Cancer-associated fibroblasts suppress CD8+ T cell infiltration and confer resistance to immune checkpoint blockade

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Statement of Significance:

Paired syngeneic models help unravel the interplay between cancer-associated fibroblasts and tumor immune evasion, highlighting the benefits of targeting fibroblast subpopulations to improve clinical responses to immunotherapy.

Key Words: Cancer-associated fibroblasts, CD8 T cells, immunotherapy, immune exclusion, Endo180, MRC2

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Abstract

Immune checkpoint blockade (ICB) promotes anti-tumor immune responses and can result in durable patient benefit. However, response rates in breast cancer patients remain modest, stimulating efforts to discover novel treatment options. Cancer-associated fibroblasts (CAF) represent a major component of the breast tumor microenvironment and have known immunosuppressive functions in addition to their well-established roles in directly promoting tumor growth and metastasis. Here we utilized paired syngeneic mouse mammary carcinoma models to show that CAF abundance is associated with insensitivity to combination αCTLA-4 and αPD-L1 ICB. CAF-rich tumors exhibited an immunologically cold tumor microenvironment, with transcriptomic, flow cytometric, and quantitative histopathological analyses demonstrating a relationship between CAF density and a CD8+ T cell-excluded tumor phenotype. The CAF receptor Endo180 (*Mrc2)* is predominantly expressed on myofibroblastic CAFs, and its genetic deletion depleted a subset of αSMA-expressing CAFs and impaired tumor progression in vivo. Addition of wild-type, but not Endo180-deficient, CAFs in co-implantation studies restricted CD8+ T cell intratumoral infiltration, and tumors in Endo180 knockout mice exhibited increased CD8+ T cell infiltration and enhanced sensitivity to ICB compared to tumors in wild-type mice. Clinically, in a trial of melanoma patients, high *MRC2* mRNA levels in tumors was associated with a poor response to αPD-1 therapy, highlighting the potential benefits of therapeutically targeting a specific CAF subpopulation in breast and other CAF-rich cancers to improve clinical responses to immunotherapy.

Introduction

The therapeutic blockade of immune checkpoint proteins such as cytotoxic T-lymphocyte associated antigen-4 (CTLA-4) and programmed death ligand-1 (PD-L1) promotes anti-tumor immunity and confers durable clinical benefit in a subset of cancer patients (1). Clinical responses to ICB are observed in multiple cancer types, but are often restricted to patients whose tumors are highly mutated, express high levels of PD-L1, and are infiltrated by a sufficiently diverse repertoire of tumor-specific CD8+ T cells (2,3). Breast cancers, which commonly lack these features, have long been considered immunologically silent and not amenable to ICB treatment. Nevertheless, evidence associating lymphocytic infiltration with better prognosis in both triple negative breast cancer (TNBC) and HER2+ disease (4), has prompted exploration of ICB as a treatment option in numerous clinical trials.

Following the reporting of the KEYNOTE-522 and -355 trials (5,6), the FDA granted approval for the PD-L1 inhibitor, pembrolizumab, in combination with chemotherapy for the treatment of patients with advanced TNBC whose tumors are PD-L1 positive, and for high-risk early-stage TNBC as neoadjuvant treatment with continued use as single agent adjuvant treatment following surgery. However, despite these advances, only a proportion of breast cancer patients treated with ICB therapy experience durable responses, even when considering mutational status or checkpoint inhibitor expression (7). Thus, elucidating the determinants of ICB response will be key to developing new treatment strategies that potentiate anti-tumor immune responses and improve outcomes in patients for whom ICB treatment is not currently effective.

Accumulating evidence has demonstrated that the overall proportion, phenotype and distribution of immune cells within the tumor microenvironment (TME) is important in determining responses to ICB (4,7). Indeed, patients with immunologically hot tumors, characterized by PD-L1 expression and CD4+ and CD8+ T cells positioned in proximity to tumor cells, exhibit better responses to anti-

PD-L1/PD-1 therapy than those whose tumors are characterized by a paucity of these effector populations, or an 'immune-excluded' phenotype (8). CAFs, a major constituent of the breast TME, are a heterogenous population of cells with an emerging role in modulating anti-tumor immunity and influencing responses to treatment (9-14). CAFs directly contribute to tumor growth, metastasis and angiogenesis, but may also promote establishment of an immunologically cold tumor phenotype, either through direct inhibition of T cell infiltration and activity, or by promoting recruitment of other immunosuppressive cell types.

Despite these advances, studies investigating the relationship between CAFs and anti-tumor immunity have been stifled by the well documented variability between CAFs in different cancers, the lack of specific CAF markers, and the paucity of suitable preclinical models (15). Here we have utilized paired syngeneic mouse mammary carcinoma models that differ in their CAF abundance to better characterize the relationship between CAF prevalence, immunomodulation, and sensitivity to ICB. We demonstrate that CAF-rich tumors exhibit an immunologically cold, CD8+ T cell excluded TME and that targeting CAF subsets via genetic deletion or downregulation of the myofibroblastic CAF (myCAF) restricted receptor Endo180 (*Mrc2*) facilitates CD8+ T cell infiltration and enhances sensitivity to ICB, findings corroborated in human clinical samples where high Endo180 expression is associated with poor responses to αPD-1. Given the heightened interest in using immunotherapy to treat breast cancer, together with the improved understanding of the diversity of CAF biology, targeting Endo180 offers a novel CAF-associated approach to improving ICB responses.

Materials and Methods

Reagents and cells

Cells were from Isacke laboratory stocks and were subject to mycoplasma testing (MycoAlert Mycoplasma Detection Kit, Lonza) on a monthly basis and used within 8 passages of thawing. Adherent cells were cultured at 37°C in a tissue culture incubator with humidified air, supplemented with CO_2 to 5%. Unless otherwise stated, cells were maintained in DMEM plus 10% FBS and 1% penicillin/streptomycin. D2A1 cells were provided by Ann Chambers (University of Western Ontario). The generation of the metastatic D2A1-m2 subline, and D2A1 and D2A1-m2 cells expressing mCherry and luciferase2 have been described previously (16,17). Whole exome sequencing of cell lines was performed and analyzed as described previously (16).

GFP+ normal mammary fibroblasts (NMFs) and 4T1 tumor-derived CAFs were isolated from Ub-GFP BALB/c mice (18), as previously described (16). CAFs were immortalized using an HPV-E6/E7-puromycin retrovirus (provided by Fernando Calvo). NMFs were immortalized using an HPV-E6/E7-neomycin lentivirus (Applied Biological Materials). Primary fibroblasts were incubated with virus-containing media (1:1 dilution with fresh complete media plus 8 μg/mL polybrene) for 48 hours. CAFs transduced with non-targeting or Endo180 targeting shRNAs have been described previously (16).

Antibodies, and the dilutions used, are detailed in Supplementary Table S1.

Cellular assays

For T cell proliferation experiments, spleens from naive BALB/c mice were dissociated through 40 μm filters. After red blood cell lysis, T cells were isolated using the EasySep mouse T cell isolation kit, and labelled with 1 mM CFSE, and plated in complete RPMI media supplemented with 50 mM β-mercaptoethanol into 96-well plates coated with 1 μg/mL αCD3e antibody. Naive CFSE-stained T cells

were cultured for 4 days at 37°C with 5 μg/mL αCD28 antibody and 10 ng/mL IL2 (BD Biosciences) in complete RPMI media or CAF-conditioned complete RPMI media. Conditioned medium was generated by culturing CAFs in complete medium for 72 hours and filtering through a 40 μm filter. After 4 days, cells were stained with an anti-mouse CD16/CD32 antibody for 10 minutes at room temperature to block non-specific binding of staining antibodies. APC-conjugated αCD45, PE-conjugated αCD4 and PerCP/Cy5.5-conjugated αCD8 antibodies were added to cells at specified dilutions (Supplementary Table S1) and incubated at 4°C for 30 minutes. Cells were stained with DAPI and the CFSE signal in gated live CD4+ and CD8+ T cells was measured by flow cytometry (LSRII flow cytometer, BD Biosciences).

For PD-L1 expression analysis, cells were cultured with 10 ng/mL recombinant mouse IFNγ (BioLegend). After 24 hours, cells were stained on coverslips and imaged on a Leica SP8 confocal microscope or detached and stained with an APC-conjugated αPD-L1 antibody, or an isotype control antibody. Live cells (DAPI-negative) were assessed for PD-L1 expression by flow cytometry (LSRII flow cytometer, BD Biosciences).

In vivo procedures

All animal work was carried out under UK Home Office Project Licenses 70/7413, P6AB1448A and PP4856884 granted under the Animals (Scientific Procedures) Act 1986 (Establishment Licence, X702B0E74 70/2902) and was approved by the "Animal Welfare and Ethical Review Body" at The Institute of Cancer Research (ICR). Mice with a genetic deletion in Endo180 (*Mrc2*) (19) were backcrossed for at least six generations with BALB/c (Charles River) mice. Genotypes were confirmed by PCR. All mice were housed in individually ventilated cages, monitored daily by ICR Biological Services Unit staff and had food and water *ad libitum*. Mice were weighed at least two times per week.

Tumor cells (5 x 10^5 4T07, 5 x 10^4 4T1, 2 x 10^5 D2A1 or 2 x 10^5 D2A1-m2) were implanted in PBS orthotopically into the 4th mammary fat pad of 6-8 week old female BALB/c or NOD scid gamma (NSG) mice (Charles River) under general anesthesia. 4T07 and D2A1 cells were either implanted alone or mixed with 6 x 10^5 NMFs or CAFs. Tumor growth was measured every 2 to 3 days, and tumor volume was calculated as 0.5236 x [(width + length)/2]^3. Tumor growth rates were calculated as previously described (20).

For immune checkpoint blockade treatment, mice received, via intraperitoneal injection, 10 mg/kg of αCTLA-4 or αPD-L1 antibodies (Supplementary Table S1) either as single agents or in combination. Control mice received 10 mg/kg of respective mouse IgG_1 kappa and/or IgG_1 D265A isotype controls antibodies. Unless otherwise stated, αCTLA-4 was given on days 7, 11, 14, 18, 21 and 25 post cell implant, and αPD-L1 was given on days 5, 7, 11, 14, 18 and 21 post cell implantation. Mice were culled individually when tumors reached 17 mm in diameter (survival analysis) or culled as a group when the first tumor reached 17 mm in diameter (tumor growth analysis).

Histology

For αSMA and CD8 immunohistochemistry, tissues were removed and fixed overnight at room temperature in 4% paraformaldehyde and embedded in paraffin wax after processing in a Tissue-Tek VIP automatic tissue processor (Sakura, Finland). 3-4 μm sections were cut from formalin-fixed paraffin-embedded (FFPE) tissue blocks, dewaxed in xylene, rehydrated through ethanol washes and stained using hematoxylin and eosin (H&E) or subjected to high-temperature antigen retrieval, depending on primary antibody requirements. Slides were cooled at room temperature before incubation with antibodies. Stained sections were scanned on the NanoZoomer Digital Pathology (Hamamatsu). All images were quantified in a blinded fashion. αSMA staining was analyzed in ImageJ from ≥ 6 randomly selected 1 mm^2

fields of view per tumor section. HRP images were color deconvoluted using the ImageJ H DAB vector and converted into 8-bit images and the % αSMA stained area quantified in a blinded fashion (threshold, 0-130). The same thresholding was applied to all images from the same experiment. For quantitative spatial analysis of CD8+ cell infiltration, QuPath software (21) was used to count the number of positively stained cells in 8 randomly selected peripheral and 8 central 1 mm^2 regions of tumor tissue. To examine intratumoral heterogeneity, 0.25 mm^2 matched regions from serial αSMA and CD8 stained sections were selected and quantified as described above. For all immunohistochemistry quantification, areas of necrosis were avoided and data shown are mean values per tumor section.

Tumor dissociation and flow cytometry

For analysis of tumor-infiltrating immune cells, tumors were removed and single-cell suspensions generated using a tumor dissociation kit in combination with a gentleMACS Octo Dissociator with the program 37C_m_TDK_2 according to the manufacturer's protocol (Miltenyi Biotec). Samples were subsequently applied to a 70 μm MACS SmartStrainer and washed in PBS and incubated in RBC lysis buffer (Sigma) for 5 minutes at room temperature. Samples were resuspended in FACS buffer for staining.

Single-cell suspensions were stained with Fixable Viability Dye eFluor 455UV (Thermo Fisher Scientific) for 20 minutes at 4°C. Cells were subsequently stained with an anti-mouse CD16/CD32 antibody for 10 minutes at room temperature to block non-specific binding to Fc receptor expressing cells. Panels of directly conjugated antibodies against cell surface markers were added to cell suspensions at specified dilutions and incubated at 4°C for 30 minutes. Cells were washed twice in PBS before being fixed and permeabilized overnight using the FoxP3/Transcription factor staining buffer set (eBioscience). Panels of directly conjugated antibodies

against intracellular markers were then added to cells for 60 minutes at 4°C. Following further washing, cells were fixed in 4% paraformaldehyde solution for 15 minutes at 4°C. Finally, cells were resuspended in FACS buffer and analyzed on a BD LSRFortessa or BD LSRII flow cytometer. Stained cells or fluorescent UltraComp eBeads (eBioscience 01-2222) were used for compensation set up. Data analysis was performed using FlowJo software (Tree Star Inc.). Gates were set using appropriate fluorescence minus one (FMO) controls.

NanoString profiling of tumors

Tumors were harvested and snap frozen in liquid nitrogen. Frozen tissue was lysed in RLT buffer (Qiagen) containing 1/100 β-mercaptoethanol in Hard-Tissue homogenizing CK28 tubes and homogenized using a Precellys tissue homogenizer (Bertin-Corp) for 2 minutes. RNA was extracted and purified using the Qiagen RNeasy kit according to the manufacturer's protocol. RNA was hybridized with the NanoString PanCancer mouse immune-oncology (IO) 360 Panel. Raw NanoString data was pre-processed using R package NanoStringNorm (v1.2.1). Differential mRNA abundance analysis was performed using voom (TMM normalization), with R package limma (v3.34.9) (22). Genes with an absolute $\log_2$ fold change of >1 and an adjusted *P* value of <0.05 were considered significant. For immune cell population abundance analysis, NanoString curated genesets representing specific cell types were used. For each cell type, genesets with more than two genes were further reduced to the largest positively correlated cluster of genes by running hierarchical clustering on Spearman's correlation distance, followed by identification of optimal number of clusters using Silhouette score. Genes were kept if they all showed pairwise Spearman's $P > 0.5$. A similar approach was used for the comparison of CD8 T effector (NanoString), fibroblast TGFβ response signature (F-TBRS) (23), TGFβ Signaling (NanoString) and Wnt Signaling signatures (NanoString). All analyses were performed in R statistical programming language (v3.4.4).

TCGA breast cancer cell type abundance estimates

Using the TCGA breast cancer (BRCA) RNA-Seq profiles (RSEM normalized) (24), relative cell type abundances estimates were created using the Consensus^{TME} (25). The statistical metric used for the estimation of scores was set to "*ssgsea*". Hierarchical clustering was performed on both rows and columns using "euclidean" as the distance measure and "complete" as the agglomeration method. Association between the cell type abundance estimates and selected marker genes was computed using the Spearman's rank correlation.

Analysis of datasets

Anti-PD-1 immunotherapy trial: Pre-processed RNA-Seq profiles of pre-treatment melanomas (26) were downloaded from GEO identifier GSE78220. Differential gene expression for selected marker genes in anti-PD-1 non-responders (patients with progressive disease) and responders (complete or partial response) was assessed using an unpaired non-parametric Wilcox rank-sum test. Single cell RNA sequencing (scRNA-Seq): scRNA-Seq data from 26 human breast cancers (27) was visualized on the Broad Institute Single Cell portal at https://singlecell.broadinstitute.org/single_cell/study/SCP1039.

Statistics

Statistical tests were performed using GraphPad Prism 8. Unless otherwise indicated, data are presented as ± standard error of the mean, and comparisons between two groups were made using two-tailed, unpaired Student's *t*-test. If more than 2 groups were compared, one-way ANOVA analysis was performed with Dunnett's test for multiple comparisons. For all correlation analysis, R^2 values were calculated from the Pearson correlation coefficient. For survival analysis, data was analyzed with a log-rank test, comparing only two groups at a time. Box plots show

median and 25^{th}–75^{th} quartiles, whiskers show minimum and maximum. *P* values are reported as follows: $P \geq 0.05$ (ns, non-significant); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Data availability

The whole exome sequencing data has been deposited at the European Nucleotide Archive (ENA) and is available under the accession number PRJEB43908 (D2A1, SAMEA8418396; D2A1-m2, SAMEA8418398; normal BALB/c, SAMEA8418401). The NanoString data has been deposited at Zenodo at https://doi.org/10.5281/zenodo.647812.

Results

CAF abundance is associated with insensitivity to ICB

To investigate the role of CAFs in modulating the breast tumor immune microenvironment, and to determine whether CAFs influence responses to ICB, the paired BALB/c-derived, 4T1 and 4T07 mouse mammary carcinoma cell lines (28) were used. As previously reported (29), when implanted orthotopically into the mammary fat pad of syngeneic BALB/c mice, 4T1 cells give rise to primary tumors defined by an abundance of intratumoral αSMA+ CAFs, whereas 4T07 tumors have significantly fewer (**Fig. 1A;** Supplementary Fig. S1A). Notably, 4T07 tumors grow poorly in immunocompetent BALB/c mice, either when implanted orthotopically (**Fig. 1B**) or subcutaneously (Supplementary Fig. S1B), but grow readily in immunodeficient NSG mice (**Fig. 1B**). By contrast, 4T1 tumors exhibit similar growth kinetics in both strains (**Fig. 1B**), indicating that an intact immune response may be a major growth-restricting factor for 4T07, but not 4T1 tumors. Moreover, when NSG-derived 4T07 tumor fragments containing stromal components are transplanted into BALB/c mice, or when 4T07 cells are co-implanted with 4T1 tumor-derived CAFs, tumor growth is enhanced (Supplementary Fig. S1B and C), prompting us to investigate whether CAFs mediate this effect through inhibition of anti-tumor immunity.

As previously reported (30,31), the 4T1 tumor model is largely insensitive to ICB, with single agent αCTLA-4 or αPD-L1 treatment promoting modest tumor growth inhibition, but ultimately failing to eradicate primary tumors (Supplementary Fig. S1D). Furthermore, despite clinical evidence suggesting that ICB combinations are more efficacious than single agent treatment (32), combination αCTLA-4 and αPD-L1 treatment fails to drive any complete tumor regression (**Fig. 1C**). Given the poor growth kinetics of 4T07 tumors in immunocompetent mice, sensitivity of this model to ICB treatment could not be determined.

To further understand the relationship between CAFs and ICB sensitivity in additional syngeneic breast cancer models, the paired BALB/c-derived D2A1 mouse mammary carcinoma cell line and its metastatic D2A1-m2 subline (17) were examined. Orthotopic D2A1-m2 tumors are abundant in αSMA+ CAFs, whereas parental D2A1 tumors are CAF-poor (**Fig. 1D**; Supplementary Fig. S1E). The sensitivity of both models to combination ICB treatment was assessed using the dosing regimen outlined in **Fig. 1E**. In contrast to the 4T07/4T1 models, orthotopic implantation of BALB/c mice with either D2A1 or D2A1-m2 cells gives rise to primary tumors with similar and reproducible growth kinetics (**Fig. 1F,** isotype controls). Combined blockade of CTLA-4 and PD-L1 has a limited effect on D2A1-m2 tumor growth rate (6% inhibition), with no statistically significant extension of median survival and no complete responders (**Fig. 1F**). By contrast, combination αCTLA-4 and αPD-L1 treatment suppresses D2A1 tumor growth (23% growth rate inhibition) and significantly extends median survival from 41 to 47 days post tumor cell implantation (**Fig. 1F**), findings reproduced in an independent experiment (Supplementary Fig. S1F). One mouse exhibited complete tumor regression and developed an immunological memory to D2A1 re-challenge, indicative of an adaptive anti-tumor immune response (**Fig. 1G**).

Insensitivity to ICB is independent of tumor cell intrinsic factors

A high tumor mutational burden (TMB), defined as the number of nonsynonymous mutations per megabase (Mb) of total genomic DNA, drives neoantigen generation and is associated with improved ICB treatment response across multiple cancer types (2,3). Whole-exome sequencing was used to identify cell-intrinsic genetic differences that may underlie the differential sensitivity of the D2A1 and D2A1-m2 models to ICB. Unsurprisingly, given that the D2A1-m2 subline is derived from parental D2A1 cells, when compared to a reference BALB/c mouse genome, the D2A1 and D2A1-m2 cells exhibit comparable copy number variation profiles (**Fig.**

2A). Moreover, despite being less sensitive to ICB treatment, D2A1-m2 cells have a higher somatic non-synonymous mutational burden (**Fig. 2B**). Many of these nonsynonymous mutations (*n*=206) are shared, but the cell lines also carry distinct mutations (**Fig. 2C**), indicating that the D2A1 line comprises a heterogeneous population, and that the D2A1-m2 cell subline likely diverges from the parental line during *in vivo* passage. Similar findings are observed when considering only exonic mutations in immune-related genes (**Fig. 2C**; Supplementary Table S2). The paucity of immune gene mutations, coupled with transcriptional profiling of the D2A1 and D2A1-m2 cell lines cultured *in vitro* failing to identify robust changes in immune regulators (17), indicates that differences in anti-tumor immune responses do not result from intrinsic mutational or transcriptional differences between these cell lines. Similarly, although flow cytometry analysis reveals significantly higher levels of PD-L1 on both immune and tumor cells within D2A1 tumors compared to D2A1-m2 tumors (**Fig. 2D**), in cultured D2A1 and D2A1-m2 cells, PD-L1 expression assessed by flow cytometry and immunostaining is similar at baseline (unstimulated) and following stimulation with IFNγ (**Fig. 2E and F**). These data indicate that elevated PD-L1 expression in D2A1 tumors reflects differences in immune activity within the TME, rather than intrinsic differences between the two cell lines.

αSMA+ CAF abundance is associated with an immunologically cold TME

To explore further the characteristics of D2A1 and D2A1-m2 tumors underlying their differential sensitivity to ICB, we utilized the NanoString PanCancer mouse Immune-Oncology (IO) 360 gene expression panel to analyze the transcriptomes of established, untreated tumors of equivalent size (Supplementary Fig. S2A). Principal component analysis shows that D2A1 and D2A1-m2 tumors cluster separately (Supplementary Fig. S2B), and differential expression analysis reveals an elevated expression in D2A1-m2 tumors of numerous genes involved in fibroblast activation including *Wnt5a*, *Loxl2*, *Edn1*, *Inhba* and *Wnt11* (**Fig. 3A**; Supplementary Fig. S2C).

By contrast, the majority of genes with higher expression in D2A1 tumors have known roles in anti-tumor immunity, including chemoattractants such as *Cxcl9* and *Cxcl10*, and the interferon-inducible gene, *Ifitm1*.

Unsupervised hierarchical cluster analysis using NanoString defined immune gene sets (Supplementary Fig. S2D) reveals clustering by tumor model (**Fig. 3B**) with D2A1-m2 tumors exhibiting a significantly lower abundance of total leukocytes (CD45), CD8+ T cells, neutrophils and NK cells (**Fig. 3C**). Similarly, a clinically-relevant gene set defining CD8+ T effector cells that is associated with enhanced response to αPD-L1 treatment in metastatic urothelial carcinoma patients (23) (Supplementary Fig. S2E), is expressed at significantly higher levels in D2A1 tumors (**Fig. 3D**), linking the effector function of tumor-infiltrating immune cells with sensitivity to ICB. Interestingly, the abundance of regulatory T cells (T_{regs}) and macrophages, both cell types with an ability to suppress T cell recruitment and function (33,34), does not significantly differ between models, suggesting that they do not directly contribute to the lack of effector immune cell activity observed in D2A1-m2 tumors (**Fig. 3E**). Finally, we examined the expression of signatures associated with stromal activation (Supplementary Fig. S2E). Consistent with the immunohistochemical analysis of the tumor stroma (**Fig. 1D**), D2A1-m2 tumors exhibit elevated expression of fibroblast activation signatures, exemplified by a fibroblast TGFβ response signature (F-TBRS) associated in both experimental models and clinical samples with an immune exclusion phenotype (23), and the NanoString TGFβ and Wnt signaling signatures (**Fig. 3F**). When considering all tumors, these TGFβ and fibroblast activation signatures were associated with a lower abundance of CD8+ T cell transcripts (**Fig. 3G).**

To confirm that αSMA+ CAF abundance is associated with an immunologically cold TME, 4T07/4T1 and D2A1/D2A1-m2 primary tumors were assessed for their immune cell content via both flow cytometry and immunohistochemistry. To control for temporal changes in immune cell composition,

tumors were collected simultaneously, resulting in analysis of 4T1 tumors that were larger than 4T07 tumors, whilst D2A1 and D2A1-m2 tumors were of a similar size (Supplementary Fig. S3A). Consistent with the NanoString profiling, CAF-rich D2A1-m2 tumors contain fewer CD8+ T cells compared to CAF-poor D2A1 tumors (**Fig. 4A** and **B**; Supplementary Fig. S3B). Equivalent findings were obtained with CAF-rich 4T1 and CAF-poor 4T07 tumors, with 4T1 tumors showing a significant reduction in CD8+ T cell content (**Fig. 4C** and **D**). Moreover, in intratumoral analysis, regions of high αSMA+ cell abundance are significantly lower in CD8+ T cells (**Fig. 4E**), together implicating a role for αSMA+ CAFs in limiting CD8+ T cell content. In addition to the paucity of CD8+ T cells, immunologically cold tumors are also characterized by T cells that lack expression of markers of cytotoxicity, such as granzyme B, and activation, such as PD-1 (35). Phenotypic flow cytometry analysis reveals that CAF-rich D2A1-m2 and 4T1 tumors contain fewer granzyme B and PD-1 expressing CD8+ T cells than the CAF-poor D2A1 and 4T07 tumors (**Fig. 4F** and **G**). Despite these marked differences in CD8+ T cell abundance and activity, consistent with earlier transcriptomic analysis (**Fig. 3E**), immunologically colder D2A1-m2 and 4T1 tumors are no more abundant in cells with recognized immunosuppressive functions including T_{regs}, macrophages and neutrophils (Supplementary Fig. S3C).

To address directly the role of CAFs in modulating the CD8+ T cell content of tumors, GFP+ CAF cultures were generated from orthotopic 4T1 tumors grown in Ub-GFP BALB/c mice (Supplementary Fig. S4A). Sorted cells express the fibroblast markers αSMA, PDGFRα and Thy1.2 (CD90.2) (36), but not the immune cell marker CD45 (Supplementary Fig. S4B). As previously reported by others (11), fibroblast conditioned media significantly inhibits *in vitro* proliferation of both CD4+ and CD8+ T cells (Supplementary Fig. S4C and D). Moreover, as previously observed with the 4T07 model (Supplementary Fig. S1C), co-implantation of D2A1 tumor cells with CAFs, but not normal mouse mammary gland fibroblasts (NMFs), promotes tumor growth (**Fig. 5A**). Neither NMFs nor CAFs form tumors when implanted alone into

syngeneic BALB/c mice (data not shown). As reported recently in a CAF 'Consensus Statement' (15), an issue with this approach is that host-derived fibroblasts outgrow co-implanted CAFs during tumor development limiting their utility in longer-term efficacy studies. Indeed GFP+ cells represent a relatively minor proportion of live cells within these tumors, whilst the proportion of CD45- Thy1.2+ cells is higher (**Fig. 5B;** Supplementary Fig. S4E). Nevertheless, flow cytometry analysis of the immune cell composition reveals that co-implantation of D2A1 tumors cells with CAFs, but not NMFs, results in reduced CD8+ T cell levels (**Fig. 5B**) and whilst not significant, across all tumors there was a significant inverse correlation between the abundance of intratumoral CAFs and CD8+ T cells (**Fig. 5C**), as observed in the D2A1/D2A1-m2 and 4T07/4T1 models (**Fig. 3G; Fig. 4E**). Interestingly, in contrast to the fibroblast-mediated inhibition of T cell proliferation observed *in vitro* (Supplementary Fig. S4C and D), co-implantation with CAFs has no effect on expression of the activation/proliferation markers PD-1 and Ki-67 on CD8+ T cells (**Fig. 5D**), nor the abundance of tumor-associated T_{regs}, neutrophils and macrophages (Supplementary Fig. S4F), indicating that within the TME, CAFs affect CD8+ T cell accumulation without directly inhibiting their proliferation, or indirectly through promoting expansion of immunosuppressive cells.

D2A1-m2 tumors exhibit a CD8+ T cell-excluded phenotype

Whilst the type and density of immune cells within tumors can predict survival across multiple cancer types (37), accumulating evidence suggests that the spatial distribution of immune cells plays an important role in determining patient survival and sensitivity to ICB treatment (38,39). Given that the presence of CAFs did not inhibit CD8+ T cell activation or proliferation within tumors (**Fig. 5D**), we sought next to determine whether CAF abundance was associated with their physical exclusion from the tumor mass. CAF-rich D2A1-m2 tumors, but not CAF-poor D2A1 tumors, are characterized by low levels of centrally-located, infiltrating CD8+ T cells and their

accumulation at the tumor periphery (**Fig. 5E** and **F;** Supplementary Fig. S5A), a phenotype also evident in spontaneous metastatic lung lesions in D2A1-m2 tumor-bearing mice (Supplementary Fig. S5B). This difference in CD8+ T cell distribution between the D2A1 and D2A1-m2 models persists upon combination αCTLA-4 and αPD-L1 treatment, suggesting that ICB treatment alone cannot reverse the CD8+ T cell-excluded phenotype (**Fig. 5E** and **F**; Supplementary Fig. S5C). Strikingly, ICB treatment significantly increases the overall density of CD8+ T cells in D2A1 tumors, but not in excluded D2A1-m2 tumors (**Fig. 5G**). In neither model does ICB treatment increase CAF abundance (Supplementary Fig. S5D).

Impairment of an Endo180+ CAF subpopulation promotes CD8+ T cell infiltration and sensitizes tumors to ICB

Characterization of CAFs based on FACS isolation of subpopulations or scRNA-Seq has revealed considerable phenotypic and functional diversity (40-43). In a recent study (16), we examined the role of the CAF receptor Endo180 (*Mrc2*) in tumor progression. Endo180 (also known as uPARAP) is a collagen-binding endocytic receptor whose expression is restricted to fibroblasts (Supplementary Fig. 5E) and upregulated on CAFs compared to normal tissue fibroblasts (16). Within CAF subpopulations, highest levels of Endo180 expression are seen on myCAFs (11) (**Fig. 6A**). Importantly, adult mice with a genetic deletion of Endo180 have no overt phenotype (19,44), however, when implanted with syngeneic tumor cells show impaired tumor progression (16) and a tumor stroma marked by reduced intratumoral fibrillar collagen content and a depletion in αSMA+ CAFs (**Fig. 6B**).

To test the hypothesis that Endo180+ CAFs play a role in the establishment of a tumor-promoting TME by contributing to CD8+ T cell exclusion, we first examined CD8+ T cell distribution in D2A1 tumors, established through orthotopic implantation of tumor cells alone or co-implanted with CAFs transduced with non-targeting (shNTC) or Endo180 targeting (shE180) shRNAs (Supplementary Fig.

S5F). Co-implantation with Endo180-expressing CAFs reduces the number of tumor-associated CD8+ T cells and significantly reduces the proportion located centrally; effects that are lost when tumor cells are co-implanted with Endo180-deficient CAFs (**Fig. 6C**; Supplementary Fig. S5G). We addressed the relevance of these findings in human breast cancers from the TCGA dataset. Cell type abundance estimates using Consensus^{TME} (25) reveal an inverse correlation between cytotoxic and CD8+ T cell abundance, and expression of *MRC2* (Endo180) and *ACTA2* (αSMA) in basal-like and HER2-enriched breast cancers, with a similar but weaker correlation with a broader fibroblast signature (Supplementary Fig. 6A). No such correlation is observed in ER+ luminal A or B subtypes, or with endothelial cell abundance in any breast cancer subtype.

Given that knockout of Endo180 results in reduced intratumoral αSMA+ staining (**Fig. 6B**) (16) it was necessary to address whether loss of Endo180 expression results in an overall reduction of CAFs, or preferentially affects a specific CAF subset. mCherry-tagged D2A1-m2 cells were implanted orthotopically into Endo180 wild-type (WT) or knockout (KO) BALB/c mice (**Fig. 6D**). Analysis of the tumors by flow cytometry reveals no differences in CD31+ endothelial cell content, as previously reported (15), or in CD45+ immune cell content (Supplementary Fig. S6B and C). Similarly, tumors do not differ in their abundance of total CAFs, defined via negative selection as mCherry-/CD45-/CD31- cells or by staining with the pan-fibroblast marker Thy1.2 (**Fig. 6D**). Importantly, whilst there is a reduction in αSMA+ cell abundance in KO tumors, there is a contrasting, non-significant increase in PDGFRα+ cells, indicating that Endo180 deletion skews the composition of fibroblast subsets, as has been reported with other interventions (45), without resulting in a general CAF depletion.

To determine whether the reduction in αSMA+ CAFs enhances sensitivity to ICB, D2A1-m2 tumors in WT and KO mice were treated with a combination of αCTLA-4 and αPD-L1 antibodies. Consistent with the data in **Fig. 1F**, ICB has no

effect on D2A1-m2 tumor growth or survival of WT mice, however, in Endo180 KO mice ICB treatment significantly suppresses D2A1-m2 tumor growth rate (20% inhibition), extends median survival by 5 days (**Fig. 7A**), and reduces spontaneous metastasis to the lungs (Supplementary Fig. S6D). Remarkably, two ICB-treated KO mice exhibited complete tumor regression and developed an immunological memory to D2A1-m2 re-challenge (**Fig. 7B**). Quantitative immunohistopathology reveals a significant reduction in αSMA+ CAFs, an increased number of tumor-associated CD8+ T cells, and a significantly increased proportion of central CD8+ T cells in the KO mice (**Fig. 7C**; Supplementary Fig. S6E), indicating that impairment of a subset of CAFs can potentiate sensitivity to ICB through optimizing CD8+ T cell positioning, limiting tumor progression.

Finally, in support of these findings, as no appropriate breast cancer dataset exists, we examined a gene expression dataset of melanomas for which clinical outcome data following treatment with αPD-1 ICB is available. Gene expression signatures of myCAFs, but not signatures of inflammatory CAFs (iCAFs) or normal fibroblasts are elevated in non-responders compared to responders (14). In keeping with this report, we show no significant difference in expression of the pan-fibroblast marker *THY1* (CD90) in tumors from responding and non-responding patients, but a significant elevation in *MRC2* and *ACTA2* expression in non-responders (**Fig. 7D**).

Discussion

The past decade has seen a rapid expansion of ICB trials in a broad range of cancer types, changing treatment paradigms (1). However, whilst ICB treatment can result in durable patient responses, there remains an urgent need to understand why success is limited to a minority of patients, and why some cancer types, such as breast cancer, respond particularly poorly. In recent years, an improved understanding of the mechanisms underpinning breast cancer's insensitivity to ICB have provided valuable insight into potential strategies to enhance clinical outcomes (3), with an increasing focus on the role of the TME.

CAFs have been reported to promote establishment of an immunologically cold TME through both direct modulation of immune cell phenotypes, and indirectly via inhibition of immune cell recruitment and infiltration into the developing tumor (9-14). Evidence that this can directly impact sensitivity to ICB has come from strategies directly targeting CAFs or related signaling pathways. For example, inhibiting the ROS-producing enzyme NOX4, which has elevated expression in CAFs, promotes CD8+ T cell infiltration and enhances response to ICB therapy (46). Similarly, it has been reported that the expression of the proline isomerase Pin1 drives formation of a desmoplastic CAF-rich stroma in pancreatic adenocarcinomas and reduces PD-L1 expression on tumor cells, and that Pin1 targeting reduces desmoplasia and enhances sensitivity to PD-1 blockade (47).

Despite these advances, studies of CAF function have been hampered by three main issues. First, limitations in *in vivo* experimental models where syngeneic models do not adequately represent the genetic and microenvironmental heterogeneity of patients tumors (48). Second, that CAFs cultured *in vitro* alter their phenotype and do not fully represent CAFs *in vivo*, and the caveats associated with the commonly used approach of co-implanting fibroblasts with tumor cells (15). Finally, CAFs, like tumor cells, represent a heterogeneous population of cells with different functional properties ascribed to different subpopulations (14,27,40-

43,49,50), including both tumor-promoting and tumor-restraining functions (12). To address these experimental limitations, we initiated this project using two pairs of mouse mammary carcinoma cell lines - 4T07/4T1 (both derived from the 410.4 cell line) and the D2A1 cell line and its metastatic derivative D2A1-m2 - which, when implanted orthotopically into syngeneic mice, give rise to tumors with strikingly different CAF content (17,29), allowing comparative investigation into the role of a CAF-rich TME on therapeutic responses.

Our findings reveal how CAF-rich models are insensitive to combination ICB treatment and, whilst poorly infiltrated with CD8+ T cells, do not differ in the infiltration of known immunosuppressive cells nor in their TMB when compared to paired CAF-poor models. The abundance of CAFs in these models is inversely correlated with CD8+ T cell content, and the CAF-rich models exhibit a CD8+ T cell-excluded phenotype, providing further evidence to support exploration of CAF targeting strategies to enhance ICB responses in breast cancer. However, optimal CAF targeting requires acknowledgment of CAF intra- and inter-tumoral heterogeneity, targeting specific CAF subpopulations to limit or eliminate CAFs that inhibit CD8+ T cell recruitment, whilst retaining CAF populations with tumor-restraining properties.

In a seminal study of the human breast cancer stroma, four CAF subsets were identified that exhibit distinct immunomodulatory properties and accumulate differentially in human BC subsets (43), with the αSMA^{high} CAF-S1 subset, shown to promote establishment of an immunosuppressive TME and to be enriched in TNBC compared to luminal breast cancers. Subsequent scRNA-Seq of CAF-S1 fibroblasts from human breast cancers identified 8 clusters, separating into iCAF and myCAF subgroups with abundance of the three myCAF clusters (ecm-myCAF, TGFβ-myCAF, wound-myCAF), but not the iCAF clusters, associated with reduced CD8+ T cell infiltration, and being significantly enriched in tumors with primary resistance to αPD-1 treatment (14). myCAF and iCAFs populations have also been defined in

other tumor types (14,41). Moreover, consistent with the established role of TGFβ in restricting T cell infiltration into tumors (23) a signature of the LRRC15+ TGFβ-driven CAF subset, which clusters with the myCAF signature, is associated with poor response to αPD-L1 (50).

Expression of the CAF receptor Endo180 (*Mrc2*) is required for the generation of a tumor-supportive TME, and in preclinical models, its genetic deletion results in the depletion of a subpopulation of αSMA+ CAFs and a reduction in collagen deposition (16). As bioinformatic analysis demonstrates elevated Endo180 (*MRC2*) expression in human breast cancer myCAFs compared to iCAFs and perivascular fibroblasts, we sought to determine whether modulating Endo180 expression in the CAF-rich, CD8+ T cell excluded D2A1-m2 syngeneic breast cancer model could reverse immunosuppression. Using tumor-CAF co-implantation approaches and analysis of tumors from Endo180 KO mice, we provide direct evidence of a role for Endo180 expressing CAFs in promoting an immunologically cold, CD8+ T cell excluded tumor phenotype. These preclinical data, combined with analysis of human clinical datasets, provide support for therapeutic modulation of Endo180 in combination with immunotherapy for improving clinical outcomes in CAF-rich breast cancers.

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Figure 1

CAF abundance is associated with insensitivity to ICB. **A,** 4T07 or 4T1 cells were implanted orthotopically into BALB/c mice (*n*=5 per group). Mice were culled at day 17. Representative αSMA stained sections (scale bar, 100 μm). Bar chart shows % αSMA+ stained area. **B,** 4T07 or 4T1 cells were implanted orthotopically into BALB/c or NSG mice (*n*=6 per group). Tumor growth curves for individual mice and tumor growth rates. **C,** 4T1 cells were implanted orthotopically into BALB/c mice (*n*=4-9 per group) and treated with αCTLA-4 or αPD-L1 antibodies alone (see Supplementary Fig. S1C), or in combination, according to the schedule shown. Control mice received isotype control antibodies. Tumor growth curves for individual mice and tumor growth rates. **D,** D2A1 or D2A1-m2 cells were implanted orthotopically into BALB/c mice (*n*=6 per group) and intratumoral αSMA staining quantified as in panel A. **E**,**F,** D2A1 or D2A1-m2 cells were implanted orthotopically into BALB/c mice and treated with αCTLA-4 and αPD-L1 antibodies in combination according to the schedule shown (*n*=8 control and 12 ICB-treated mice per group). Tumor growth curves for individual mice (CR, complete responder), tumor growth rates and Kaplan-Meier survival analysis (log-rank test). **G,** D2A1 cells were implanted bilaterally into naive BALB/c mice (*n*=2 mice) or into the opposite mammary fat pad of the surviving mouse from the D2A1 arm of Fig. 1F (Re-challenged, *n*=1 mouse).

Figure 2

Insensitivity to ICB *in vivo* is independent of tumor cell intrinsic factors. D2A1 and D2A1-m2 cell lines and BALB/c mouse germline DNA (as reference) were subjected to whole exome sequencing. **A,** Copy number variation plots (log_2 ratio). **B,** Number of exonic non-synonymous mutations per megabase (Mb) of exome. **C,** Venn diagrams illustrating the number of total mutations, non-synonymous exonic mutations and nonsynonymous exonic immune mutations in common between the D2A1 and D2A1-m2 cell lines. Immune mutations refer to mutations in the 750 genes represented in the NanoString mouse PanCancer IO 360 panel (see Supplementary Table S2). **D,** PD-L1 expression in CD45+ immune cells and CD45- tumor cells from dissociated tumors (MFI values). **E,F,** Cultured cells with or without IFNγ stimulation were (**E**) stained with APC-conjugated αPD-L1 or isotype control antibody and analyzed via flow cytometry or (**F**) stained *in situ* with αPD-L1 antibody followed by Alexa488-conjugated anti-rat Ig and visualized by confocal microscopy (scale bar, 50 μm).

Figure 3

NanoString transcriptomic profiling. D2A1 or D2A1-m2 cells were implanted orthotopically into BALB/c mice (*n*=5 or 6 per group). Mice were culled at day 24 (see Supplementary Fig. S2A for tumor weights). **A,** Profiling of tumors was performed using the NanoString mouse IO 360 panel. Heatmap of significant differentially expressed genes. **B,** Unsupervised hierarchical clustering based on expression of NanoString immune cell population abundance signatures (see Supplementary Fig. S2D). **C,** Significantly differentially expressed NanoString immune cell population abundance signatures. **D,** CD8 T effector signature expression. **E,** T_{reg} and macrophage signature expression. **F,** Expression of fibroblast TGFβ response (F-TBRS) signature (23) and NanoString TGFβ and Wnt signaling signatures (see Supplementary Fig. S2E). **G,** Correlation between NanoString 'CD8 T cells' and F-TBRS (left panel) or NanoString TGFβ signaling (right panel) signature expression.

Figure 4

CAF-rich tumors exhibit an immunologically cold TME. Tumor cells were implanted orthotopically into BALB/c mice. Mice were culled at day 19 (D2A1/D2A1-m2; *n*=7-8 per group for flow cytometry, *n*=6 per group for immunohistochemistry) or day 16 (4T07/4T1; *n*=6-8 per group for flow cytometry, *n*=5 per group for immunohistochemistry) (see Supplementary Fig. S3A for tumor weights at necropsy). Left panels, % CD8+ T cells assessed by flow cytometry (see Supplementary Fig. S3B for gating strategy). Right panels, immunohistochemistry analysis. CD8+ T cells per mm^2 tumor section. Representative images (scale bar, 100 μm). **E,** Quantification of αSMA staining and CD8+ T cell number in matched 0.25 mm^2 regions from serial sections of D2A1 and D2A1-m2 tumors (*n*=3 tumors per group; *n*=18-25 regions per section). Right panel, correlation of all regions sampled. **F**,**G,** Left panels, representative pseudocolor dot plots showing proportion of granzyme B+ CD8+ cells and PD-1+ CD8+ cells. Right panels, granzyme B+ or PD-1+ cells as a proportion of CD8+ T cells.

Figure 5

CD8+ T cell abundance and distribution in CAF-rich and CAF-poor tumors. **A,** D2A1 cells alone or with GFP+ NMFs or CAFs were implanted orthotopically into BALB/c mice (*n*=6-8 mice per group). Tumor growth curves and tumor growth rates. **B-D**, Primary tumors from panel A were analyzed via flow cytometry. **B,** % live GFP+, CD45-/Thy1.2+ and CD8+ T cells. **C,** Correlation between CD8+ and CD45-/Thy1.2+ cell number in all tumors. **D,** % Ki67+/CD8+ T cells and PD-1+/CD8+ T cells. **E-G,** D2A1 or D2A1-m2 cells were implanted orthotopically into BALB/c mice (*n*=15-18 per group) and treated with αCTLA-4 and αPD-L1 antibodies or isotype controls according to the schedule in Fig. 1E. **E,** Representative images of peripheral and central regions of tumors from isotype control and ICB treated mice stained for CD8. Dotted line indicates tumor-stroma boundary (scale bar, 250 μm). **F,** % of centrally-located CD8+ T cells (see Supplementary Fig. S5A for methodology of central and peripheral CD8+ T cell quantification). **G,** CD8+ T cell density in control (C) or ICB-treated tumors.

Figure 6

Functional characterization of Endo180+ CAFs. **A**, Expression of *MRC2* and *EPCAM* in stromal cells from scRNA-Seq of 26 human breast cancers (27). **B,** D2A1-m2 cells were implanted orthotopically into Endo180 WT or KO BALB/c mice (*n*=5 per group). Mice were culled on day 35. Bar chart shows % αSMA+ stained area (mean values per mouse ± SEM, unpaired *t*-test). Representative images (scale bar, 100 μm). **C,** D2A1 tumor cells were implanted alone or co-implanted with shNTC or shE180 CAFs (*n*=8 per group) (16). Mice were culled at day 32. Representative images of CD8 immunohistochemistry in peripheral and central tumor regions (scale bar, 200 μm). CD8+ T cells per mm^2 and % centrally located CD8+ T cells. **D**, mCherry-tagged D2A1-m2 cells were implanted orthotopically into Endo180 WT or KO BALB/c mice (*n*=5 per group) (16). Mice were culled on day 27. Left panel, tumor growth curves. Remaining panels, flow cytometric analysis of indicated stromal cell populations.

Figure 7

Stromal Endo180 depletion sensitizes tumors to ICB. **A,** D2A1-m2 cells were implanted orthotopically into Endo180 WT or KO BALB/c mice. Mice were treated with combination αCTLA-4/αPD-L1 therapy or isotype control antibodies, according to the Fig. 1E schedule (*n*=8 control and 19 ICB treated mice per group). Left, tumor growth curves for individual mice. Note, two Endo180 KO ICB-treated mice show complete tumor regression (CR). Middle , tumor growth rates**.** Right , Kaplan-Meier survival analysis (log-rank test). **B,** D2A1-m2 cells were implanted into the opposite mammary fat pad of the two surviving E180 KO mice from panel A (Re-challenged) or into 5 naive BALB/c mice. **C,** Tumors from ICB-treated mice from panel A stained for αSMA and CD8. Representative CD8 stained images (scale bar, 100 μm). Right panels show total number of CD8+ T cells per mm^2 and % centrally located CD8+ T cells. **D**, mRNA abundance profiles (log_2 (FPKM+1)) of selected marker genes in melanomas from anti-PD-1 non-responders (NR) and responders (R) (26) (Wilcox rank-sum test).