cells were used to performed different staining. The expression of different molecules was then analysed with the multi-parameter flow cytometer (BD Fortessa flow cytometer, BD Biosciences). FITC channel was used to detect C₁₂FDG.

**Apoptosis Assays**

Apoptosis assays were performed using Annexin V staining according to the manufacturer's instructions. The cells were stained with PE-conjugated/eFluor™ 450-conjugated Annexin V (eBioscience) according to manufacturer's recommendations. Annexin V-FITC was diluted in the manufacturer's Hepes-buffer (containing 2.5 mM CaCl₂), added to the cultures, and incubated for 15 min at room temperature. Further, the cells were then stained with other antibodies. The cells stained were subjected to FACS.

**In vitro T cell suppression assay**

In vitro suppression assays were carried out in RPMI/10% FCS in 96-well U-bottom plates (Corning, NY). Naive splenocytes were labelled with 5μM CFSE (Molecular Probes) and activated in vitro with anti-CD3 and anti-CD28 beads (Invitrogen) according to the manufacturer's instructions. BM-MDSCs or Senescent-like BM-MDSCs were added to the culture. After 3 days, the proliferation of CFSE-labelled CD⁸⁺ T cells was analysed by BD LSR Fortessa.

**Transwell assay for migration and chemotaxis**

The migration of unstimulated cells and chemotaxis of cells were measured with Falcon Permeable Support for 24 Well Plate with 8.0μm Transparent Polyester (PET) Membrane. RPMI 10%FBS 1%PS in different conditions was placed in the bottom of the support. Conditions: untreated, canonical BM-MDSCs (1 × 10⁶ cells), BM-SL-MDSCs (1 × 10⁶ cells). Canonical BM-MDSCs were labelled with 5μM CFSE (Molecular Probes) were plated on top of the support (1 × 10⁶ cells) and were incubated at 37 °C, 5% CO₂ for 16 h. The medium was taken from the bottom wells and cells were acquired on a BD Fortessa flow cytometer (BD Biosciences). Data were analysed using FlowJo software (TreeStar, Ashland, OR).

**Arginase I Activity Assay**

For *in vitro* experiment, Arginase I Activity Assay was performed using Arginase Activity Assay Kit (Sigma-Aldrich, Cat. No MAK112) according to the manufacturer’s instructions.

**Metabolic Phenotyping**
Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XFp bioanalyser. 1x10^5 cells per were spun onto poly-D-lysine coated Seahorse XFp Cell Culture Miniplates. Before the assay the plates were centrifuged 1min 300g w/o break and preincubated in Seahorse XF media (non-buffered DMEM + 10 µM L-glutamine + 10 µM sodium pyruvate + 25 mM glucose) at 37°C for a minimum of 30 min in the absence of CO2. OCR and ECAR were measured under basal conditions, and after the addition of the following drugs: 2 µM oligomycin, 2 µM fluorocarbonyl cyanide phenylhydrazone (FCCP) and 2.5 µM rotenone + antimycin A as indicated. Measurements were taken using a Seahorse XFp Analyzer (Seahorse bioscience).

**ROS detection**

Oxidation-sensitive dye Dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes/Invitrogen, Carlsbad, CA), was used to measure ROS production by MDSC. Cells were incubated at 37°C in prewarmed PBS in the presence of 2.5 µM DCFDA for 30 min. For induced activation, cells were simultaneously cultured, along with DCFDA, with 120ng/ml phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO) and 1 µg/ml ionomycin. Analysis was then conducted by flow cytometry as described above. mtROS detection Superoxide Detection Agent from Mitochondrial Superoxide Indicator (Red) Assay Kit (ab228567, abcam), was used to measure mtROS production by MDSC, according to the manufacturer's instructions. Cells were incubated at 37°C in prewarmed HBSS buffer in the presence of the suggested amount of the Superoxide Detection Agent working solution for 10 minutes in the dark. Analysis was then conducted by flow cytometry as described below. PE-TexasRed channel was used to detect Mitochondrial Superoxide.

**Murine tumour-infiltrating PMN-MDSCs characterization**

Tumours were disaggregated and digested in collagenase D and DNase for 45 minutes at 37°C to obtain single-cell suspension. For the assessment of B-galactosidase activity cells were stained with C12FDG. To assess the viability the cells were stained with Live/dead™ Fixable Aqua (cat # L34965, thermofisher). The Apoptosis assays were performed using Annexin V. For intracellular cytokine detection cells were stimulated for 5 hours with PMA/ionomycin. After neutralization of unspecific binding with αCD16/CD32 (clone 93), single-cell suspensions were stained with specific mAb (primary antibodies directly conjugated) to assess the phenotype. The antibodies used were: αCD45 (clone 30-F11); αLy-6G (clone 1A8); αLy6C (clone HK1.4), αCD11b (clone M1/70); αCD3 (clone 145-2C11), αCD8 (clone 53-6.7), αTREM2 (clone 237920), αIFNg (XMG1.2), αCCR7 (4B12), αF4/80 (T45-2342), αCD19 (1D3), αB220 (RA3-6B2), αCXCR2 (SA045E1), αCD3 (145-2C11).

All the antibodies were purchased from eBioscience or Biolegend or RnD or BD. Samples were acquired on a BD Fortessa flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR).

**High-Dimensional Single-Cell Data Preprocessing and Analysis by UMAP and FlowSOM**
27-Parameter Flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version 9 or 10 and left untreated or biexponentially transformed (the same transformation for all files, performed in version 10) prior to UMAP analysis.

Following transformation, 15-Flow Cytometry Standard (FCS) 3.0 files were assigned with a computational barcode for their unique identification, concatenated and visualized with UMAP in FlowJo. The following parameters were used: Euclidean; nearest neighbors: 10; minimum distance: 0.01; number of components: 2. All parameters except for CD45 and Aqua dead cell marker, were included in the analysis.

The clusterization was automatically define by the FlowSOM algorithm. The default parameters were used to run the algorithm except for the number of meta clusters. The identity of the clusters was determined by the heatmap generated by FlowSOM.

**Immune tumour microenvironment characterization of tumours from patients with prostate cancer**

Tumours were disaggregated and digested in collagenase I and DNase for 30 min at 37 °C to obtain single-cell suspensions. Single-cell suspensions were stained with specific monoclonal antibodies (primary antibodies directly conjugated) to assess the phenotype. The antibodies used were: CD45 (clone HI30), CD33 (clone WM-53), CD11b (clone M1/70), HLA-DR (clone L243), TREM2 (Clone 237920), CD66b (G10F5). To draw the gates, we used isotype controls or fluorescence minus one. All antibodies were purchased from BD Bioscience, eBioscience or Biolegend. Samples were acquired on a BD Fortessa flow cytometer (BD Biosciences). Data were analysed using FlowJo software (TreeStar, Ashland, OR).

**Immunohistochemistry and Immunofluorescence**

For immunohistochemistry (IHC), tissues were fixed in 10% formalin (ThermoScientific, 5701) and embedded in paraffin in accordance with standard procedures. Preceding immunohistochemical staining, tumour sections (4μm) were exposed to two washes with OTTIX plus solution (Diapath, X0076) and subsequent hydration with OTTIX shaper solution (Diapath, X0096) followed by deionized water. Antigen unmasking was performed by heating sections in the respective pH solutions based on the antibodies used at 98°C for 20 minutes. Subsequently the sections were blocked for peroxidases and nonspecific binding of antibodies using 3% H₂O₂ (VWR chemicals, 23615.248) and Protein- Block solution (DAKO Agilent technologies, X0909) respectively for 10mins each split by 0.5% PBST washing. H&E staining was performed according to standard procedures. Sections were stained for anti-Ki67 (Clone SP6; Lab Vision Corporation). Images were obtained using objectives of 5x, 10x, 40x magnification and Pixel image of 1.12μm and 0.28μm respectively. All the quantifications have been done using the public online software ImmunoRatio (153.1.200.58:8080/iimunoratio/) or counted manually in a double-blind manner. For the immunofluorescence (IF) staining, tissue paraffin embedded sections were stained for 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (#70238421, Roche), For immunofluorescence, prostate samples were snap frozen in OCT and 4 μm cryosections were cut and fixed with 4% PFA for 10 mins before staining. Then slides were permeabilized with Tween 0.1% for 10 min, washed twice with 1xPBST (0.1%
Tween 20), and incubated with blocking solution (10 % FBS in 1xPBST). Sections were stained with anti p16 antibody (Abcam, cat # ab211542, dilution 1:100) at 4°C overnight, followed by 3 washes with 1xPBST. Alexa Fluor 488-conjugated anti rabbit IgG secondary antibody (Thermo Scientific, cat # A-11008, dilution 1:300), anti-CD11b 594 (clone M1/70; Biolegend, cat # 101254, dilution 1:50), were incubated at room temperature for 1 hour. The nuclei were counterstained with Hoechst 33342 1ug/ml (Invitrogen, cat # H3570) and slides were mounted with ProLong (Invitrogen, cat # P36931).

For p21 staining, an antigen retrieval step with pH6 solution (Citrate, Company: Diapath, Cat No. T0050) was performed in water bath at 98° C for 20-25 mins. Then slides were allowed to cool at room temperature for 20-25 mins, washed twice with 1xPBST (0.5% Tween20), and incubated with blocking solution. Sections were stained with anti p21 antibody (Abcam, cat # ab107099, dilution 1:50) and anti CD45.1 (Abcam, cat ab25078, dilution 1:50) at 4°C, followed by 3 washes with 1xPBST. Alexa Fluor 594-conjugated anti rat IgG secondary antibody (Thermo Scientific, dilution 1:300), Alexa Fluor 488-conjugated anti mouse IgG secondary antibody (Thermo Scientific, dilution 1:300) were incubated at room temperature for 1 hour. The nuclei were counterstained with Hoechst 33342 1ug/ml (Invitrogen, cat # H3570) and slides were mounted with ProLong (Invitrogen, cat # P36931).

Images were acquired by a confocal microscope Leica SP5, with an oil-immersion objective (63×/1.4 NA Plan-Apochromat; Olympus), using laser excitation at 405, 488, or 594 nm. Images were processed using ImageJ software. Confocal images were obtained with the Leica TCS SP5 confocal microscope using ×10/1.25 oil.

**Multiplex IF in formalin fixed paraffin embedded (FFPE) tissue section**

**TREM2, CD15 and PanCK Immunofluorescence**

Immunofluorescence was performed on 4µm FFPE tissue sections using an automated staining platform (Bond-RX, Leica Microsystems). Briefly, antigen retrieval was achieved using ER2 (pH 9.0) (#AR9640, Leica Biosystems) for 10 minutes. Sections were blocked in 10% normal goat serum for 30 min at room temperature. Primary antibodies mouse monoclonal (IgM) anti-CD15 (#M3631, Dako, clone Carb-3, dilution 1:200), rabbit monoclonal (IgG) antibody anti-TREM2 (#91068, Cell Signaling Technology, clone D8I4C, dilution 1:100) and mouse monoclonal (IgG1) anti Pan-Keratin antibody (#4545, Cell Signaling Technology, clone C11, dilution 1:500) were incubated for one hour. Primary antibodies were detected with goat anti-rabbit (H+L) Alexa Fluor® 555-conjugated (#A21429, Life Technologies), goat anti-mouse IgM Alexa Fluor® 488-conjugated (#A21042, Life Technologies) and goat anti-mouse IgG1 Alexa Fluor® 647-conjugated (#A21240, Life Technologies) for 30 minutes. Nuclei were counterstained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (#70238421, Roche) and tissue sections were mounted with ProLong Gold antifade reagent (#P36930, Molecular Probes).

**Tissue image acquisition and analysis**
After staining, slides were scanned using the VS200 slide scanner (Olympus). Quantification of the immune cell densities was performed using Halo v3.0 software (Indica Labs). Tissue segmentation algorithm based on PanCK positivity was used to separate tumour from adjacent stroma. Phenotype determination was based on positivity for CD15 and TREM2. Immune cell densities are presented as number of cells per mm². All tissue segmentation, cell segmentation and phenotype determination maps were reviewed by a pathologist (BG).

**RNA expression/quantitative real-time PCR**

RNA isolation (TRIzol, Qiagen) and retro-transcription with ImProm-II (Promega, A3800) were performed according to the manufacturer's instructions. Quantitative PCR (qPCR) reactions were performed using GoTaq® qPCR (Promega, A6001) and the specific primers reported below. The primer sequences used were as follows: p21 forward 5'-TTCCCTCACAGGAGCAAAGT-3', reverse 5'-CGGCGCAACTGCTCACT-3'; p16 forward 5'-CGCAGGTTCTTGGTCACTGT-3', reverse 5'-TGTTACGAAAGCCAGAGCG-3'; Pai1 forward 5'-TTCAAGCCCTTGCTTGCTC-3', reverse 5'-ACACTTTTACTCGAAGTCG-3'.

**Western blot analyses and protein detection**

Tissue and cell lysates were prepared with RIPA buffer (1x PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail; Roche). Total protein concentration was measured using BCA Protein Assay Kit (Cat: 23225; Pierce, Rockford). Equal amounts of proteins were separated by SDS-PAGE and western blotted onto a 0.45 μm nitrocellulose membrane. Membranes were blocked in 5% defatted milk or 5% BSA in Tris-buffered saline containing 0.1% Tween-20 (TBST), probed with diluted antibodies and incubated at 4°C overnight. The following primary antibodies were utilized: Rabbit monoclonal C45G5 anti-HSP90 (1:1000 dilution, Cell Signaling, #4877), rabbit polyclonal Anti-Syk (phospho Y323) (1:500 dilution, ab63515), Rabbit monoclonal [EP573Y] Anti-Syk antibody (1:500 dilution, ab40781), Rabbit monoclonal D9E anti-phospho-Akt (Ser473) (1:2000 dilution, Cell Signaling, #4685), Rabbit monoclonal 11E7 anti-Akt (pan) (1:1000 dilution, Cell Signaling, #4695), Rabbit monoclonal D13.14.4E anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (1:1000 dilution, Cell Signaling, #4370), Rabbit monoclonal 137F5 anti-p44/42 MAPK (Erk1/2) (1:1000 dilution, Cell Signaling, #4695), mouse monoclonal AC-74 anti-β-Actin (1:5000 dilution, Sigma-Aldrich, A2228), Rabbit monoclonal D17C4 anti-Bcl-2 (1:1000 dilution, Cell Signaling, #3498), Rabbit monoclonal C5B11 anti-Acetyl-Histone H3 (Lys9) (1:1000 dilution, Cell Signaling, # 9649), Rabbit monoclonal D10A8 anti-β-Catenin (1:1000 dilution, Cell Signaling, # 8480), Rat monoclonal [HUGO291] Anti-p21 (1:1000 dilution, ab107099), Rabbit monoclonal [EPR20418] Anti-CDKN2A/p16INK4a (1:2000 dilution, ab211542). The protein bands were visualized using the ECL Western Blotting Substrate (Pierce).

**Human prostate samples**

Patients were identified from a cohort of men with CRPC treated at the Royal Marsden NHS Foundation Trust. All patients had given written informed consent and were enrolled in institutional protocols approved by the Royal Marsden hospital (London, UK) ethics review committee (reference no.
04/Q0801/60). Eligible patients (n = 13) had matched histologically confirmed formalin-fixed paraffin-embedded (FFPE) diagnostic (archival) and metastatic castration-resistant prostate cancer (mCRPC) biopsies. Castration-sensitive prostate cancer (CSPC) samples were obtained from primary prostate tumours and included needle core biopsies (n = 9) and transurethral resections of the prostate (TURP) (n = 4). mCRPC biopsies were obtained from primary (n = 2) and metastatic sites including lymph nodes (n = 5), bone (n = 4), liver (n = 1) and soft tissue (n = 1). Patients had at diagnosis a median age of 64 years. Tissue samples were collected from prostatic needle biopsies, transurethral resections of the prostate or prostatectomies. All tissue blocks were re-sectioned and reviewed by a pathologist who confirmed adequacy of the material.

**Bioinformatics analysis**

Different bulk RNA-seq experiments were performed: senescent MDSCs compared to not-senescent MDSCs; senescent MDSCs treated with or without Romidepsin. For both, the overall quality of sequencing reads was evaluated using a variety of tools, namely FastQC (Andrews S., 2010), RSeQC\(^{36}\), AfterQC\(^{37}\) and Qualimap\(^{38}\). Sequence alignments to the reference mouse genome (GRCm38) was performed using STAR\(^{39}\) (v.2.5.2a). Gene-expression was quantified at gene level by using the comprehensive annotations made available by Gencode\(^{40}\). Specifically, we used v20 release of the Gene Transfer File (GTF). Raw counts were further processed in the R Statistical environment and downstream differential expression analysis was performed using the DESeq2 pipeline. Genes characterized by zero expression were removed and genes with low mean normalized counts were filtered out by the Independent Filtering feature embedded in DESeq2\(^{41}\) (alpha = 0.05). Two different types of gene-set analysis were performed: gene-set enrichment analysis was performed using Camera\(^{42}\); while over-representation analysis was performed using enrichGO (clusterProfiler package) and egsea.ora function. Statistical enrichments were determined for gene-sets obtained from the KEGG collection and Gene Ontology, which are curated by the Molecular Signature DataBase (MSigDB)\(^{43,44}\). For RNA-seq data of senescent and canonical MDSCs, two different runs were performed and to remove the batch effect, removeBatchEffect of limma\(^{45}\) package was used. The database used for secreted factors derived from protein atlas secretome (https://www.proteinatlas.org). For the transmembrane protein-encoding genes we refer to Martinez-Martin et al work\(^{46}\).

To investigate the ligand-receptor interactions, we used as database CellTalkDB\(^{47}\). 9 couples were selected: for the ligands we filtered based on secreted factors up-regulated in PTEN\(^{PC/-}\) compared to WT mice (E-MTAB-9624)\(^{48}\); for the receptors we considered transmembrane proteins-encoding genes found up-regulated in senescent MDSCs. Only couples with both ligand and receptor significantly differentially expressed were considered. All graphs were produced using ggplot2 package\(^{49}\).

For single cell-RNA sequencing data, we used published data from “http://www.pradcellatlas.com”\(^{17}\). The original strategy was kept identifying the different cell populations. By expression of PMN-MDSCs
signature\textsuperscript{18}, calculated using \textit{AddModuleScore} function, we defined this population inside the “Monocytic” cluster.

Senescent-like signature of C12FDG+ MDSCs signature was composed by genes up-regulated in C12FDG+ MDSCs from our RNA-seq data. Correlation analysis were performed between Senescent-like signature and TREM2 expression. Pearson's correlation coefficient is the test statistics used for the analysis.

The human RNA-Seq datasets used were obtained from the TCGA database, which includes 481 primary prostate cancer patients and 51 normal prostate patients, and from SU2C effort\textsuperscript{50}, which includes metastatic castration resistant patients (SU2C/PCF Dream Team, PNAS 2019; cBioPortal). Correlation analysis were performed between expressions of different genes of interest. Pearson's correlation coefficient is the test statistics used for the analysis. Plots of correlation were designed using \textit{ggscatter} function for single correlation and \textit{corrplot} function for multiple comparisons. Expression of APOE and TREM2 in cancer patients was analysed based on Gleason score and based on prostate cancer subtypes (normal tissue, primary tumour, metastatic tumour). Senescence PMN-MDSCs signature was composed by Senescent-like signature of C12FDG+ MDSCs signature and PMN-MDSCs signature and was calculated using \textit{gsva} function\textsuperscript{51} (method = ssgsea). Senescence PMN-MDSCs signature was correlated with expression of TREM2 in primary tumours (TCGA) and metastatic tumours (SU2C effort). Statistical significance between two groups was determined using wilcox test, while comparison among three or more groups was evaluated with two-way ANOVA followed by Tukey’s post-hoc test.

Analysis of survival was performed using \textit{survival} R package and, specifically, using Kaplan-estimator and Cox-regression model. Log-rank test was used to calculate statistical significance in survival curves. The groups compared in survival analysis (both disease free and overall survival) were classified into quartiles based on mRNA expression level of APOE: high level corresponds to forth quantile, low level to first quantile while medium levels include second and third quantiles. The datasets used for survival analysis were TCGA database, SU2C effort\textsuperscript{50} and data already reported\textsuperscript{52}.

\textbf{Statistical analysis and reproducibility}. Data analyses used GraphPad Prism version 9. The data are presented as mean ± standard error of the mean, individual values as scatter plot with column bar graphs and were analyzed using Student’s t-tests (unpaired) by a two-sided and, when indicated, followed by Wilcoxon posttest. One-way ANOVA was used to compare three or more groups in time point analyses. Differences were considered significant when $P < 0.05$ and are indicated as NS, not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Non-parametric tests were applied when variables were not normally distributed using the SPSS statistical software. N values represent biological replicates. For animal studies, sample size was defined on the basis of past experience with the models\textsuperscript{7}, to detect differences of 20% or greater between the groups (10% significance level and 80% power). For ethical reasons the minimum number of animals necessary to achieve the scientific objectives was used. Animals were allocated randomly to each treatment group. Different treatment groups were processed identically and animals in different
treatment groups were exposed to the same environment. For bioinformatic analyses, the data were considered statistically significant with FDR<0.05.

**Declarations**

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**Author Contributions**

A.C. and A.A. developed the concept and designed the experiments. A.C., A.R.E., R.A.A., and E. Z. performed in vivo experiments. G.A., performed surgical castration in all the animals and helped with in vivo treatments. N.B., M.dA, C.S., performed in vitro experiments. B.C., established and carried out fluorescence microscopy on murine specimens. B.G., R.P., C.G., M.C., identified the patients, established and carried out fluorescence microscopy on human samples and analysed the results. A.R., checked the quality of the RNA and performed RNA sequencing. E.P., took care of the transgenic mouse model husbandry. M.T. and M.B. performed bioinformatic analyses. S.M., performed immunohistochemical experiments and analysis. S.dL., R.P.M., F.M., selected and provided human samples. M.M., helped with the supervision of students. J.D.B. supervised human experiments and interpreted the data. A.C. and A.A. interpreted the data and wrote the paper.

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**Figures**
Figure 1

Senescent-like MDSCs accumulate in the tumour microenvironment of prostate cancers. a, Experimental scheme. Briefly, Ptenpc-/- mice were injected i.p. with 20x106 BM-MDSCs differentiated from BMPs collected from C57/BL6 CD45.1 mice (Y.1). Analyses of CD45.1+ BM-MDSCs were performed at different time points in the prostate of the mice by flow cytometry. Representative dot plot of CD45.1+ cells (donor cells; Y.1) and CD45.2+ cells (host cells; Y.2) are reported. Gated on singlets. b, Representative dot plots at
different time points. Gated on CD45.1+ cells. c, Tumour CD45.1 BM-MDSCs frequencies determined by flow cytometry. d, Representative confocal images of CD11b+ (red) p16+ (green) cells in Ptenpc-/− mice prostate lesions (nuclei, blue (Dapi). Scale bar, 5μm). e, Representative dot plot (left panel) and quantification (right panel) of C12FDG+ and C12FDG- cells gated on CD45+ CD11b+ Ly6Gbright Ly6Clow cells within the prostate of Ptenpc+/+ and Ptenpc-/− mice at different stage of disease. f, Experimental scheme and gate strategy. g, qRT-PCR analyses of the indicated genes in C12FDG+ or C12FDG- tumour-sorted CD11b+ Ly6G+ cells. h, Beta Galactosidase (βGal) staining of C12FDG+ or C12FDG- tumour-sorted CD11b+ Ly6G+ cells. i, Bar plot showing enrichment pathway analysis of Gene Ontology collection in C12FDG+ tumour-sorted CD11b+ Ly6G+ cells from Ptenpc-/− mice and compared to C12FDG- tumour-sorted CD11b+ Ly6G+ cells. Statistical up-regulated pathways are reported. j, Volcano plot of secreted factors differentially expressed (306 up-regulated genes depicted in yellow and 216 down-regulated genes in blue) analysed in C12FDG+ tumour-sorted CD11b+ Ly6G+ cells from Ptenpc-/− mice and compared to C12FDG- tumour-sorted CD11b+ Ly6G+ cells. k, Bar plot showing over-representation pathway analysis in Gene Ontology collection of the up-regulated secreted factors identified in C12FDG+ tumour-sorted CD11b+ Ly6G+ cells from Ptenpc-/− mice. l, Representative dot plot and quantification of C12FDG+ and C12FDG- cells gated on CD45+ CD11b+ CD33+ cells within the tumours of patients affected with prostate cancers at diagnosis (n=12 biologically independent patients). c, e, g, Aggregated data from at least three independent experiments are reported as mean ± SE. Each dot represents an individual mouse. c, e, Statistical analyses: Two-way ANOVA followed by Tukey’s multiple comparisons test. c, Upper Statistic analyses referred to total CD45.1+ cells, lower Statistic analyses referred to C12FDG+ MDSCs. g, Statistical analyses: Two-way ANOVA followed by Šídák's multiple comparisons test. i, j, k, FDR <0.05.
Figure 2

Tumour cells producing ApoE induce senescence to MDSCs through TREM2. a, Scheme of bioinformatic analysis to obtain ligand-receptor interactions. b, Heatmap of transmembrane proteins differentially expressed between Ptenpc-/- tumour-sorted C12FDG+ MDSCs and C12FDG- MDSCs. Normalized counts are reported. c, Circle plot showing ligand-receptor interactions. The receptors are transmembrane protein encoding genes found up-regulated in Ptenpc-/- tumour-sorted C12FDG+ MDSCs. The ligands are
secreted factor genes found up-regulated in Ptenpc-/- prostate tumours compared to Ptenpc+/+ prostate tissue. The arrows connect the 9 couples identified: from ligand to receptor. d, Annotated heatmap of the correlation matrix between each ligand-receptor couple from primary tumour patients (TCGA database). The value reported is the R coefficients. e, Gate strategy (left panels) and quantification (right panel) of TREM2 expression on C12FDG+ CD11b+ Ly6Gbright and C12FDG- CD11b+ Ly6Gbright cells within the prostate of Ptenpc-/- mice. f, Experimental scheme. Briefly, Ptenpc-/- mice were injected i.p. with 20x106 BM-MDSCs differentiated from BMPs collected from C57/BL6 CD45.1 mice. Analysis of TREM2 expression on CD45.1+ cells was performed at different time points in the prostate of the mice by flow cytometry. g, Representative histograms showing TREM2 expression on C12FDG+ (Blue histogram) and C12FDG- (green histogram) BM-MDSCs treated (Senescent-like MDSCs) or not (Canonical MDSCs) with conditioned media (C.M.) from TRAMP-C1. h, Representative western blot and quantification of protein phosphorylation analysed on C12FDG+ (Blue histogram) and C12FDG- (green histogram) BM-MDSCs treated (Senescent-like MDSCs) or not (Canonical MDSCs) with C.M. from TRAMP-C1 i, Schematic representation showing C.M. fractionation (left panel) and ApoE concentration in the different fractions (right panel). j, Quantification of BM-MDSCs treated for 72h with normal media, total TRAMP-C1 C.M., TRAMP-C1 C.M. fraction >100kDa, TRAMP-C1 C.M. fraction <100kDa, TRAMP-C1 C.M. fraction >100kDa heat-inactivated. k, Quantification of BM-MDSCs treated for 72h with normal media, C.M. collected from TRAMP-C1 infected with an empty vector, C.M. collected from TRAMP-C1 infected with shApoE plasmids. l, Representative western blot on BM-MDSCs under the reported conditioning. Numbers indicate fold change in phosphorylation level compared to the untreated conditions. m, Representative quantification of BM-MDSCsWT and BM-MDSCsTREM2mut treated or not for 72h with C.M. TRAMP-C1. n, Representative western blot. Numbers indicate fold change in phosphorylation level compared to the untreated conditions. o, Experimental scheme. Briefly, Ptenpc-/- mice were lethally irradiated, transplanted with BM precursors from C57 or TREM2mut mice. p, Tumour MDSCs frequencies determined by flow cytometry. q, Tumour volume of the anterior prostate lobes is reported. r, Representative dot plot of TREM2 expression within MDSC populations in single cells (sc) RNA-seq data from human biopsies. s, Correlation plot between TREM2 expression and senescence-like signature within MDSC populations in sc RNA-seq data from human biopsies e, p, q, Each dot represents an individual mouse. h, i, j, k, m, Aggregated data from at least three independent experiments are reported as mean ± s.e.m. Each dot represents an individual sample. e, h, i, p, q, Statistical analyses: Unpaired t test. j, k, m Statistical analyses: Two-way ANOVA followed by Tukey's multiple comparisons test.
Figure 3

Senescent-like MDSCs inhibition reduces prostate cancer progression in vivo. a, Ptenpc-/- mice were lethally irradiated, transplanted with BMPs from C57 or 3MRp16 mice and treated with Ganciclovir. b, Representative dot plots of CD11b+ Ly6Gbright cells gated on CD45+ cells and C12FDG+ and C12FDG- cells gated on CD11b+ Ly6Gbright cells within the prostate of Ptenpc-/-;C57 and Ptenpc-/-;3MRp16 mice. c, Quantification of C12FDG+ and C12FDG- CD11b+ Ly6Gbright Ly6Clow cells gated on CD45+ cells determined by flow cytometry within the prostate of Ptenpc-/-;C57 and Ptenpc-/-;3MRp16 mice. d, Tumour volumes of the anterior prostate lobes. e, Representative haematoxylin and eosin and Ki-67 stainings at the endpoint. Scale bars, 100 μm. f, Quantification of Adenocarcinoma or prostatic intraepithelial neoplasia (PIN)-affected glands or normal glands in Ptenpc-/-;C57 and Ptenpc-/-;3MRp16 mice. One
tumour per mouse, three sections per mouse, ≥ 3 fields per section. g, Quantification of Ki-67 positive cells is reported as a percentage of total within the glands. One tumour per mouse, mean of three sections per mouse. h, Schematic representation of the experiment. Six-week-old NOD/SCID males were challenged subcutaneously with TRAMP-C1 cells and treated weekly with BM-MDSCs differentiated in vitro from C57/BL6 or 3MRp16 or TREM2mut mice. i, Representative dot plots of CD11b+ Ly6Gbright cells gated on CD45+ cells and C12FDG+ and C12FDG- cells gated on CD11b+ Ly6Gbright cells within the tumours of the reported groups. j, Area under the curve of the tumour and tumour weight (±s.e.m.) for each experimental group. d, g, j, Statistical analyses: Unpaired t test Student’s t-test followed by Wilcoxon signed-rank test
Figure 4

Romidepsin increases the efficacy of prostate cancer standard of care and anti-CXCR2 therapy in vivo. a, Graphical representation of the senolytic screening cascade using senescent-like BM-MDSCs. The assay is based on the assessment of cellular viability using compounds administered at single dose. b, Dose-response of the indicated drugs. Viability was measured after 3 days. The IC50 for each drug and condition is shown in the respective boxes. Data from at least two experiments are shown. c, Heatmap of
differentially expressed genes between senescent-like BM-MDSCs treated or not with Romidepsin (0.5μM for 6 h). Normalized counts are plotted. d, Bar plot of enrichment gene-set analysis performed in KEGG dataset between senescent-like BM-MDSCs treated or not with Romidepsin (0.5μM for 6 h). e, Expression levels of the reported genes analysed by RNA-seq of senescent-like BM-MDSC treated or not with Romidepsin. Statistical analyses: DeSeq2 pipeline. f, Representative western blot on senescent-like BM-MDSCs treated or not with Romidepsin (0.5μM for 6 h). g, Experimental set-up. Ptenpc-/- mice were surgical castrated, when castration resistance occurred (12 weeks after castration), they were randomly enrolled in the preclinical trial. Treatments: Enzalutamide (E, n=15), Enzalutamide in combination with Romidepsin (E+R, n=12), Enzalutamide in combination with CXCR2 antagonist (E+αC, n=12), and Enzalutamide in combination with Romidepsin and CXCR2 antagonist (E+R+αC, n=12). Tumour MDSCs frequencies determined by flow cytometry. h, Representative hematoxylin and eosin and Ki-67 staining at the endpoint. Scale bars, 60 μm. i, Quantification of Adenocarcinoma or prostatic intraepithelial neoplasia (PIN)-affected glands or normal glands at endpoint. One tumour per mouse, mean of three sections per mouse. j, Quantification of Ki-67 positive cells is reported as a fold change of positive nuclei compared to the control with single treatment. One tumour per mouse, mean of three sections per mouse. k, Experimental set-up. Tumour growth of RM1 allografts under the reported treatments. Treatments: Enzalutamide (E, n=5), Enzalutamide in combination with Romidepsin (E+R, n=4), Enzalutamide in combination with CXCR2 antagonist (E+αC, n=5), and Enzalutamide in combination with Romidepsin and CXCR2 antagonist (E+R+αC, n=4). l, Representative UMAP clustering of intra-tumoural CD45+ cells (aggregates of all the events of every condition). m, UMAP clustering of intra-tumoural CD45+ cells. Each dot plot represents the same number of aggregate events for each condition. The colours identify the different populations. n, UMAP clustering of intra-tumoural CD45+ cells. Each plot represents the density of the same number of aggregate events for each condition. o, Quantification of Apoptotic-like (Annexin+), Senescent-like (C12FDG+ and Annexin-), and Live (Annexin- and C12FDG-) CD45+ CD11b+ Ly6Gbright Ly6Clow cells within the prostate. g, One-way ANOVA followed by Holm-Šidák's multiple comparisons test. j, k, Two-way ANOVA followed by Tukey's multiple comparisons test. *p <0.05 **p <0.01 ***p <0.001 ****p <0.0001

Supplementary Files

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