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TITLE: From Cold to Hot: Leveraging RNA Splicing Therapeutics to Unleash Antitumor Immunity for Breast Cancer Patients

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Although immune checkpoint blockade therapy (ICBT) emerged as an effective treatment on a subset of breast cancer patients, it remains a daunting challenge to make more patients respond. Our preliminary studies suggest that inhibition of splicing, a process to join the protein-coding parts of RNAs during gene expression, leads to immunostimulatory responses in triple negative breast cancers (TNBCs). We hypothesized that splicing inhibition accumulates mis-spliced RNAs, which may activate innate immune responses and lead to expression of neo-antigens. In this application, we aim to test this hypothesis and examine if a clinically available splicing inhibitor can synergize with ICBT in immunocompetent models. We will also elucidate specific immune cells that may mediate such synergy.
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1. Introduction

This application aims to address the overarching challenges that 1) eliminate the mortality associated with metastatic breast cancer and 2) identify what drives breast cancer growth; determine how to stop it. Escaping immunosurveillance is a prerequisite of tumor growth and metastasis. This application aims to revigorated anti-tumor immunity to stop breast cancer growth and eliminate the metastasis.

Immunotherapies, especially immune checkpoint blockade therapies (ICBTs), have achieved remarkable successes in several cancer types including metastatic melanoma and lung cancers, demonstrating the power of anti-tumor immunity when appropriately activated. However, recent clinical trials revealed less impressive efficacies of these treatments in breast cancer, suggesting that ICBTs are not sufficient to activate the immune system. Possible reasons behind this insufficiency include the lack of immune cell cytotoxicity and/or the paucity of neo-antigens. Therapeutic solutions to overcome these roadblocks are urgently needed.

Our previous work has demonstrated an unexpected vulnerability of cancer cells to the perturbation of splicing. Although splicing is almost universally needed for gene expression in all cell types, cancer cells under oncogenic stresses are particularly sensitive to spliceosome inhibition (SI). Our preliminary experiments uncovered that mis-spliced RNAs could accumulate in cytoplasm upon SI, and cause anti-viral responses that are known to further enhance immune cell cytotoxicity. Moreover, proteomic profiling detected peptides resulted from translation of intronic RNAs, which provide a potential source of neo-antigens. Thus, SI appears to be a suitable solution to abovementioned roadblocks of ICBTs.

In this application, we will use a splicing inhibitor with defined pharmacological properties to tackle the hypothesis that SI may convert “cold” tumors to “hot” tumors and enhance ICBT. We will mechanistically investigate how this inhibitor may stimulate innate immune response and enhance immune cell activities using both patient-derived xenograft and genetically engineered mouse models (GEMMs). We will also obtain evidence for activation of antigen-specific adaptive immunity presumably by translation of mis-spliced RNAs. Finally, we will perform proof-of-principle experiments to test if SI sensitizes tumors to ICBT using various syngeneic tumor models. Taken together, these data will likely provide strong rationale for further clinical trials using combined SI and ICBT.

The proposed studies will be jointly carried out by Dr. Thomas Westbrook’s laboratory and Dr. Xiang Zhang’s laboratory. Dr. Westbrook’s group will leverage on their knowledge in splicing and RNA metabolism to investigate tumor-intrinsic signaling pathways that may lead to enhanced innate immune response. Their experience in PDX models will make it possible to establish the connection between splicing and cell-intrinsic innate immune response in human cells. Dr. Zhang’s group will take advantage of their expertise in immune cell profiling to characterize how SI’s impact may extend beyond tumor cells and stimulate the immunity of host. They will also use the GEMMs to functionally study various immune compartments and delineate cellular mechanisms. Taken together, the joint efforts of both groups will powerfully fuel the proposed experiments that span multiple fields.

2. Keywords:
Splicing, spliceosome inhibitor, cellular stress, immune-checkpoint blockade therapy, triple negative breast cancer, immune microenvironment.

3. Accomplishment

Major Task 1: Evaluate the activation of tumor innate immune signaling in TNBC PDXs in response to spliceosome inhibition (Month 1-36)
Westbrook Lab.

Major Task 2: Evaluate the activation of tumor innate immune signaling in immunocompetent syngeneic murine TNBC models in response to spliceosome inhibition (Month 1-36)
Westbrook Lab.
Major Task 3 Test whether deficiencies of various host immune cells modulate the efficacy of SI.Tumor models (1-30).

We have been breeding animals as proposed in our application to obtain tumor hosts with specific deficiencies in various lineages of immune cells. The progress has been summarized in the following table.

<table>
<thead>
<tr>
<th>Model</th>
<th>Targeted cell population</th>
<th>Progress</th>
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<tbody>
<tr>
<td>Cd8 knockout</td>
<td>CD8+ T cells</td>
<td>Successfully bred to C57B/6 and Balb/c background. Tumor transplantation and SI treatment will start as soon as the targeted group size is achieved.</td>
</tr>
<tr>
<td>Cd4 knockout</td>
<td>CD4+ T cells</td>
<td>Successfully bred to C57B/6 and Balb/c background. Tumor transplantation and SI treatment will start as soon as the targeted group size is achieved.</td>
</tr>
<tr>
<td>Igh-J knockout</td>
<td>B cells</td>
<td>Backcrossing is ongoing…</td>
</tr>
<tr>
<td>Fox3P-DTR</td>
<td>T regulatory cells</td>
<td>Mice have been obtained and are being maintained. Backcrossing is ongoing.</td>
</tr>
<tr>
<td>Ccr2 knockout</td>
<td>mMDSCs and ΜΦ</td>
<td>Successfully bred to C57B/6 and Balb/c background. The first in vivo experiments have been performed to examine baseline responses tumors to ICBT in these hosts. In one model (T11), Ccr2 knockout alone led to complete response to ICBT (Figure 1, below).</td>
</tr>
</tbody>
</table>

As can be seen from above table, the progress is smooth and we do not anticipate significant problem in achieving the proposed goals timely. One interesting finding is that the baseline responses of Ccr2 knockout mice to ICBT in T11 model, a model enriching M2-like macrophages. Specifically, combination of Ccr2 knockout and ICBT appeared to regress T11 tumors completely without recurrences (Figure 1) – indicating a strong role of M2-like macrophages in creating an immunosuppressive microenvironment. This data has been included into our recent publication (attached). Although intriguing and important, this data does exclude the usage of T11 for future SI treatment as the baseline sensitivity to ICBT is already high.

![Figure 1. T11 tumors in CCR2 KO mice exhibited complete response to ICBT.](image)

Left: growth curves of individual T11 tumor in wild type (WT) or CCR2 knockout (CCR2 KO) mice with treatment of ICBT (anti-CTLA4 and anti-PD1) or IgG control. Right: Kaplan-Meier curves show the progression-free survival of animals in the four experimental groups. * P < 0.05; ** P < 0.01. P value was determined by log rank test, comparing each experimental group to WT + IgG control group.
Besides breeding GEMM as host of transplantable tumors, we also proposed to use acute pharmacological treatment to ablate specific immune cell lineages. The proposed experiments and the corresponding progress are shown in the following table.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Targeted cell population</th>
<th>Progress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ASGM1</td>
<td>NK cells</td>
<td>Not started yet.</td>
</tr>
<tr>
<td>Csf1R inhibitor</td>
<td>mMDSCs and MΦ</td>
<td>We have performed baseline treatment (without SI) and validated the effects of the drug.</td>
</tr>
<tr>
<td>Anti-Ly6G</td>
<td>Granulocytic MDSCs or neutrophils</td>
<td>This experiment has been extensively performed in combination with ICBT. A surprising compensation from monocytes has been observed (Figure 2).</td>
</tr>
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</table>

We had to combine anti-CXCR2 with anti-Ly6G to achieve a relatively complete depletion of neutrophils. This is consistent with the literature. One noteworthy finding from above experiments is that depletion of neutrophils led to accumulation of monocytes in the tumor microenvironment, which may represent a compensation pathway that maintains immunosuppressive microenvironment. We will follow up with this phenomenon as it might have implications in the proposed SI treatments.

<table>
<thead>
<tr>
<th></th>
<th>PyMT-N</th>
<th>2208L</th>
</tr>
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<tbody>
<tr>
<td>Absolute number per 1000 total cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mono</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>TIN</td>
<td>90</td>
<td>80</td>
</tr>
<tr>
<td>TIM</td>
<td>80</td>
<td>70</td>
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**Figure 2. Depletion of neutrophils resulted in compensatory recruitment of monocytes.**
Quantification of monocytes, TIN (tumor-infiltrating neutrophils) and TIM (tumor-infiltrating macrophages) in PyMT-N and 2208L tumors upon treatment of combined anti-CXCR2 and anti-Ly6G to deplete TINs. P values are computed by Student’s t tests without assumption of equal variations.

Major Task 4: Test whether the effects of spliceosome inhibition on tumor growth, metastasis, and immune infiltration are through tumor intrinsic signaling. (Month 6-18).

We have successfully obtained tumors expressing drug-insensitive mutant cDNA (SF3B1-R1074H). Drug treatment experiments are ongoing and will be reported after next grant period.

Major Task 5: Identify and characterize the neo-peptidome resulting from translation of intron-retained RNAs in TNBCs treated with spliceosome inhibitor. (Month 12-24).

Samples have been submitted for label-free proteomic quantification (LC-MS/MS). Results will be updated in next progress report.

Major Task 6. To determine if specific T cell clones are amplified in tumor-bearing animals – an indication of neo-antigen recognition. (Month 24-36).

Not started yet.

Major Task 7. To determine if spliceosome inhibition potentiates ICBT in multiple syngeneic models. (Month 12-36).

Experiments have been initiated. Results will be updated in next progress report.
4. Impact

Immunotherapies are revolutionizing the treatment of many cancers. Immune checkpoint blockade therapies (ICBT) have been remarkably effective in several cancer types including metastatic melanoma and non-small cell lung cancer. These treatments target negative regulators of the immune system, thereby unleashing anti-tumor immunity. Durable responses are reported in 20-40% of patients with limited toxicity. These successes exemplify the power of the immune system against malignancies. However, ongoing clinical trials suggest that although some breast cancer patients may also benefit remarkably from ICBT, the percentage is disappointingly low as compared to melanoma and lung cancers. In fact, most breast cancers are considered immunologically “cold”, i.e., lacking infiltration of immune cells and not expressing enough proteins that are “foreign” to the immune system.

Mistakes in splicing can result in aberrant RNA sequences. Our preliminary studies indicate that these aberrant RNA sequences may trigger two different responses. First, these RNAs tend to form double-strands, a structure mimics some virus genome. As a result, they can stimulate cells’ defense against virus, which has evolved to cause death of affected cells, and enhance the entire organism’s immune system including T cells and NK cells. Second, some of the aberrant RNAs may be translated into aberrant proteins. Because these proteins are nothing like existing human proteins, they may be recognized as foreign “antigens” and stimulate further immune reactions. Interestingly, both outcomes may be effectively utilized to fight tumors, especially when combined with immunotherapies that require a strong and activated immune system.

Intriguingly, targeting splicing seems to be the exact kind of solution to improve the efficacy of immunotherapy. As mentioned above, it enhances the immune system in general and triggers expression of more foreign proteins. Our preliminary study provides strong evidence supporting this hypothesis. In this application, we will further explore this possibility. Importantly, we will evaluate a clinical grade splicing inhibitor. This inhibitor (H3B-8800) has already been granted orphan drug status in AML/CMML. We will apply this inhibitor to a broad range of patient-derived xenografts (PDXs) and genetically engineered mouse models (GEMMs) in order to drive rational strategies to select TNBC patients who may best respond to the treatment. We will investigate the molecular and cellular mechanisms underlying various models’ responses. We will also test the hypothesis that the inhibition of splicing sensitizes TNBC and may allow more breast cancer patients to benefit from immunotherapies such as ICBT.

Taken together, our proposed research will provide the first broad assessment of this therapeutic approach (targeting RNA splicing) in breast cancer patients. Our goal is to provide a conceptual and pre-clinical framework for initiating clinical trials for breast cancer patients.

5. Changes/Problems

So far, we have not encountered any significant problems or need to change the proposed plans.

6. Products


7. Participants & Other Collaborating Organizations

<table>
<thead>
<tr>
<th>Name:</th>
<th>Weijie Zhang</th>
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<tbody>
<tr>
<td>Project Role:</td>
<td>Postdoctoral Fellow</td>
</tr>
<tr>
<td>Researcher Identifier:</td>
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### Contribution

**Nearest person month worked**: 12

Dr. Zhang is responsible for all SI-treatment experiments proposed in the application.

<table>
<thead>
<tr>
<th>Name:</th>
<th>Jun Liu</th>
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<tr>
<td>Project Role:</td>
<td>Research Technician</td>
</tr>
<tr>
<td>Researcher Identifier:</td>
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<tr>
<td>Nearest person month worked</td>
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</tr>
<tr>
<td>Contribution</td>
<td>Ms. Liu is responsible for mouse breeding. She also assists Dr. Zhang in proposed experiments.</td>
</tr>
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<table>
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<tr>
<th>Name:</th>
<th>Xiang Zhang</th>
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<td>PI/PD</td>
</tr>
<tr>
<td>Researcher Identifier:</td>
<td>N/A</td>
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<td>Nearest person month worked</td>
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</tr>
<tr>
<td>Contribution</td>
<td>Dr. Zhang designed and supervised the experiments described in this report.</td>
</tr>
<tr>
<td>Funding Support</td>
<td>Dr. Zhang is also supported by NIH/NCI, Breast Cancer Research Foundation, and McNair Medical Institute.</td>
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All collaborators and participants are at Baylor College of Medicine.

8. **Special Reporting Requirements**

None.

9. **Appendices**

A copy of the publication on NCB.
Immuno-subtyping of breast cancer reveals distinct myeloid cell profiles and immunotherapy resistance mechanisms

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Cancer-induced immune responses affect tumour progression and therapeutic response. In multiple murine models and clinical datasets, we identified large variations of neutrophils and macrophages that define ‘immune subtypes’ of triple-negative breast cancer (TNBC), including neutrophil-enriched (NES) and macrophage-enriched subtypes (MES). Different tumour-intrinsic pathways and mutual regulation between macrophages (or monocytes) and neutrophils contribute to the development of a dichotomous myeloid compartment. MES contains predominantly macrophages that are CCR2-dependent and exhibit variable responses to immune checkpoint blockade (ICB). NES exhibits systemic and local accumulation of immunosuppressive neutrophils (or granulocytic myeloid-derived suppressor cells), is resistant to ICB, and contains a minority of macrophages that seem to be unaffected by CCR2 knockout. A MES-to-NES conversion mediated acquired ICB resistance of initially sensitive MES models. Our results demonstrate diverse myeloid cell frequencies, functionality and potential roles in immunotherapies, and highlight the need to better understand the inter-patient heterogeneity of the myeloid compartment.

Immune cells participate in every aspect of tumour progression. Many immune cells may play disparate roles—anti-tumorigenic in some situations, pro-tumorigenic in others. For instance, macrophages undergo different activation and polarization pathways, the classically activated subsets potentiate anti-tumour immunity whereas the alternatively activated subsets promote tumours through multiple mechanisms. Neutrophils also play opposing roles in different settings, probably due to plasticity and heterogeneity. Therefore, it is critical to understand how immune cell functions vary in different tumour contexts.

Solid tumours also induce systemic immune alterations. Immature neutrophils and monocytes may accumulate in blood and immune organs, develop immunosuppressive activity, and alter tumour progression either by infiltrating tumours or via homing to distant organs to establish pre-metastatic niches. It remains elusive how these local and systemic immune aberrations are related to inter-tumoural heterogeneity. This has been predominantly characterized based on tumour-intrinsic features, where different subtypes of breast cancer exhibit distinct developmental programs, metastatic behaviours and molecular landscapes.

Variations in immune profiles have been linked to prognosis, therapeutic responses and breast cancer subtypes. However, it remains a challenge to dissect the causal effects and mechanistic functions of different immune cells based solely on clinical data. The current study overcomes these limitations by integrating the immunological characterization of a variety of murine syngeneic mammary tumour models with the analyses of human breast cancer datasets.

Results

Immune cell profiling of murine tumour models reveals a dichotomous distribution of macrophages and neutrophils. We chose eight syngeneic murine tumour models derived from either a BALB/c or C57BL/6 background, and maintained as cell lines or primary tissues. MMTV-PyMT tumours express oestrogen receptor (ER) in early tumorigenesis, but the tumours progressively lose ER as they develop. A MES-to-NES conversion mediated acquired ICB resistance of initially sensitive MES models. Our results demonstrate diverse myeloid cell frequencies, functionality and potential roles in immunotherapies, and highlight the need to better understand the inter-patient heterogeneity of the myeloid compartment.
previous results indicate that, by definition, the eight models represent triple-negative breast cancer (TNBC), which is a heterogeneous group of diseases. Expression of characteristic genes suggested that these models resemble luminal-like (2208L and PyMT-N), basal-like (4T1 and AT3) and the claudin-low (PyMT-M, E0771 and 67NR) subtypes (Supplementary Fig. 1d), covering a spectrum of differentiation and metastatic propensity (Supplementary Fig. 1e). Thus, these models may collectively represent heterogeneous TNBC.

Major immune cell populations were profiled (Supplementary Fig. 1f) when tumours reached a similar size (Supplementary Fig. 1g). Hierarchical clustering was performed to display FACS-determined cell frequencies (Supplementary Fig. 1h). We prioritized different cell types based on inter-model variations and median frequencies (Supplementary Table 1). Tumour-infiltrating neutrophils (TINs) and macrophages (TIMs) were the most frequent and variable cell types across models, as confirmed by immunofluorescence staining of Ly6G and CD68 (Supplementary Fig. 1i).

TINs are defined as CD45+Ly6G+Ly6Cmed-low cells, and TIMs are defined as CD45+CD11b+Ly6G-Ly6C-F4/80+ cells (Fig. 1a,b) — note that F4/80+ cells are CD64+ (ref. 29) (Fig. 1c). Wright-Giemsa staining confirmed their polymorphonuclear/multi-lobed and mononuclear morphologies, respectively (Fig. 1d).

Three clusters were observed (Fig. 1e), driven mainly by the total number of CD45+ cells and the TIN/TIM ratio (Fig. 1f). PDX tumours in SCID/Beige mice fell mostly into cluster II with low numbers of CD45+ cells. However, some PDXs were sorted to other clusters, and some murine tumours fell into cluster II and exhibited low T cell infiltration (Supplementary Fig. 1f). Note that F4/80+ cells are CD64+ (ref. 29) (Fig. 1c).

The TIN/TIM ratio is another cluster-driving factor. Clusters I and IV represent tumours with increased TINs, whereas clusters II and III represent those with increased TIMs. The TIN/TIM ratio exhibited a bimodal distribution (Fig. 1f).

The TIN/TIM frequency strongly correlated with PBNs (Fig. 1g), indicating that systemic neutrophil accumulation accompanied local TIN enrichment, which was also evidenced by splenomegaly (Supplementary Fig. 1k) and alterations in the bone marrow (Supplementary Fig. 1l). This was opposed to the weak correlations among TIMs, PBMs and tumour-infiltrating monocytes (Fig. 1h,i).

Taken together, we divided pre-clinical models into immunologically cold, macrophage-enriched (MES) or neutrophil-enriched (NES) subtypes (NES). MES features local accumulation of macrophages with few neutrophils and little systemic impact. In contrast, NES features local and systemic neutrophil accumulation. In NES, macrophages are still present, sometimes as frequently as neutrophils.

As well as immunosuppressive activities, CD45+CD11b+ Ly6G-Ly6Cmed-low cells in tumour-bearing hosts were also defined as granulocytic myeloid-derived suppressor cells (gMDCs)15,29, tumour-associated neutrophils (TANs)15,29,401 or immunosuppressive neutrophils; CD45+CD11b+Ly6G-Ly6Chigh and CD45+CD11b+Ly6G-Ly6C-F4/80+ cells were also termed monocytic MDCS15,29 and tumour-associated macrophages40, respectively; together these cells were also called immature myeloid cells41. However, the suppressive and immature properties of neutrophils and macrophages vary widely among the models in this study. Thus, we prefer the more generic terms TIN and TIM to describe the comparisons among various models.

Inter-tumoural variation of neutrophils and macrophages across human TNBCs. To examine TIM/TIN ratios in human tumours, we first analysed a TNBC dataset with matched tissue microarray (TMA) and NanoString assays of 750 immunity-related genes. Using the human Primary Cell Atlas of BioGPS (http://biogps.org/dataset/BDS_00013/primary-cell-atlas)45, we identified subsets of NanoString genes differentially expressed between macrophages and neutrophils (Fig. 2a). Thirty-three macrophage-specific genes (MSGs) and 45 neutrophil-specific genes (NSGs) were identified to cluster TNBCs (Fig. 2b). Four clusters were uncovered, ranging from MSG-enriched to NSG-enriched (Fig. 2b). One cluster lacks both, and may represent ‘cold’ tumours. The expression of Elastase and G-CSF genes (both highly relevant for neutrophil biology)15,29 confirmed the neutrophil variation, and CD68 expression appears consistent with MSGs (Fig. 2b). Immunohistochemical (IHC) staining of CD68 in matched TMA data revealed variable macrophage infiltration across tumours (Fig. 2b). TMA sections with strong CD68 staining (>10 positive cells) express a higher level of MSGs (Fig. 2c), supporting the NanoString-based analysis. Furthermore, the MSG/NSG ratio exhibited a bimodal distribution (Fig. 2d), consistent with observations in murine models.

Next, we analysed larger datasets using CIBERSORT and TIMER45,46 to deconvolute immune infiltration from bulk tumour transcriptomes. Hierarchical clustering of TIMER scores of six cell types revealed distinct clusters among the TCGA TNBC dataset (Fig. 2e): a ‘cold’ cluster with overall low immune infiltration, another cluster with higher infiltration of TIM and CD68+ T cells, and the rest with TIM and other immune cells. In confirmation, we conducted t-distributed stochastic neighbour embedding (tSNE) using TIMER scores, resulting in three clusters corresponding to tumours that are cold, TIM-enriched and TIN-enriched (Fig. 2f). Analysis of non-TNBCs in TCGA and TINBC in METABRIC42 led to similar results (Supplementary Fig. 2a–c). We next analysed CIBERSORT outputs of >1,000 TNBCs (Fig. 2g) and observed clusters with heavy neutrophil infiltration (and some macrophages) or tumours enriched with macrophages of various subtypes.

Taken together, using different approaches and patient cohorts, we observed heterogeneous tumour immune microenvironments characterized by the divergent infiltration of neutrophils and macrophages.

Tumour-intrinsic factors contribute to myeloid cell profiles. The variations of TIM/TIN frequency within each model are much smaller compared to overall variations across all models (Fig. 3a), suggesting that TIM/TIN frequency is a relatively stable trait. Some other immune cells are also enriched or depleted in specific models (Supplementary Fig. 3a), but exhibited less variation and lower overall frequencies (Supplementary Table 1).

We co-transplanted T11 (MES) and 2208L (NES) tumours into contralateral mammary glands of the same animals (Fig. 3b). This did not alter TIN/TIM frequency (Fig. 3b), further supporting that the TIN/TIM frequency is in part determined by tumour-intrinsic factors. Interestingly, T11 tumours displayed minimal neutrophil infiltration even in the presence of systemic neutrophil accumulation (induced by the contralateral 2208L tumours) (Supplementary Fig. 3b), suggesting an active neutrophil-repelling mechanism.

MMTV-PyMT is an exception: 28 spontaneous tumours derived from 6 animals exhibited diverse TIN/TIM frequency (Fig. 3c). We chose one tumour with intermediate levels of TIN and TIM, and performed animal-to-animal transplantation of small tumour fragments (one per animal). This operation resulted in two primary tumour lines with stable TIN/TIM frequency, named PyMT-M (macrophage-enriched) and PyMT-N (neutrophil-enriched) (Fig. 3c
Fig. 1 | Diverse immune cell profiles in murine mammary tumour models. a, FACS analyses showing dichotomous infiltration of Ly6G−Ly6Cmed-low cells (neutrophils) and CD11b+F480+ cells (macrophages) in two representative tumour models. Plots are gated on CD45+CD11b+ cells (top) and CD45+ cells (bottom). b, FACS analyses show variable systemic accumulation of Ly6G−Ly6Cmed-low cells (neutrophils) in peripheral blood of tumour-bearing mice. Plots are gated on CD45+CD11b+ cells. For a,b, the experiments were repeated at least five times with similar results. c, CD64 staining of CD45+CD11b+Ly6G−Ly6C−F4/80+ tumour-infiltrating macrophages (TIMs) shows positive signals (pink curve) as compared to the unstained control (black curve) in the T11 and 2208L model. d, Wright-Giemsa staining of purified CD45+CD11b+Ly6G−Ly6C−F4/80+ TIMs (left) and CD45+CD11b+Ly6C−Ly6G−F4/80+ TIMs (right). Scale bar, 10 μm. For c,d, the experiments were done once. e, The heatmap shows unsupervised clustering of 19 breast tumour models (n = 70 biologically independent animals) based on the frequency of total numbers of CD45+ cells in tumours (T-CD45), TIM, TIN, peripheral blood Ly6Chigh monocytes (PBM) and peripheral blood neutrophils (PBN). All cell frequencies are normalized to totals, log-transformed and then z-transformed. The genetic background of each model is encoded by a different colour: pink, BALB/c; blue, FVB; yellow, C57BL/6; green, SCID/Beige. Experimental systems are indicated by letters: C, cell lines; N, p53-null tumour (primary tissue); G, genetically engineered spontaneous tumours; P, PDX models (human tumours in mouse). f, Top: scatter plot of total CD45+ cells against TIN/TIM ratios with the four clusters in e indicated by circles. Bottom: histogram of the log10-transformed TIN/TIM ratio of the tumour models/biological replicates shown in e, n = 70 biologically independent animals. g-i, Scatter plots show the correlations among the indicated immune cells. Linear smoothed lines (blue lines) and confidence intervals (grey shade) are shown based on linear regression analyses. n = 70 biologically independent animals. The Pearson correlation coefficients and corresponding P values are based on two-sided t-tests. For f-i, genetic background and individual tumour models are indicated by distinct point shape and colour, and are shown below f.
Fig. 2 | Myeloid cell profiles in human TNBC. a, A schematic showing the derivation of NSGs and MSGs based on the human Primary Cell Atlas of BioGPS. FC, fold change; FDR, false discovery rate. b, Unsupervised clustering of a human TNBC NanoString dataset (n = 72 patients) using MSGs (blue) and NSGs (yellow). Boxplots of CD68 IHC quantitation, and the gene expression levels of CD68, Elastase and G-CSF are indicated. The P value on the row-side was computed by two-sided Fisher's exact test. The P value along the column side with boxplots was computed based on one-way ANOVA. The colour scale indicates z-scores of log2-transformed, normalized counts. c, Left: IHC image of negative/weak (top; representative of 60 patients) or strong (bottom; representative of 12 patients) CD68 staining. Scale bar, 100 µm. Right: boxplots (defined in Methods) show normalized expression of MSG in tumours whose TMA sections exhibit negative or weak CD68 staining (positive cell count <10, n = 60 patients) or strong staining (>10, n = 12 patients). The P value is computed by two-sided t-tests with Welch correction. d, Histogram of log-transformed ratio of NSG over MSG. The approximated bimodal distribution is shown by solid lines. e, Unsupervised clustering of TIMER scores of six indicated immune cells in TNBC of the TCGA dataset (n = 112 patients) after z-transformation. Potential cold, NES and MES clusters are indicated by grey, yellow and blue rectangles, respectively. DC, dendritic cells. f, tSNE clustering of the same cohort of tumours (n = 112 patients) as in e. Tumours that are 'cold' as determined in e are coloured grey. Others are coloured in gradient according to the z-score difference between TIN and TIM. Clusters representing potential NES and MES subtypes are indicated by yellow and blue circles, respectively. g, CIBERSORT output of TNBC was obtained from a previous study (n = 973 patients) and reanalysed (top). The contribution of macrophages and neutrophils to the clustering is highlighted in the middle panel. The predicted proportions of neutrophils and macrophages are displayed in stacked bars following the same order. Yellow and blue rectangles indicate clusters of divergent ratios of neutrophils versus macrophages, respectively.
Fig. 3 | The TIN/TIM frequencies are relatively stable for individual tumour models. a, Boxplots (defined in Methods) of TIN and TIM frequencies (relative to total cells) within individual models and across all models (far right). Each dot represents one tumour from a biologically independent animal, and the sample size of each model is indicated in parentheses. Values were computed by one-way ANOVA. b, Quantification of TIM and TIN in primary tumours. The sample size of each group is indicated in parentheses. Data are shown as mean ± s.d. Values were computed by two-sided t-tests, not adjusted for multiple comparisons. c, Development of MMTV-PyMT sublines that stably maintain divergent myeloid cell profiles. An experimental schematic is shown on the left and quantification of myeloid cells is on the right. Spontaneous tumours risen from the same animal are coded with the same colour and shape. Red or blue dotted circles indicate potential MES or NES tumours, respectively. Tumours used for the next round of transplantation are indicated by solid circles. Points of the resulting tumours are outlined with the same colour as their parental tumour. d, TIM/TIN profiles in orthotopic tumours of eight models are presented as pie charts. Other characteristics are shown by colours as indicated by annotations above the table. e, Quantification of migrated neutrophils (left) and monocytes (right) towards tumour-conditioned medium. f, Heatmap showing the expression level of chemokines and cytokines known to regulate chemotaxis (chemo) of neutrophils and monocytes. Tnfαip6 is a neutrophil-repelling molecule. Z-scores are based on regularized log2-transformed RNA-seq data. Statistical difference of the sum of each group of genes between NES (n = 4 biologically independent models: 2208L, 4T1, PyMT-N and AT3) and MES (n = 4 biologically independent models: T11, 67NR, PyMT-M and E0771) is assessed by two-sided t-tests. g, h, Correlation between in vitro migration data and in vivo tumour infiltration data of neutrophils and monocytes (n = 8 biologically independent models). The TIM/TIN profile of each model is indicated as a mini pie chart. Smoothed trend lines are shown. Pearson correlation coefficients and corresponding P values (by two-sided t-tests) are indicated.
The frequency of TIN is determined by tumour-derived chemoattractants. To investigate whether the TIN/TIM dichotomy in vivo is recapitulated by in vitro chemotaxis of neutrophils or monocytes, we assessed chemo-attraction of bone marrow neutrophils and monocytes by tumour conditioned medium (CM) of the eight models. The variations in chemotaxis (Fig. 3d,e) can be partially explained by expression of chemokines and cytokines known to attract these cells as assessed by RNA-seq (Fig. 3d,f) or qPCR (Supplementary Fig. 3f). In particular, *Tnfaip6* encodes TSG6, which binds CXCL1/2 and inhibits neutrophil migration. It is expressed in three out of four MES models, and may mediate neutrophil repulsion.

Neutrophil migration in vitro tightly correlated with TIM frequency in vivo (Fig. 3g), suggesting that tumour-cell-derived chemokines or cytokines contribute to TIM accumulation. In contrast, monocyte migration only weakly correlated with monocyte frequencies in vivo (Fig. 3h), and did not correlate with TIM frequencies (Supplementary Fig. 3g).

Alteration of epithelial–mesenchymal transition tilts the TIN/TIM balance. When cultured in vitro, the eight tumour models exhibited different cell morphologies. Three NES models, 2208L, 4T1 and PyMT-N, were cobblestone/epithelial-like, whereas all MES models were spindle/mesenchymal-like. AT3 was unique: single cells scatter but are not spindle-like (Fig. 4a). Transcriptomic profiling largely confirmed the epithelial and mesenchymal properties, and classified AT3 as mesenchymal (Fig. 4b). Expression of the key genes *Zeb1* and *Cdh1* was validated by qPCR (Supplementary Fig. 4a). Thus, epithelial–mesenchymal transition (EMT) is associated with the TIN/TIM frequency and NES/MES subtyping.

*Zeb1* was upregulated in all mesenchymal lines (Fig. 4b). The reciprocal inhibition between the miR-200 family and *Zeb1* regulates EMT. miR-200c expression is higher in epithelial NES tumours (Supplementary Fig. 4b), miR-200c overexpression in MES reduced *Zeb1* (Fig. 4c), shifted cells towards an epithelial phenotype as assessed by an EMT reporter and cell morphology (Supplementary Fig. 4c, d), increased neutrophil-recruiting chemokines including CXCL1, and decreased the neutrophil-repelling molecule *Tnfaip6* (Fig. 4c). Short hairpin RNA-mediated *Zeb1* knockdown elicited similar changes (Supplementary Fig. 4e). miR-200c expression in human MDA-MB-231 cells also reduced TGFβ and increased the functional *Ccx1* homologue *IL8* (Fig. 4c). Consistently, miR-200c overexpression promoted in vitro neutrophil migration in trans-well assays (Fig. 4d), but slightly (statistically significant in one of four models examined) decreased monocyte-related chemokine/cytokine *CCL2* and M-CSF levels (Supplementary Fig. 4f) and corresponding in vitro monocyte migration (Supplementary Fig. 4g). Finally, miR-200c expression in T11 tumours in vivo caused a TIM increase but a TIM decrease (Fig. 4e). Thus, perturbation of EMT reprograms the tumour microenvironment.

In the TCGA dataset, we used GSEA to identify pathways correlating with TIM/TIM derived and Supplementary Fig. 3c). Like other NES tumours, PyMT-N induced systemic neutrophil accumulation (Supplementary Fig. 3d). Thus, the original MMTV-PyMT tumour harboured separate TIM- and TIN-enriched regions, which may be explained by the polyclonality of PyMT tumours. Importantly, the TIM/TIN frequencies of transplanted tumours are within the spectrum of spontaneous tumours, suggesting that the MES/NES phenotypes are unlikely to be a result of transplantation-induced inflammation (Fig. 3c). We also compared spontaneous and transplanted MMTV-WNT1 tumours, and observed no significant differences in TIM/TIN frequencies (Supplementary Fig. 3e).

TIN-associated pathways include PI3K-AKT-mTOR (Supplementary Fig. 4i), consistent with previous findings that the mTOR signalling causes gMDSC accumulation. Gene set variation analysis (GSVA) reinforced the connection between EMT/mTOR pathways and TIM/TIN. Two different EMT signatures are associated with the monocyte/macrophage-recruiting cytokines *CSF1* and *CCL2*, the neutrophil-recruiting molecule TSG6 (Fig. 4g) and TIM scores (Supplementary Fig. 4j). The PI3K-AKT-mTOR pathway is associated with the neutrophil-recruiting chemokines *CXCL1* and IL8, as well as with TIM scores (Fig. 4g). Finally, miR-200c expression inversely correlated with TIM scores (Fig. 4h). Thus, tumour-intrinsic pathways contribute to the development of a diverse myeloid cell compartment. In particular, EMT may simultaneously drive monocyte/macrophage recruitment and neutrophil exclusion.

TINs in MES and NES exhibit different CCR2-dependency and interactions with TINs. We profiled TIM transcriptomes in four models representative of different genetic backgrounds and immune subtypes. Principle component analysis suggested multi-polar TIM polarization (Fig. 5a). GSVA indicated that T11-TIMs overexpress multiple immunosuppressive hallmark pathways including TGF-β, reactive oxygen species (ROS) and mTOR54, whereas E0771-TIMs overexpress pro-inflammatory pathways including IFN-γ and TNF-α (Fig. 5b and Supplementary Fig. 5a). Similarly to T11-TIMs, AT3-TIMs overexpress several immunosuppressive pathways such as Myc and ROS, but also highly express pro-inflammatory pathways such as TNF-α.

The functional impact of TIMs was evaluated by transplantation of various tumours into CCR2 knockout (CCR2-KO) mice. As expected, Ly6C++ monocytes were reduced by 3- to 7-fold in all models (Fig. 5c). However, a significant TIM reduction (fold-change >2 and *P* < 0.05) was only seen in MES tumours (Fig. 5c), indicating MES-specific CCR2-dependency. In contrast, NES-TIMs were not affected by CCR2-KO.

The impact of CCR2-KO was heterogeneous on tumour growth (Supplementary Fig. 5b), T cell infiltration (Supplementary Fig. 5c), proliferation, apoptosis or angiogenesis (Supplementary Fig. 5d). Nevertheless, the inverse relationship between TIMs and TINs was evident—that is, whenever TIMs were reduced, TINs increased (Fig. 5c–e). This effect was systemic in animals bearing MES tumours (Supplementary Fig. 5e), but did not occur in tumour-free animals (Supplementary Fig. 5f). Because in two NES models CCR2-KO failed to reduce TIMs, we used combined CSF1-neutralizing antibody (anti-CSF1) and clodrosome to deplete TIMs. This approach can eliminate tissue-resident macrophages, and indeed depleted TIMs in all models tested including NES (Fig. 5f). Interestingly, whereas anti-CSF1 and clodrosome treatment in MES resulted in increased TINs, confirming the CCR2-KO results, it failed to induce a similar increase in NES (Fig. 5f). Thus, the negative impact on TINs appears specific to CCR2-dependent TIMs.

Neutrophils in NES are immunosuppressive. TIM transcriptomes of E0771, T11, 2208L and AT3 tumours were also profiled. We used previously identified gene signatures to distinguish normal neutrophils from gMDSCs and/or tumour-associated neutrophils (TANs)9, NES-TINs (2208L and AT3) express a substantial proportion of TAN/MDSC genes, whereas MES-TINs (T11 and E0771) are more related to normal neutrophils (Fig. 6a). GSVA of the hallmark pathways further uncovered differentiations of TINs between NES and MES. The former displayed enhanced expression of several immunosuppressive pathways, including STAT3, TGFB-β and ROS (Fig. 6b and Supplementary Fig. 6a). In addition, the NOTCH pathway is elevated, supporting a previously reported feedback loop between MDSCs and tumour-initiating cells (Fig. 6b). A search in additional gene sets revealed adenosine metabolism (Supplementary Fig. 6b) as another immunosuppressive pathway.
Fig. 4 | Perturbation of EMT tilts the balance between TIM and TIN. a, Cell morphology of eight tumour models in 2D culture. The pie charts are shown to indicate each model’s in vivo TIM/TIN profiles. Scale bars, 100 μm. b, Heatmap showing the expression level of a panel of EMT-related genes across the eight models (each in technical triplicate). Colour scale indicates z-scores of regularized log-transformed data across columns. c, Relative expression of indicated genes in T11 (n = 4), E0771 (n = 3) and MDA-MB-231 (n = 4) models following miR-200c induction with doxycycline. Data are shown as mean ± s.d. P values were determined by two-sided t-tests. d, Quantification of neutrophil migration towards tumour-conditioned medium of T11 (n = 4), E0771 (n = 4) and MDA-MB-231 (n = 3) models following miR-200c induction with doxycycline. Data are shown as mean ± s.d. P values were determined by paired two-sided t-tests. For c, d, n values indicate numbers of independent experiments. e, Quantification of TIM and TIN in orthotopic T11 tumours with or without miR-200c induction. Data are shown as mean ± s.d. P values were determined by two-sided Student’s t-tests. f, Venn diagram showing the hallmark pathways associated with TIM and TIN frequencies in the TCGA TNBC dataset (n = 112 patients) gauged by TIMER using GSEA. Significant pathways (FDR < 0.05 empirically determined by random permutations) are shown. g, Heatmap showing unsupervised hierarchical clustering of indicated genes and GSVA scores of indicated pathways (in bold) on TNBC of the TCGA dataset. h, Negative correlation between TIM/TIN scores (TIMER-TIM) and miR-200c expression in the TCGA TNBC dataset (n = 112 patients). Both scatter plots (grey dots) and boxplots (defined in Methods) are shown to treat TIMER-TIM as continuous and categorical variables (cutoffs indicated, respectively). A two-sided t-test (for the Pearson correlation coefficient) and one-way ANOVA are applied to compute P values.
Fig. 5 | Inter-tumoural heterogeneity of TIMs and inverse change of TINs following TIM depletion. a, Principle component analysis (PCA) of TIMs of four tumour models (n = 3 biologically independent samples for each model). b, Heatmap shows unsupervised clustering of TIMs purified from four tumour models (n = 3 biologically independent samples for each model) using GSVA of the 50 hallmark pathways from MSigDB. Pathways related to immunosuppressive or immunostimulatory activities are shown. The heatmap with complete pathway annotations is shown in Supplementary Fig. 5a. c, Heatmaps showing the impact of CCR2-KO-mediated Ly6C<sup>high</sup> monocyte depletion on the frequency of TIM and TIN in the indicated tumour models. Numbers in parentheses show the specific n values of biologically independent mice per group denoted by different colours. The absolute cell numbers were quantified by flow cytometry. Arrows to the right of the heatmaps show the direction of changes. Numbers beside the arrows indicate fold changes of immune cell infiltration in CCR2-KO compared to WT. Numbers in parentheses indicate P values computed by two-sided t-tests. d, Example FACS plots showing the alteration of TIMs (CD11b<sup>+</sup>Ly6C<sup>−</sup>Ly6C<sup>−</sup>F4/80<sup>+</sup>) in WT and CCR2-KO hosts bearing T11 and 2208L tumours as representatives of MES and NES, respectively. e, Example FACS plots showing the alteration of TINs (CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>med-low</sup>) in WT and CCR2-KO hosts bearing T11 and 2208L tumours as representatives of MES and NES, respectively. For d, e, the results are representative of at least eight biologically independent animals. f, Quantification of tumour-infiltrating monocytes, TIM and TIN in the indicated tumour models following treatment of anti-CSF1 and clodrosome. Numbers in parentheses show the specific n values of biologically independent mice per group denoted by different colours. Data are shown as mean ± s.d. P values were determined by two-sided t-tests.
in NES-TINs, CD11b^+Ly6G^+ cells in the bone marrow of NES-tumour-bearing animals suppressed T cell proliferation in vitro (Fig. 6c), thereby meeting the definition of gMDSCs. Thus, TINs in different immune subtypes differ in both frequency and immunosuppressive activity. Moreover, NES tumours induce systemic accumulation of gMDSCs.

Given the negative impact of TIMs on TINs recruitment in MES, we asked if a reciprocal regulation occurs in NES. By applying anti-CXCR2 and anti-Ly6G, we reduced NES-TINs by 2- to 10-fold. This result in an increase of Ly6C^- monocytes. In contrast, monocytes in MES readily differentiate into TIMs (Fig. 5c).

Thus, the definition of MES includes tumours enriched with TIMs as a representative of a ‘cold’ tumour to immune checkpoint blockade (ICB) therapy (anti-PD1 and anti-CTLA4). The NES and cold tumour did not respond (Fig. 7a), even when the dosage was escalated to the maximum tolerable level (Supplementary Fig. 7a). MES showed largely variable responses (Fig. 7a and Supplementary Fig. 7a). We also observed that MES tumour-derived cell lines (for example, PyMT-M), when transplanted, gave rise to tumours exhibiting stronger ICB responses than tumours derived from tissue fragments of the same model (Fig. 7a and Supplementary Fig. 7b). However, NES-derived cell lines (for example, PyMT-N) remained resistant (Supplementary Fig. 7b). TIMs may create an immunosuppressive microenvironment independent of checkpoints, rendering these tumours non-responsive to ICB. ICB responses do not correlate with baseline tumour-infiltrating CD8^+ T cells (Supplementary Fig. 7c) or PD1L expression (Supplementary Fig. 7d). However, MES, but not NES, exhibited increased CD8^+ T cell infiltration post-treatment, and sometimes a reduction of the percentage of PD1^+ T cells (Supplementary Fig. 7e), suggesting that ICB restores CD8^+ T cell activity in MES.
Although MES exhibited better responses than NES, the extent varied. T11 is enriched with immunosuppressive TIMs (Fig. 5b), and CCR2-KO improved ICB responses: 5/5 T11 tumours regressed completely (Fig. 7b). The same treatment in E0771 and PyMT-M did not significantly alter responses (Supplementary Fig. 7f).

We wanted to know if immunosuppressive TINs mediate de novo ICB resistance. TIN reduction by combined anti-CXCR2 and anti-Ly6G treatment did not lead to improved ICB responses in NES (Supplementary Fig. 7g), perhaps due to the compensatory increase of immunosuppressive monocytes (Fig. 6d), as indicated by T cell proliferation assay (Supplementary Fig. 7h). ICB resistance was confirmed by lack of alterations in Tcell frequencies or PD1+ proportion (Supplementary Fig. 7i).

MES accumulates immunosuppressive TINs when acquiring resistance to ICB. E0771 tumours (>90%) exhibited durable responses to ICB, even after treatment cessation. One tumour recurred with increased TINs, which was designated E0771-ICBR. When further transplanted, neutrophil accumulation persisted both locally and systemically (Fig. 8a). Moreover, E0771-ICBR expresses higher levels of Cxcl1 and lower levels of Tnfaip6 (Fig. 8b). Neutrophils from E0771-ICBR-bearing animals potently...
Fig. 8 | TINs mediate acquired resistance to ICB. a, Quantification of peripheral blood neutrophils and TINs in animals transplanted with parental (n = 5) or recurrent (ICBR, n = 5) E0771 tumours. Data are shown as mean ± s.d. P values were determined by a two-sided t-test. b, Relative expression of the indicated genes in parental (n = 3) and ICBR (n = 3) E0771 cell lines. Data are shown as mean ± s.d. P values were determined by a two-sided t-test. c, An in vitro immunosuppression assay was performed by co-culturing bone marrow neutrophils from parental and ICBR E0771 tumour-bearing animals and splenic T cells. T cell proliferation was determined using CFSE intensity as measured by FACS. Data are shown as mean ± s.d. of three biological replicates (neutrophils from three different mice). P values were computed using two-sided t-tests. d, Therapeutic responses of parental and ICBR E0771 tumours to ICB (anti-PD1 and anti-CTLA4) and/or neutrophil depletion (anti-Ly6G). A treatment schematic is shown below the growth curves. Recurrence rate post-ICB is shown as bar graphs under the curves. e, Kaplan–Meier curves show the progression-free survival of parental or ICBR E0771 tumour-bearing animals subjected to either ICB alone or ICB and anti-Ly6G. P values were determined by two-sided log likelihood test. f, Heatmap showing the TIMER scores of indicated immune cells in a metastatic melanoma dataset. Red, orange and green bars indicate progressive diseases (PD), partial response (including stabilized diseases, PR/SD) and complete response (CR), respectively. g, Boxplots (defined in Methods) of TIN scores in patients with different responses to nivolumab. The sample size of each group is indicated in parentheses. P values were determined by two-sided t-test. h, The same as f except for a different metastatic melanoma dataset.
suppressed T-cell proliferation, displaying features of gMDSC (Fig. 8c), and were Ly6G高等 (Supplementary Fig. 8a), representing immature neutrophils9,16. The recurrent tumours accumulated neutrophils in the bone marrow (Supplementary Fig. 8b,c) and led to splenomegaly (Supplementary Fig. 8d). These alterations mirrored NES, and suggest an MES-to-NES switch following acquisition of ICB resistance. Combination of anti-Ly6G and ICB reduced recurrence by 50% and significantly improved progression-free survival of the tumour-bearing animals (Fig. 8d,e).

Similar results were obtained in PyMT-M. Cell-line-derived PyMT-M tumours exhibited tumour stasis or regression following ICB (Supplementary Fig. 7b). A recurrent derivative (PyMT-M-ICBR) showed significantly increased accumulation of TINs locally and systemically (Supplementary Fig. 8e). Cxcl1/2 expression was increased, whereas Tnfai/p6 expression was decreased (Supplementary Fig. 8f).

Thus, accumulation of immunosuppressive TINs or gMDSCs is associated with acquired ICB resistance in MES, and targeting these cells may alleviate resistance to ICB.

Exceptional neutrophil accumulation is associated with poor patient outcome. We used published metastatic melanoma datasets to query TIN roles in ICB response in human tumours, as relevant datasets are not yet available for TNBC. TIMER was applied to predict immune cell infiltration (Fig. 8f,h). In one dataset (Fig. 8i)9, TIN scores were significantly higher in patients with progressive disease (PD) or partial response (SD/PR) than in those exhibiting a complete response (CR) (Fig. 8g). In another dataset10, 70% of patients with PD were either top- or bottom-ranked according to the TIN score (Fig. 8h). We observed a significant inverse correlation between TIM and TIN scores (R = −0.53, P = 0.0033). Low-TIN tumours might enrich TIMs that attenuate ICB efficacy, similarly to T11 (Fig. 7b). Normality tests revealed 20% of tumours beyond a normal distribution, representing a distinct TIN-enriched group (Supplementary Fig. 8g,h). Applying the 20% cutoff and combining both datasets, we found a PR depletion and PD enrichment in NES-like melanoma (Supplementary Fig. 8i), supporting the correlation between heightened neutrophil accumulation and ICB resistance.

Discussion

One possible limitation of our study is the usage of transplantable tumours (cell lines or primary tissues). Tissue injury during transplantation and the absence of natural tumorigenesis may influence immune cell profiles. In two models, we compared spontaneous tumours with their transplantable derivatives, and found no significant difference in TIM/TIN frequency (Fig. 3c and Supplementary Fig. 3e). Genetically engineered models with spontaneous tumours also have caveats. Thus, it is important to compare the immune landscape of pre-clinical models to human tumours, and ideally at a single-cell level.

Different immune subtypes may co-exist intra-tumorally as demonstrated in PyMT. NES and MES tumours can be co-transplanted to a single host without affecting one another. This mutual exclusivity may result from the strong attraction of neutrophils in NES, whereas MES seems to repel neutrophils through EMT-mediated inhibition of neutrophil chemotaxis. Furthermore, neutrophils and monocytes/macrophages appear to negatively regulate each other, consistent with a previous report14. These mechanisms may cause spatial segregation between MES and NES within the same tumour.

Our data suggest different TIM biology between MES and NES tumours. In MES, TIMs are derived from CCR2+ monocytes, they may be polarized to M1-like or M2-like, and they negatively regulate TIM recruitment. In NES, TIMs are not clearly M1-M2 polarized, they are not impacted by CCR2-KO, nor do they regulate TINs. These observations suggest a more complicated biology of NES-TIMs, perhaps involving different cells of origin, differentiation, proliferation or activation.

NES tumours drive systemic gMDSC accumulation. However, MES-TINs are more similar to normal neutrophils, and might even perform anti-tumorigenic functions as previously shown1. Thus, the functions of TIN are determined by the entire myeloid compartment, further highlighting the importance of investigating the interactions among multiple cell types.

Previous studies linked EMT to immunosuppression, as EMT upregulates checkpoint molecules in cancer cells49,50. Here, we show that reversion of EMT may be accompanied by an influx of neutrophils, thereby switching the source of immunosuppression to neutrophils.

A recent study suggested that loss of p53 dictates systemic accumulation of pro-metastasis neutrophils in breast cancer11. One of our models (T11) lacks p53 but did not induce neutrophil accumulation, indicating more complicated mechanisms. The present study and a previous study from our laboratories11 suggest that additional tumour-intrinsic pathways (e.g., EMT and mTOR) and interplay between different immune cell populations (e.g., neutrophils and macrophages) need to be considered.

Overall, our studies highlight systematical characterization of microenvironmental heterogeneity by integrating multiple cell types in multiple tumour models, and show that the heterogeneity of breast cancer extends to the immune microenvironment. Therefore, in addition to mutation load and antigenicity, the tumour myeloid compartment should be examined to tailor immunotherapies.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at https://doi.org/10.1038/s41556-019-0373-7.

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Author contributions

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Methods

Mice. All animal experiments were conducted in accordance with a protocol approved by Institutional Animal Care and Use Committee of Baylor College of Medicine. The study is compliant with all relevant ethical regulations regarding animal research.

Female animals of 6–8 weeks of age were used as the recipients of tumour tissue or cell line transplantation. Age-matched mice (10–12 weeks old) were subjected to euthanization for immune profiling in all experiments. BALB/cAnN(Hsd) (BALB/c), C57BL/6N(Hsd) (C57BL/6, or B6), FVB and C.B-17/IcrHsd-PrkdcscidLystbg (C57BL/6 or B6), FVB, B6-Tg(MMTV-PyVT)634Mul/Lelj (MMTV-PyMT), B6.Cg-Foxp3tm1Wtsi (B6.FOXP3-GFP), C.Cg-Foxp3tm1Wtsi (B6.Cg-FOX3-GFP) and C57BL/6j (WT) mice were purchased from The Jackson Laboratory and bred in our facilities. To generate CCR2-KO mice in a BALB/c background, CCR2-KO mice were crossed with WT BALB/c mice for five generations.

Breast tumour models and transplantation. Primary tumour tissue lines were maintained by implanting 1–2 mm³ tumour pieces and cryopreserving following tumour harvest. Tumour models include T11 (BALB/c, p53-null tumour), 2208L (Balb/c, p53-null tumour), T1 (BALB/c, p53-null tumour), T2 (BALB/c, p53-null tumour), 215IR (BALB/c, p53-null tumour), 224AR (BALB/c, p53-null tumour), PyMT-M and -N (B6, MMTV-PyMT sub-lines), MMTV-WNTI (FVB) and PyT3-PEN DKO (FVB). The PyT3-PEN DKO tumour tissue used for transplantation was derived from the MMTV-cre;Trp53F/F;PtenF/F strain, which was directly bred by J. Xu’s lab at Baylor College of Medicine.

Cell lines were derived from above models and maintained as described in the ‘Cell lines and cell culture’ section. For inoculation into animals, cells were collected from culture with 0.25% trypsin (HyClone), washed with PBS (Lonza), was directly bred by J. Xu’s lab at Baylor College of Medicine.

Transplantation was derived from the MMTV-cre;Trp53F/F;PtenF/F strain, which was directly bred by J. Xu’s lab at Baylor College of Medicine.

in vivo drug treatment for immune checkpoint blockade and combination therapy. On day 0 of experiments, tumour tissue pieces or cell lines were implanted orthotopically as specified in the previous section. Animals were randomized when tumours reached a similar size (4–5 mm in diameter), which was day 5–7 for cell line inoculation (AT1, AT3, E0771-Parental and -ICRBM) and given treatments with the following regimen for each drug. To size-match tumours, MMTV-Wnt1 tumour-bearing animals received initial treatment at various time points following transplantation due to variable tumour latency. 67NR was not included for this analysis due to its distinct growth kinetics, which makes it difficult to compare to all other models with regards to therapeutic response.

In Fig. 7a, 100 μg anti-CTLA4 (clone 9D9) and 200 μg anti-CD123 (clone RPMI-14) antibody were delivered every three days until the end-point (a total of 4–6 doses). In Supplementary Fig. 7a, treatment was initiated on day 1 post-transplantation and continued every other day until the end-point.

In Fig. 7b and Supplementary Fig. 7b, on day 0 of the experiment, 0.5 × 10⁵ cells of the T11 tissue-derived cell line, E0771 cell line, PyMT-M and PyMT-N derived cell lines were inoculated orthotopically and randomized on day 5 for treatment initiation. ICB treatment was given every three days with the same dosage as described above (a total of 8 doses for T11, 4 doses for E0771, PyMT-M and PyMT-N).

In Fig. 8d, for the first 4 doses animals were given 100 μg anti-Ly6G (clone 1A8) every three days together with ICB (same dosage as described above), and for the next 8 doses given 200 μg anti-Ly6G without ICB every three days.

In Supplementary Fig. 6d and Fig. 7g, 100 μg (5 mg kg⁻¹) of anti-Ly6G (clone 1A8), 50 μg (10 mg kg⁻¹) of anti-CD123 (clone 200G) and 200 μg (100 mg kg⁻¹) of CXCR2 inhibitor (Selleckchem, SB225002) were administered intraperitonially every three days from tumour palpation until the end-point. ICB treatment was given every three days with the same dosage as described above. SR225002 was dissolved in DMSO and with 30% PEG300 (Sigma), 5% Tween 80 (Sigma) in distilled water.

In Figs. 5, 61, 5.5 mg of anti-CSF1 (clone 5A1) and 10 μl of clodrosome (Encapsula Nanosciences) were delivered through intraperitonial and intravenous (retro- orbital) injection respectively and administered every five days, with different treatments spaced out by two to three days. Treatment was initiated on day 2 post-tumour transplantation for T11, PyMT-N and 2208L, and following tumour palpation for PyMT-M, and continued until the end-point (a total of 3–6 doses).

Control animals received an equal amount of isotype-matched antibodies (mouse IgG2b (clone MPC-11), rat IgG2a (clone 2A3) and rat IgG1 (clone IG1 (clone HRPN))). All antibodies were delivered intraperitonially and were purchased from Bioxcel. Tumours were measured with a caliper and the volume was calculated using the formula π×width²×length.

Spontaneous pulmonary metastasis assay. The assay was performed using eight tumour models implanted as either tissues or cell lines (10³–2 × 10⁶ cells) in 100 μl PBS by orthotopic transplantation to mammary fat pads, followed by tumour resection when tumours reached ~1 cm³. The mice were closely monitored for one of the following end-points: (1) recurrent tumours reaching 2 cm³; (2) significant signs of morbidity; or (3) four months after resection. Lungs were extracted for evaluation of macroscopic metastases as previously described.¹⁴

Tissue harvest and dissociation. Tumours were sectored when they reached approximately the size of 1 gram. For RNA-seq and immune cell profiling, orthotopic breast tumours were collected in ice-cold PBS and subjected to dissociation using the mouse Tumour Dissociation Kit (Miltenyi Biotec) according to the manufacturer’s protocol. Tumours (0.2–0.4 g) were cut into small pieces (around 1 mm³) and transferred to gentleMACS Tubes (Miltenyi) containing 2.35 ml of RPMI-1640, 100 μl of enzyme D, 50 μl of enzyme E and 25 μl of enzyme A. Tissues were mechanically dissociated on a gentle MACS dissociator (Miltenyi). Three consecutive ‘in_Lung_02’ programs were run on the dissociator, with 10 min shaking incubation at 37°C in between each program run. The dissociation reaction was stopped by ice-cold RPMI-1640 and a single-cell suspension was obtained by filtering through a 70 μm cell strainer (Greiner Bio-One). The single-cell suspension was centrifuged for 5 min at 350g, re-suspended in 1 ml RBC lysis buffer (eBioscience), incubated on ice for 1 min, and washed with 10 ml FACs buffer (PBS containing 1% FBS). Samples with >90% cell viability were used for further analyses.

were treated with doxycycline (DOX) (Sigma) for 1–2 weeks: 2 μg ml⁻¹ for T11 and PyMT-M, 50 μg ml⁻¹ for E0771 and 100 μg ml⁻¹ for MDA-MB-231. To induce miR-200c/141 in T11 in vivo, cells were first treated with 2 μg ml⁻¹ DOX for 2 weeks in vitro prior to inoculation to mice. DOX was administered to animals from day 1 post-tumour implantation until tumour harvest (2 weeks). Harvested tumours were immune-profiled as well as re-transplanted to another batch of mice for further DOX induction (another 2 weeks) and immune profiling. Data were combined and analysed from both batches. A DOX solution of 1 mg ml⁻¹ was freshly prepared once a week, using 5% sucrose in water as the vehicle.

Lentivirus transduction of tumour cells. T11 cells were transduced with mouse ZEB1-specific shRNA (5’-ATATGTCAGGCTATAGAAGC-3’) or a scramble-control non-specific shRNA using the lentiviral pGIPZ vector system that allows puromycin selection to obtain pure transduced cell populations.
Articles

Blood was drawn and collected in 0.5 M EDTA-coated tubes. To separate plasma, bone marrow and spleen were flushed through 1.95 g/L and 4°C was performed. Whole bone marrow and splenic immune cells were isolated by crushing the respective organ, and a single-cell suspension was obtained by filtering through a 70-μm cell strainer. Erythrocytes were lysed with RBC lysis buffer (Tonbo, Cat. no. TMB-4300-L100) by incubating on ice for 10 min, after which cells were washed with FACS buffer.

Flow cytometry. Single-cell suspension was prepared as described in the 'Tissue harvest and dissociation' section. Cells were incubated for 10 min on ice with FC block (1:100, clone 2.4G2, Tonbo) in FACS buffer. Cells were subsequently stained with directly conjugated antibodies in FACS buffer for 25 min on ice in the dark, followed by two washes with FACS buffer. PDPX* regulatory T cells were identified by endogenous GFP signal from reporter mice (B6.FOXP3-GFP, BALB/c, FOXP3-GFP). Stained cells were immediately analysed or fixed with 0.5% PFA in PBS. All data were acquired using BD LSR Fortessa or LSRII Analyzer, and analysed with FlowJo v10.0. The absolute number of tumour-infiltrating immune cells (over total number of cells in single-cell suspension) was determined by using the liquid counting beads (BD Biosciences). The following antibodies were used for FACS sorting as well as immune profiling:

Myeloid cell phenotyping panel 1: CD45-violetFluor450 (clone 30-F11, Tonbo), CD11b-APC-Cy7 (clone M170, Tonbo), Ly6G-PerCpCy5.5 (clone 1A8, Ly6c-PE-CF594 (clone AL-21, BD Biosciences), F4/80-BV605 (clone BM8, Biocytex), I-A/I-E-BV510 (clone M5/114.15.2, Biocytex), CD11c-PE-Cy7 (clone N418, Biocytex), CD64-APC (clone X54-5/7.1, Biocytex), CD103-PE-Cy7 (clone 2E7, Biocytex), PD-L1-BV711 (clone MIHS, BD Biosciences) and DAPI (NucBlue Fixed Cell ReadyProbes Reagent).

Myeloid cell phenotyping panel 2: CD45-violetFluor450 (clone 30-F11, Tonbo), CD11b-APC (clone M170, Tonbo), Ly6G-PerCpCy5.5 (clone 1A8, Ly6c-PE-CF594 (clone AL-21, BD Biosciences), F4/80-BV605 (clone BM8, Biocytex), I-A/I-E-BV510 (clone M5/114.15.2, Biocytex) and CCRL2-PE (R&D Systems). Lymphoid cell phenotyping panel 1: CD45-violetFluor450 (clone 30-F11, Tonbo), B220-APC-Cy7 (clone RA3-6B2, Biocytex), CD3e-PerCpCy5.5 (clone 145-2C11, Tonbo), CD4-APC (clone GK1.5, Tonbo), CD8-FTTC (clone 53-6-7, Ly6C-PE-CF594 (clone AL-21, BD Biosciences), F4/80-BV605 (clone PC61, BD Biosciences), PDL1-BV605 (clone 29F1A2, Biocytex), γδ TCR-PE (clone GL13, Biocytex) and DAPI (NucBlue Fixed Cell ReadyProbes Reagent).

Cell sorting and library preparation for RNA-seq. To obtain pure tumour-infiltrating neutrophils (TINs) and macrophages (TIMs), breast tumours were dissociated into single-cell suspension by the same method as described in the 'Tissue harvest and dissociation' section. FACS sorting was performed using the Aria Cell Sorter (BD Biosciences) to purify TINs (DAPI-CD45-CD11b-Ly6G-Ly6Cint) and TIMs (DAPI-CD45CD11bLy6G-Ly6C−F4/80−). Cells were directly sorted into TEEtoll (Invitrogen) and kept at 80°C until further processing. RNA was extracted using the Direct-Zol RNA microprep kit (Zymo Research).

Following RNA isolation, MAT-OSeq was performed to amplify the whole transcriptome TINs and TIMs as previously described23. Raw RNA-seq was performed for cancer cells’ RNA. The paired-end reads were mapped to the mouse genome (NCBI mm10 STAR genome) using STAR (https://github.com/alexbglee/STAR) with NCBI RefSeq gene as the reference.

RNA isolation and quantitative real-time polymerase chain reaction. We followed procedures described in our previous publication for RNA isolation and qPCR24. The primers are listed in Supplementary Table 4.

In vitro trans-well migration assay. Bone marrow immune cells were harvested immediately from euthanized 8-week-old naive WT (BALB/c and B6) mice as described in the 'Tissue harvest and dissociation' section. FACS sorting was performed on the eight cell line models in technical triplicates as shown in Supplementary Fig. 1a. Genes corresponding to different properties of these cells were used for (1) TNBC heterogeneity (Supplementary Fig. 1b), (2) cytotoxic response (Fig. 3s), and (3) EMT states (Fig. 4). In particular, in Supplementary Fig. 1d, PyMT-N expresses GATA3, K8 and K18, whereas PyMT-M expresses vimentin, Zeb1 and Snail, suggesting that the two models represent luminal-like and claudin-low TNBC models, respectively.

Bioinformatics analyses. Characterization of the eight syngeneic murine tumour models. RNA-seq was performed on the eight cell line models in technical triplicates as shown in Supplementary Fig. 1a. Genes corresponding to different properties of these cells were used for (1) TNBC heterogeneity (Supplementary Fig. 1b), (2) cytotoxic response (Fig. 3s), and (3) EMT states (Fig. 4). In particular, in Supplementary Fig. 1d, PyMT-N expresses GATA3, K8 and K18, whereas PyMT-M expresses vimentin, Zeb1 and Snail, suggesting that the two models represent luminal-like and claudin-low TNBC models, respectively.

Nanosting dataset. Formalin-fixed paraffin-embedded (FFPE) tissue was first examined with hematoxylin and eosin staining to localize invasive tumour cells and surrounding area. A Roche High Pure FFPE RNA Isolation Kit was then used to purify RNAs. Macrodissection was performed when needed. Approximately 50ng of total RNA was used to measure the expression of 730 immune-related genes and 40 housekeeping genes using the nCounter platform (NanoString Technologies) and the PanCancer Immune Profiling Panel. Data were log2-transformed and normalized using housekeeping genes selected using the nSolver 2.6 package. The normalized data are provided in Supplementary Table 2c.
We then examined the Q-Q plot and noted that approximately 20% of points fall in the first dataset (Supplementary Table 2a). This subset of samples was used for subsequent analyses. The distribution of this score was examined as a histogram. The median was selected as a cutoff to define a distinct group of tumours with exceptionally high TIN (Supplementary Fig. 8i).

Therefore, the top 20% was determined as a cutoff to define a distinct group of tumours with exceptionally high TINs. (Supplementary Fig. 8e).

To further investigate the potential role of neutrophils in cancer progression, we downloaded 86 gene sets from MSigDB that are related to adenosine or immune cell infiltration (https://github.com/Xiang-HF-Zhang/Dichotomous-of-innate-immune-landscape).

For GO-enrichment analyses, FDR < 0.05 were used to define TIN-enriched genes. GOstats was then carried out to calculate GO terms enriched in up-regulated and down-regulated genes in neutrophils of TAN.

Biostatistics analysis of macrophage and neutrophil transcriptomes from different tumour models. RNA-seq data of macrophages and neutrophils were obtained from GEO for the 50 hallmark gene sets (http://software.broadinstitute.org/goes/metab/genesets.jsf?collection=I10). Using the same tools described in previous section. The results are exhibited by hierarchical clustering in Figs. 5a and 6b. Principle component analysis (PCA) was used to analyse macrophage data shown in Supplementary Fig. 6d.

For GO-enrichment analyses, FDR < 0.05 were used to define TIN-enriched genes. GOstats was then carried out to calculate GO terms enriched in up-regulated and down-regulated genes in neutrophils of TAN. We focused on GO terms that contain more than five genes, P < 0.001 and odds ratio > 3.250. Identified GO terms are listed in Supplementary Table 3a. Four representative pathways are shown in Supplementary Fig. 6b.

Statistics and reproducibility. Data were analysed with Microsoft Excel functions, Prism 7 software (GraphPad) or R programming language. Statistical analysis was performed using unpaired or paired two-tailed Student’s t-test (with unequal variations if an F-test ruled out the equal variation assumption), ANOVA analysis, log-rank test (survival analysis), Fisher’s exact test or Shapirow test, as appropriate for the dataset. Statistical details (for example, sample size and specific test performed) for each experiment are denoted in the corresponding figure or figure legends. Individual mouse and independent in vitro samples (independent batch experiments, different tumour models and different animals) were considered biological replicates. All biologically independent samples were included and combined for statistical analyses. Experimental findings were reliably reproduced.

In this study, the sampling size was determined based on the results of preliminary experiments and no statistical method was used to predetermine sample size. Data are shown as means ± standard deviation (s.d.) unless otherwise specified. In box and whisker plots, the middle line is plotted at the median, the upper and lower hinges correspond to the first and third quartiles, and the upper and lower whiskers extend no further than 1.5 × IQR from the hinges (IQR, interquartile range or distance between first and third quartiles). P values lower than 0.05 were considered statistically significant. The statistical source data are included in Supplementary Table 5.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The RNA-seq data for cancer cells, tumour-infiltrating macrophages and tumour infiltrating neutrophils have been submitted to the Gene Expression Omnibus under accession number GSE104765. The normalized RNA-seq data for human TNBC nanostar datasets are provided in Supplementary Table 2c. Other secondary datasets used in this study include

1. TCGA dataset, available from https://portal.gdc.cancer.gov/. The sample IDs used in this study are provided in Supplementary Table 2d.


3. BioGPS Primary Cell Atlas, available from http://biogps.org/dataset/BDSD_00013/primary-cell-atlas/. The specific samples used in this study are listed in Supplementary Table 2a.


5. Metastatic melanoma dataset26 available from GEO: GSE78220.

Code availability

Key codes for data analyses and major intermediate data are available at Github: https://github.com/Xiang-HF-Zhang/Dichotomous of innate immune-landscape.