Immuno-subtyping of breast cancer reveals distinct myeloid cell profiles and immunotherapy resistance mechanisms

Ik Sun Kim1,2,3,4, Yang Gao3,4, Thomas Welte1,3,4, Hai Wang1,3,4, Jun Liu1,3,4, Mahnaz Janghorban3,4, Kuanwei Sheng2,3,5, Yichi Niu3,5, Amit Goldstein1,3,4, Na Zhao3,4, Igor Bado1,3,4, Hin-Ching Lo1,2,3,4, Michael J. Toneff3,4,6, Tuan Nguyen3,4,7, Wen Bu1,3,4, Weiyu Jiang1,3,4, James Arnold3,8, Franklin Gu3,8, Jian He9, Deborah Jebakumar9, Kimberly Walker6, Yi Li1,3,4, Qianxing Mo5,10, Thomas F. Westbrook3,5,8, Chenghang Zong3,5,11, Arundhati Rao9, Arun Sreekumar3,4, Jeffrey M. Rosen3,4 and Xiang H.-F. Zhang1,3,4,11*

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 neutrophils (or granulocytic myeloid-derived suppressor cells), is resistant to ICB, and contains a minority of macrophages that

Immune cells participate in every aspect of tumour progression1. Many immune cells may play disparate roles—anti-tumorigenic in some situations, pro-tumorigenic in others2. For instance, macrophages undergo different activation and polarization3,4; the classically activated subsets potentiate anti-tumour immunity5,6 whereas the alternatively activated subsets promote tumours through multiple mechanisms7,8. Neutrophils also play opposing roles in different settings9–12, 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 alterations1,3,14. Immature neutrophils and macrophages may accumulate in blood and immune organs, develop immunosuppressive activity, and alter tumour progression either by infiltrating tumours11,13 or via homing to distant organs to establish pre-metastatic niches14–18. 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 features19–21, where different subtypes of breast cancer exhibit distinct developmental programs, metastatic behaviours and molecular landscapes22–25. Variations in immune profiles have been linked to prognosis, therapeutic responses and breast cancer subtypes26–30. 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 (Supplementary Fig. 1a). In particular, PyMT-M and PyMT-N were derived from the same C57/BL6 tumour but exhibited different properties. MMTV-PyMT tumours express oestrogen receptor (ER) in early tumorigenesis, but the tumours progressively lose ER as they develop31. We confirmed the lack of ER, progesterone receptor (PR) and ErbB2 expression in PyMT-M and PyMT-N tumours (Supplementary Fig. 1b,c). These and
previous results\textsuperscript{12–14} indicate that, by definition, the eight models represent triple-negative breast cancer (TNBC), which is a heterogeneous group of diseases\textsuperscript{21}. 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\textsuperscript{13} 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\textsuperscript{+}CD11b\textsuperscript{+}Ly6G\textsuperscript{−}Ly6C\textsuperscript{med-low} cells, and TIMs are defined as CD45\textsuperscript{+}CD11b\textsuperscript{+}Ly6G\textsuperscript{−}Ly6C\textsuperscript{F4/80\textsuperscript{+}} cells (Fig. 1a,b)—note that F4/80\textsuperscript{+} cells are CD64\textsuperscript{+} (ref. \textsuperscript{22}) (Fig. 1c). Wright-Giemsa staining confirmed their polymorphonuclear/ multi-lobed and mononuclear morphologies, respectively (Fig. 1d).

We next extended TIM/TIN analyses to an additional seven syngeneic murine and five patient-derived xenograft (PDX) TNBC models. PDXs informed residual immune cells infiltrating human tumours in SCID/Beige mice. Circulating immune cells were examined to evaluate systemic alterations. We performed unsupervised clustering using TIM, TIN, peripheral blood neutrophils (PBNs, CD45\textsuperscript{+}CD11b\textsuperscript{+}Ly6G\textsuperscript{−}Ly6C\textsuperscript{F4/80\textsuperscript{+}}) and peripheral blood monocytes (PBMs, CD45\textsuperscript{+}CD11b\textsuperscript{+}Ly6G\textsuperscript{−}Ly6C\textsuperscript{CD11b\textsuperscript{+}}) and total tumour-infiltrating CD45\textsuperscript{+} cells. PBNs and PBMs are potential sources of TINs and TIMs, respectively.

Four clusters were observed (Fig. 1e), driven mainly by the total number of CD45\textsuperscript{+} cells and the TIM/TIN ratio (Fig. 1f). PDX tumours in SCID/Beige mice fell mostly into cluster II with low numbers of CD45\textsuperscript{+} 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. 1j), arguing against a specific link between immunodeficiency and the ‘cold’ phenotype.

The TIM/TIN 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 TIM/TIN ratio exhibited a bimodal distribution (Fig. 1f).

The TIM frequency strongly correlated with PBNs (Fig. 1g), indicating that systemic neutrophil accumulation accompanied local TIM 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) tumour environments 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 TIM/TIN frequency (Fig. 3b), further supporting that the TIM/TIN 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-M is an exception:** 28 spontaneous tumours derived from 6 animals exhibited diverse TIM/TIN frequency (Fig. 3c). We chose one tumour with intermediate levels of TIM 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 TIM/TIN 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\(^+\)Ly6C\(^{\text{med-low}}\) cells (neutrophils) and CD11b\(^+\)F4/80\(^+\) cells (macrophages) in two representative tumour models. Plots are gated on CD45\(^+\)CD11b\(^+\) Ly6G\(^+\)Ly6C\(^{\text{med-low}}\) F4/80\(^+\) cells 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\(^+\)Ly6C\(^{\text{med-low}}\)TINs (left) and CD45\(^+\)CD11b\(^+\)Ly6C\(^{\text{med-low}}\)TIMs (right). Scale bar, 10 µm. For **c,d**, the experiments were repeated at least five times with similar results. **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 Ly6C\(^{\text{high}}\) 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 log2-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)\(^47\) 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. P-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. P-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. Quantification of migrated neutrophils (left) and monocytes (right) towards tumour-conditioned medium. e, Heatmap showing the expression level of chemokines and cytokines known to regulate chemotaxis (chemo) of neutrophils and monocytes. Tnfaip6 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 TIN/TIM 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.
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).

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, \( \text{Ifn}\alpha \) 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 (Fig. 3b).

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 TIM/TIN 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 \( \text{Ifn}\beta \) (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 TGF\( \beta \) and increased the functional CXCL2 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 myeloid microenvironment.

In the TCGA dataset, we used GSEA to identify pathways correlating with TIMER-derived TIM and TIM scores (Fig. 4f). EMT is the top pathway specifically associated with TIM (Supplementary Fig. 4h), supporting our observations in mouse models. The TIM-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 chemokines CSF1 and CCL2, the neutrophil-repelling 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 pathway contribute to the development of a diverse myeloid cell compartment. In particular, EMT may simultaneously drive monocye/macrophage recruitment and neutrophil exclusion.

TIMs 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-\( \beta \), reactive oxygen species (ROS) and mTOR, whereas E0771-TIMs overexpress pro-inflammatory pathways including IFN-\( \gamma \) and TNF-\( \alpha \) (Fig. 5b and Supplementary Fig. 5a). Similar to T11-TIMs, AT3-TIMs overexpress several immunosuppressive pathways such as Myc and ROS, but also highly express pro-inflammatory pathways such as TNF-\( \alpha \).

The functional impact of TIMs was evaluated by transplantation of various tumours into CCR2 knockout (CCR2-KO) mice. As expected, Ly6C\( \text{med} \) 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 clodosome to deplete TIMs. This approach can eliminate tissue-resident macrophages, and indeed depleted TIMs in all models tested including MES (Fig. 5f). Interestingly, whereas anti-CSF1 and clodosome 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). NES-TINs (2208L and AT3) express a substantial proportion of TAN/MDCS genes, whereas MES-TINs (T11 and E0771) are more related to normal neutrophils (Fig. 6a). GSVA of the hallmark pathways further uncovered differences of TINs between NES and MES. The former displayed enhanced expression of several immunosuppressive pathways, including STAT3, TGF-\( \beta \) 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 = 3) and E0771 (n = 4) cell lines following miR-200c induction with doxycycline. The bar graph represents the mean value of biological replicates. e, Quantification of neutrophil migration towards tumour-conditioned medium of T11 (n = 9) miR-200c (n = 10) and vehicle (n = 9) treated models. 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 TIMER scores (TIMER-TIM) and miR-200c expression in the TCGA TNBC dataset (n = 112 patients).
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 showing the 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 Ly6Chigh 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+Ly6C−F4/80+) 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+Ly6G+Ly6Cmed-low) 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 with 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 mMDSCs. Thus, TINs in different immune subtypes differ in both frequency and immunosuppressive activity. Moreover, NES tumours induce systemic accumulation of mMDSCs.

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 resulted in an increase of Ly6C+ monocytes (Fig. 6d,e), suggesting a negative regulation of TIN on monocyte recruitment. The increased monocytes did not differentiate into TIMs. In contrast, monocytes in MES readily differentiate into TIMs (Fig. 5c). Thus, the definition of MES includes tumours enriched with TIMs and their precursor monocytes.

NES and MES respond differently to immune checkpoint blockade. We subjected eight models (4 NES, 3 MES, and MMTV-WNT1) 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 NES-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. 7a). However, NES-derived cell lines (for example, PyMT-N) remained resistant (Supplementary Fig. 7a). MES showed largely variable responses (Fig. 7a and Supplementary Fig. 7a). TIMs (CD11b+Ly6G−Ly6C−) in hosts bearing PyMT-N tumours (NES) treated with anti-Ly6G and anti-CXCR2, or IgG control. The results are representative of at least five biologically independent animals.

**Fig. 6 | TINs in NES tumours express multiple immunosuppressive pathways, and negatively regulate Ly6C+ monocyte recruitment.** a, Heatmap showing unsupervised hierarchical clustering of TINs purified from four tumour models (n = 3 biologically independent samples for each model) using gene characteristic of normal neutrophils or TANs and/or MDSCs as published previously59. b, Heatmap showing unsupervised clustering of TINs of four tumour models (n = 3 biologically independent samples for each model) using GSVA of the 50 hallmark pathways from MSigDB. Pathways related to immunosuppressive activities are shown. The heatmap with all pathways annotated is shown in Supplementary Fig. 6a. c, In vitro immunosuppression assay by co-culturing bone marrow neutrophils from indicated NES-tumour-bearing animals and splenic T cells. Proliferation of T cells was determined based on CFSE intensity as measured by FACS. A left-shift of CFSE intensity histogram indicates dilution of signals by proliferation. Data are shown as mean ± s.d. of three biological replicates (neutrophils from three different mice). p values were determined by two-sided t-tests. d, Quantification of monocytes, TIM and TIN in NES tumours (PyMT-N and 2208L) following treatment of anti-CXCR2 and anti-Ly6G. 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 computed by two-sided Student’s t-tests. e, Example FACS plots showing the alteration of TINs (CD11b+Ly6G−Ly6C−), monocytes (CD11b+Ly6G+Ly6C+), TIMs (CD11b+Ly6G−Ly6C−F4/80+) in hosts bearing PyMT-N tumours (NES) treated with anti-Ly6G and anti-CXCR2, or IgG control. The results are representative of at least five biologically independent animals.
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 T cell 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. 7 | Heightened accumulation of immunosuppressive TINs or gMDSCs is associated with de novo resistance to ICB. a. Tumour growth curves show responses of indicated tumour models to ICB therapy (anti-PD1 and anti-CTLA4). A treatment schematic is shown below the growth curves. Numbers in parentheses show the specific n values of biologically independent mice per group denoted by different colours. Dotted lines indicate the time point at which tumour sizes were compared between control and treatment groups. P values were computed by two-sided t-test. b. Left: growth curves of T11 tumour in WT or CCR2-KO mice with or without treatment of ICB (anti-PD1 and anti-CTLA4). Right: Kaplan–Meier curves show the progression-free survival of the animals in four groups. Numbers in parentheses show the specific n values of biologically independent mice. P values were determined by two-sided log rank test, comparing each experimental group to WT and IgG control group.
**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. at each time point. **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 on the right. Because experimental groups without ICB never regressed (parental IgG, ICBR IgG and ICBR anti-Ly6G), they were not further analysed for recurrence rate or progression-free survival. The sample size of each group is provided in parentheses. Growth curves of parental IgG, ICBR IgG and ICBR anti-Ly6G are homogeneous, and therefore are summarized as mean ± s.d. at each time point: **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. Recurrence rate post-ICB is shown as bar graphs under the curves. **f**. Heatmap showing the TIMER scores of indicated immune cells in a metastatic melanoma dataset. **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<sup>+</sup> (Supplementary Fig. 8a), representing immature neutrophils<sup>3,8</sup>. 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 systematically (Supplementary Fig. 8e). Cxcl1/2 expression was increased, whereas Tnfαip6 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. 8f)<sup>48</sup>, 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 dataset<sup>7</sup>, 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<sub>− = 0.53, P = 0.0033</sub>). 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-tumourally 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 report<sup>48</sup>. 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<sup>+</sup> 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-TIMs are more similar to normal neutrophils, and might even perform anti-tumorigenic functions as previously shown<sup>7</sup>. Thus, the functions of TIMs 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 cells<sup>8,50</sup>. 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 cancer<sup>41</sup>. 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 laboratories<sup>39</sup> 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 systematic 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.

Received: 30 June 2018; Accepted: 4 July 2019; Published online: 26 August 2019.

**References**

1. Joyce, J. A. & Pollard, J. W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 9, 239–252 (2009).
2. Kim, I. S. & Zhang, X. H. F. One microenvironment does not fit all: heterogeneity beyond cancer cells. *Cancer Metas. Rev. 35*, 601–629 (2016).
3. Porta, C. et al. Macrophages in cancer and infectious diseases: the good and the bad. *Immunotherapy* 3, 1185–1202 (2011).
4. Mantovani, A., Sica, A. & Locati, M. Macrophage polarization comes of age. *Immunity* 23, 344–346 (2005).
5. Schreiber, R. D., Old, L. J. & Smyth, M. J. Cancer immunoediting: integrating immunoLOGY's roles in cancer suppression and promotion. *Science* 331, 1565–1570 (2011).
6. Pyonteck, S. M. et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat. Med.* 19, 1264–1272 (2013).
7. Qian, B. Z. & Pollard, J. W. Macrophage diversity enhances tumour progression and metastasis. *Cell* 141, 39–51 (2010).
8. Gallina, G. et al. Tumours induce a subset of inflammatory monocytes with immunosuppressive activity on CD8<sup>+</sup> T cells. *J. Clin. Invest.* 116, 2777–2790 (2006).
9. Granot, Z. et al. Tumour entrained neutrophils inhibit seeding in the premetastatic lung. *Cancer Cell* 20, 300–314 (2011).
10. Wulèk, S. K. & Malanchi, I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature* 528, 413–417 (2015).
11. Fridlender, Z. G. et al. Polarization of tumor-associated neutrophil phenotype by TGF-beta: 'N1' versus 'N2' TAN. *Cancer Cell* 16, 183–194 (2009).
12. Park, J. et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci. Transl. Med.* 8, 361ra138–361ra138 (2016).
13. Redig, A. J. & McAllister, S. S. Breast cancer as a systemic disease: a view of metastasis. *J. Int. Med.* 274, 113–126 (2013).
14. Egéblad, M., Nakasone, E. S. & Werb, Z. Tumors as organs: complex tissues that interface with the entire organism. *Dev. Cell* 18, 884–901 (2010).
15. Gabrilovich, D. I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* 9, 162–174 (2009).
16. Kaplan, R. N. et al. VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 438, 820–827 (2005).
44. Semerad, C. L., Liu, F., Gregory, A. D., Stumpf, K. & Link, D. C. G-CSF is an essential regulator of neutrophil trafficking from the bone marrow to the injured tissue [1].

42. Mabbott, N. A., Baillie, J. K., Brown, H., Freeman, T. C. & Hume, D. A. An immune infiltration in breast cancer and their clinical implications: a gene-expression-based retrospective study. PLoS Med. 10, e31524 (2013).

40. Hanahan, D. & Coussens, L. M. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell 21, 309–322 (2012).

38. Kustrametz, S. & Gabrilovich, D. I. Role of immature myeloid cells in mechanisms of immune evasion in cancer. Cancer Immunol. Immunother. 55, 237–245 (2006).

36. Mabbott, N. A., Baillie, J. K., Brown, H., Freeman, T. C. & Hume, D. A. An expression atlas of human primary cells: inference of gene function from expression profiles. Nat. Methods. 7, 12150 (2016).

34. Tian, L. et al. Mutual regulation of tumour vessel normalization and immune infiltration in breast cancer and their clinical implications: a gene-expression-based retrospective study. PLoS Med. 13, e1002194 (2016).

Du, Z. et al. Introduction of oncopines into mammary glands in vivo with an avian retroviral vector initiates and promotes carcinogenesis in mouse models. Proc. Natl Acad. Sci. USA 103, 17396–17401 (2006).

Dyer, D. P. et al. TGF-β inhibits neutrophil migration via direct interaction with the chemokine CXCL8. J. Immunol. 192, 2177–2185 (2014).

Korpal, M., Lee, E. S., Hu, G. & Kang, Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. J. Biol. Chem. 283, 14910–14919 (2008).

Gregory, P. A. et al. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat. Cell Biol. 10, 593–601 (2008).

Burk, U. et al. A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep. 9, 588–593 (2008).

Toneff, M. J. et al. The Z-cad dual fluorescent sensor detects dynamic changes between the epithelial and mesenchymal cellular states. BMC Biol. 14, 47 (2016).

Hänzelmann, S., Castelo, R. & Guinney, J. GSA: gene set variation analysis for microarray and RNA-Seq data. BMC Bioinformatics 14, 7 (2013).

Taub, J. H. et al. Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with claudin-low and metastatic breast cancer subtypes. Proc. Natl Acad. Sci. USA 107, 15449–15454 (2010).

Subramanian, A. et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102, 15545–15550 (2005).

Kaneda, M. M. et al. PI3Kγ is a molecular switch that controls immune suppression. Nature 539, 437–442 (2016).

De Henau, O. et al. Overcoming resistance to checkpoint blockade therapy by targeting PI3Kγ in myeloid cells. Nature 539, 443–447 (2016).

Fridlender, Z. G. et al. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. PLoS One 7, e31524 (2012).

Condamme, T. & Gabrilovich, D. I. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol. 32, 19–25 (2011).

Gabrilovich, D. I., Ostrand-Rosenberg, S. & Bronte, V. Coordinated regulation of myeloid cells by tumours. Nat. Rev. Immunol. 12, 253–268 (2012).

Deaglio, S. et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J. Exp. Med. 204, 1257–1265 (2007).

Sitkovsky, M. & Lukashev, D. Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. Nat. Rev. Immunol. 5, 712–721 (2005).

Netherby, C. S. & Abrams, S. I. Mechanisms overseeing myeloid-derived suppressor cell production in neoplastic disease. Cancer Immunol. Immunother. 66, 989–996 (2017).

Maruyama, K. et al. The transcription factor Jdp2 controls bone homeostasis and antibacterial immunity by regulating osteoclast and neutrophil differentiation. Immunity 37, 1024–1036 (2012).

Riaz, N. et al. Tumor and microenvironment evolution during immunotherapy with nivolumab. Cell 171, 934–949.e15 (2017).

Hugo, W. et al. Genomic and transcriptomic features of response to anti-PD-1 therapy in metastatic melanoma. Cell 165, 35–44 (2016).

Pahler, I. C. et al. Plasticity in tumor-promoting inflammation: impairment of macrophage recruitment evokes a compensatory neutrophil response. Neoplasia 10, 329–339 (2008).

Dongre, A. et al. Epithelial-to-mesenchymal transition contributes to immunosuppression in breast carcinomas. Cancer Res. 77, 3982–3989 (2017).

Lou, Y. et al. Epithelial-mesenchymal transition is associated with a distinct tumor microenvironment including elevation of inflammatory signals and multiple immune checkpoints in lung adenocarcinoma. Clin. Cancer Res. 22, 3630–3642 (2016).

Wellenstein, M. et al. Loss of p53 triggers WNT-dependent systemic inflammation to drive breast cancer metastasis. Nature https://doi.org/10.1038/s41586-019-1450-6 (2019).
Author contributions
Conception and design: X.H.-EZ, I.S.K. and J.M.R. Development of methodology: I.S.K., X.H.-EZ, T.W., M.J.T., H.W., J.L., K.S., Y.L., Q.M., T.F.W., C.Z., A.R., and A.S. Acquisition of data: I.S.K., Y.G., T.W., M.J., N.Z., A.G., Y.N., H.C.L., I.R., T.N., W.B., W.J., J.A., F.G., J.H., D.I., K.W., and X.H.-EZ. Analysis and interpretation of data: I.S.K., X.H.-EZ, and J.M.R. Writing and review of manuscript: X.H.-EZ, I.S.K., and J.M.R. Study supervision: X.H.-EZ.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41556-019-0373-7.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to X.H.-EZ.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

© The Author(s), under exclusive licence to Springer Nature Limited 2019
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/cAnNcHsd (BALB/c), C57BL/6NcHsd (C57BL/6 or B6), FVB and C3H/HeNcLac-PrdcRiSlac-Ly5cRiSlac-Ly5c (SCID) mice were purchased from Enivgo and either directly used for experiments or bred in our facilities. The study is compliant with all relevant ethical regulations regarding animal research.

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. Age-matched mice (10–12 weeks old) were subjected to euthanization for immune profiling in all experiments. BALB/cAnNcHsd (BALB/c), p53-null tumour), T1 (BALB/c, p53-null tumour), T12 (BALB/c, p53-null tumour), 2151R (BALB/c, p53-null tumour), 2245R (BALB/c, p53-null tumour), PyMT-M and -N (B6, MMTV-PyMT sub-lines), MMTV-WNT1 (FVB) and PyMT-PDENT KDO (FVB). The study is compliant with all relevant ethical regulations regarding animal research.

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), counted, resuspended in 1:1 solution of PBS and Matrigel (Pheno Red-free and 200 μg/ml of ant-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 Fig. 6f, Supplementary Fig. 7f, 3–4 weeks as described above. SB225002 was dissolved in DMSO and with 30% PEG300 (Sigma), 5% Tween 80 (Sigma) in distilled water.

In Fig. 5f, 0.5 mg of anti-CSF1 (clone 5A1) and 100 μl of cdloocluscd (Encapsula Nanosciences) were delivered through intraperitoneal 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 HRPN)). All antibodies were delivered intraperitoneally and were purchased from BioXcell. Tumours were measured with a caliper and the volume was calculated using the formula π/6 × width² × length.

Spontaneous pulmonary metastasis assay. The assay was performed using eight tumour models implanted as either tissues or cell lines (10⁻² × 2 × 10⁹ 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 examination, and macroscopic metastases were counted as previously described.

Tissue harvest and dissociation. Tumours were resected 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 Dissection 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 C tubes (Miltenyi) containing 2.33 ml of RPMI-1640, 100 μl of enzyme D, 50 μl of enzyme R 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 (eBiосience), 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.
Blood was drawn and collected in 0.5 M EDTA-coated tubes. To separate plasma, red blood cells (RBC) and white blood cells (WBC), blood samples were centrifuged at 1,500 g and 4°C for 5 min. 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 FcR blocker (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. POP3+ regulatory T cells were identified by endogenous GFP signal from reporter mice (B6.FOXP3-GFP, BALB/c.FOX3-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 (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-PerCP-Cy5.5 (clone 1A8, LyteC), CD45-PE-CF594 (clone AL-21, BD Biosciences), F4/80-BV605 (clone BM8, Biotech), I-A/E-BV510 (clone M5/114.15.2, Biotech), CD11c-AlexaFluor700 (clone N418, Biotech), CD64-APC (clone X54-57.1, Biotech), CD103-PE-Cy7 (clone 2E7, Biotech), PD1-BV711 (clone @MHS, 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-PerCP-Cy5.5 (clone 1A8, LyteC), CD45-BV711 (clone HK1.4, Biotech), F4/80-FTTC (clone BM8, Biotech), I-A/E-PE (clone M5/114.15.2, Biotech) and CR2-PE (R&D systems).

Lymphoid cell phenotyping panel 1: CD45-violetFluor450 (clone 30-F11, Tonbo), B220-APC-Cy7 (clone RA3-6B2, Biotech), CD3e-PerCP-Cy5.5 (clone I45-2C11, Tonbo), CD4-APC (clone GK1.5, Tonbo), CD8-FITC (clone 53-6.7, Biotech), CD25-BV510 (clone PC61, Biotech), PD1-BV605 (clone 29F1A12, Biotech), γδTCR-PE (clone GL1, Biotech) and DAPI (NucBlue Fixed Cell ReadyProbes Reagent).

Cell sorting and library preparation for RNA-seq. To obtain pure tumour-infiltrating neutrophils (TIDNs) and macrophages (TIDMs), 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 TIDNs as described in the ‘Tissue harvest and dissociation’ section. FACS sorting as well as immune profiling: TCR-PE (clone GL3, Biolegend) and DAPI (NucBlue Fixed Cell ReadyProbes Reagent).

RNA isolation and quantitative real-time polymerase chain reaction. Following RNA isolation, MATQ-seq was performed to amplify the whole transcriptome TIDMs and TIDMs as previously described [10]. Raw RNA-seq was performed for cancer cells’ RNA. The paired-end reads were mapped to the mouse genome (UCSC mm10) using STAR (https://github.com/alexdobin/STAR) with NCBI RefSeq genes as the reference.

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. Following RBC lysis, neutrophils were enriched by positive selection using biotinylated anti-mouse Ly6G (clone 1A8, Biotech) and Ly6C+ cells were enriched by negative selection using biotinylated anti-mouse antibodies against B220, CD3ε (BD PharMingen, cat. no. 559971) and Ly6G (clone 1A8, Biotech) as per the protocol of the EasySep Mouse Biotin Positive Selection Kit (STEMCELL Technologies). For the trans-well migration assay, tumour-conditioned medium (TCM) containing 0.2% FBS (cultured for 24 hours) was added to the bottom of a 24-well plate. 1X 10^6 cells of either neutrophils or monocytes (in the same medium used to generate TCM) were added to the upper chamber of trans-well inserts: 3×10^5 neutrophils per transwell (Cat. No. 3422). Cells were incubated at 37°C for 2 hours (neutrophils) or 4 hours (mononuclear cells) before inserts were removed, and cells in the bottom well were harvested. Flow cytometry was used to quantify migrated cells using liquid counting beads as a reference (BD Biosciences).

In vitro T cell proliferation (suppression) assay by co-culture with neutrophils and monocytes. CD3+ T cells of naive BALB/c or C57BL/6 mice (6-8 weeks old) were enriched by negative selection using biotinylated anti-mouse antibodies against B220, CD11b, Gr1 (BD PharMingen, cat. no. 559971) and CD11c (BD PharMingen, cat. no. 553800) followed by magnetic separation using EasySep Mouse Biotin Positive Selection Kit (STEMCELL Technologies). Bone marrow neutrophils and monocytes from either naive or relevant tumour-bearing mice were harvested as described in the ‘In vitro trans-well migration assay’ section. Magnetically sorted CD3+ T cells were labelled with CFSE (5μM, Molecular Probes) as per the manufacturer’s instructions. T cells were cultured alone or admixed with neutrophils or monocytes (at a 1:3 ratio) in a 96-well plate. T cell proliferation was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay performed by the BCM Breast Center Pathology Core. Briefly, 3–4μm thick sections were deparaffinized in xylene and graded alcohols, and treated with a heat-induced antigen retrieval buffer Tris-HCL 9.0 in a pressure cooker. Slides were blocked in 3% hydrogen peroxide solution for 5 min at room temperature. Primary antibodies were incubated for 1 hour at room temperature in antibody diluent solution (1% BSA in TBS-20) and subsequently stained with Envision Labelled Polymer-HRP anti-rabbit (Dako) for 30 min at room temperature. Slides were incubated with DAB (Diaminobenzidine) solution for 5 min over a gentle warm water bath at 37°C, then counterstained in Harris Hematoxylin. Images were obtained at a magnification of 10× using a Leica DM8 microscope with DMC-A500 camera.

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. 1d), (2) cytokine expression (Fig. 3f), and (3) EMT states (Fig. 4b). 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.

Cell morphology analysis. Cancer cell images were captured at a magnification of 10x using a Leica confocal microscope. 50,000 FACS-purified neutrophils (CD45+ CD11b+ Ly6G+ Ly6Cmed-low) and macrophages (CD45+CD11bLy6G+Ly6C+ ‘F4/80’) were cyto-spun and dried slides were stained with Wright-Giemsa Stain (Sigma-Aldrich). Images were obtained at a magnification of 40x using a Leica DM1200 microscope.

Derivation of macrophage-specific and neutrophil-specific genes for analysis of TNBC NanoString data. Over 700 gene expression profiles of a variety of human cell types were obtained from Primary Cell Atlas of BioGPS (http://biogps.org/)…
We then examined the Q-Q plot and noted that approximately 20% of points fall in the first dataset (Fig. 8g), in which only pre-treated tumours were included. Z-scores displayed as heatmap shown in Fig. 8f,h with samples ordered according to the TIN scores.

Macrophages were also displayed as stacked bar graphs using the 'ggplot2' package. The distance function and the option of 'Ward.D2' of the 'hclust' function for dendrogram computation. The predicted relative abundance of neutrophils and TNBC tumours were isolated based on associated annotations and displayed in Supplementary Table 3a. Four representative pathways are shown in Supplementary Fig. 6b.

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

Bioinformatics analysis of macrophage and neutrophil transcriptomes from different tumour models. RNA-seq data of macrophages and neutrophils were analysed by GSEA for the 50 hallmark gene sets (http://software.broadinstitute.org/gsea/mghd/geneSets.jsp) collected to identify NSG. Specific arrays used in comparisons are listed in Supplementary Table 2a. These genes were then intersected with genes contained in the nanostring TNBC dataset. 33 MSGs and 45 NSGs were identified, as listed in Supplementary Table 2b.

Unsupervised hierarchical clustering was performed using the 'heatmap.2' function in the 'gplots' package of R. Ward clustering algorithm was used and the sample distance was defined by the Manhattan approach (absolute distance between two vectors). The resulted cluster structures were then superimposed with the expression of the CSF3, ELANE and CD68 genes, as well as IHC staining results of CD68 of the same dataset.

In Fig. 2d, Σlog(NSG) − Σlog(MSG) was used to compute a single score for each tumour. The distribution of this score was examined as a histogram. The bimodal distribution was approximated by the 'normalmixEM' function in the 'mixtools' package of R.

**TIMER and CIBERSORT analysis of immune cell infiltration in RNA-seq/microarray datasets of bulk tumours.** We obtained RAN-seq profiles of breast cancer from TCGA data portal in June 2016. To avoid any potential batch effects, we only chose samples profiled at UNC and stored in a folder named ‘RNASeqV2’. 1,073 profiles were collected. We then used associated IHC-determined ER/PR/HER2 statuses to extract 112 triple-negative breast cancer samples. We did not perform any additional filtration of samples. Barcodes or IDs of all samples included in our analyses are listed in Supplementary Table 2d.

TIMER output of TCGA tumour specimens was downloaded from the algorithm website (https://cistrome.shinyapps.io/timer/). Immune cell infiltration estimates of the corresponding 1,073 TCGA breast tumours were isolated by matching the barcodes and IDs. The distribution of all six types of immune cells across TNBC and non-TNBC is displayed in Fig. 5e and Supplementary Fig. 2a by using the ‘heatmap.2’ function with the option of ‘Manhattan’ of the distance function and the option of ‘Ward.D2’ of the ‘hclust’ function for dendrogram computation. The TIN scores (Fig. 21 and Supplementary Fig. 2b, c) were implemented by the ‘tsne’ package of R with perplexity = 10 and other parameters in default setting.

CIBERSORT output was downloaded from website provided in the reference52. TNBC tumours were isolated based on annotated annotations and displayed by hierarchical clustering using ‘heatmap.2’ with the option of ‘Manhattan’ of the distance function and the option of ‘Ward.D2’ of the ‘hclust’ function for dendrogram computation. The predicted relative abundance of neutrophils and macrophages were also displayed as stacked bar graphs using the ‘ggplot2’ package of R in Fig. 2p.

In Fig. 4f and Supplementary Fig. 4h,i, GSEA was applied to the TCGA TNBC dataset, using TIM-TIMER scores and TIN-TIMER scores as continuous phenotypic values and hallmark pathway as gene sets. Pathways with P < 0.05 and FDR < 0.1 were selected and displayed in Fig. 4f. The graphic output depicting correlation between TIM and EMT and between TIM and P13K-akt-mTOR were shown in Supplementary Fig. 4h,i, respectively.

In Fig. 4g and Supplementary Fig. 4j, we applied gene set variation analysis (GSVA) to three hallmark pathways and an EMT signature defined previously53. GSVA was implemented using the ‘gsva’ package of R, and under default settings except for ‘RNAseq=TRUE’. The EMT signature was obtained from the supplementary material of the original paper54, and contains 91 upregulated and 160 downregulated genes. There are 32 genes in common between the 91 upregulated genes and the 200 hallmark EMT genes. The correlation between TIMER scores and pathway GSVA scores were assessed by Pearson correlation coefficients (Supplementary Fig. 4i).

In 4h, miR-200c expression TCGA TNBC was obtained from the TCGA data portal and compared against TIM scores predicted by TIMER.

In Fig. 8d and Supplementary Fig. 8i, we analysed two datasets. The normalised transcriptome profiles (regularized log-transformed) were uploaded to the TIMER website to generate estimates of immune cell infiltration, which were displayed as heatmap shown in Fig. 8d with samples ordered according to the TIM scores. Z-scores of TIM were compared between different therapy response groups in the first dataset (Fig. 8g), in which only pre-treated tumours were included.

The distribution of TIM scores was examined by histogram (Supplementary Fig. 8k), which exhibits an asymmetric pattern with long right tail. Non-parametric tests (Shapiro test and Skew test implemented in R) confirmed this observation. We then examined the Q-Q plot and noted that approximately 20% of points fall above the normality line (Supplementary Fig. 8g). Indeed, removal of the top 20% tumours renders the rest following normal distribution (Supplementary Fig. 8h).

**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 each experiment, the group sizes were 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 nanostaining 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.

2. METABRIC dataset, available from https://ega-archive.org/datasets/EGA_D000100000266.

3. BioGPS Primary Cell Atlas, available from http://biogps.org/dataset/BD5_00013/primary-cell-atlas/. The specific samples used in this study are listed in Supplementary Table 2a.

4. Gene expression profiles of TAN, gMDSC and normal neutrophils. Data available from GEO, dataset GSE25284.

5. Metastatic melanoma dataset†,† 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.
Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Research policies, see Authors & Referees and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- The statistical test(s) used AND whether they are one- or two-sided
- Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on Statistics for Biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: FACS data were acquired using BD FACSDIVA v8.0.

Data analysis: FACS data were analyzed using FlowJo v10.0. Experimental data were analyzed using Excel 2016, Graphpad v7.0 and v8.0, and R v3.3. For RNA-seq data analyses, The pair-ended reads were mapped to the mouse genome (UCSC mm10) using STAR v2.7 with NCBI RefSeq genes as the reference. Further analyses were performed using R v3.3, including packages ggplot2, gplots, Rtsne, gdata, gsva, prcomp, and GOstats were used. Customized R codes were provided at https://github.com/Xiang-HF-Zhang/Dichotomous-of-innate-immune-landscape.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The RNA-seq data for cancer cells, tumor-infiltrating macrophages, tumor-infiltrating neutrophils have been submitted to the Gene Expression Omnibus under accession number GSE104765. The normalized RNA-seq data for human TNBC nanostring 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.
2. METABRIC dataset, available from https://ega-archive.org/datasets/EGAD00010000266.
3. BioGPS Primary Cell Atlas, available from http://biogps.org/dataset/BDS_00013/primary-cell-atlas/. The specific samples used in this study are listed in Supplementary Table 2a.

4. Gene expression profiles of TAN, GMDSC and normal neutrophils. Data available from GEO, dataset GSE43254.

5. Riaz et al., metastatic melanoma dataset: https://github.com/riazn/bms038_analysis

6. Hugo et al., metastatic melanoma dataset available from GEO: GSE78220.

Statistical Source Data of all other figures are available in Supplementary Table 5.

Field-specific reporting
Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

Sample size
The group sizes were determined based on the results of our previous experiments of similar types. They are sufficient to reproducibly discern biologically meaningful difference according to our previous experience and the general standard in the field.

Data exclusions
No data was excluded from the analyses.

Replication
All animal experiments included at least n=3 (up to n=22) per group. The majority of animal experiments was repeated with at least two (up to five) independent cohorts with similar results. All in vitro experiments were performed with at least three biological replicates (either independent experiments, different tumor models, different animals). All results are reproducible.

Randomization
In all animal experiments involving treatments of exogenous reagents, animals were randomly allocated to ensure similar tumor sizes between different groups for treatment or other analyses.

Blinding
All samples were not blinded to the authors. Blind experiments were not necessary as all measurements were objective.

Reporting for specific materials, systems and methods
We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

- n/a
- Antibodies
- Eukaryotic cell lines
- Palaeontology
- Animals and other organisms
- Human research participants
- Clinical data

Methods

- n/a
- ChIP-seq
- Flow cytometry
- MRI-based neuroimaging

Antibodies

Antibodies used

1. Flow cytometry
   - FcR blocker (1:100, clone 2.4G2, Tonbo, cat # 40-0161-M001)
   - Myeloid cell phenotyping panel I: CD45-violetFluor450 (1:100, clone 30-F11, Tonbo, cat # 75-0451-U100), CD11b-APC-Cy7 (1:100, clone M1/70, Tonbo, cat # 25-0112-U100), Ly6G-PerCPcy5.5 (1:100, clone 1A8, Tonbo, cat # 65-1276-U100), Ly6C-PE-CF594 (1:100, clone AL-21, BD Biosciences, cat # 562728), F4/80-BV605 (1:100, clone BM8, Biolegend, cat # 123133), I-A/I-E-BV510 (1:100, clone M5/114.15.2, Biolegend, cat # 107635), CD11c-AlexaFluor700 (1:200, clone N418, Biolegend, cat # 117320), CD64-APC (1:100, clone X54-5/7.1, Biolegend, cat # 139306), CD103-PE-Cy7 (1:100, clone 2E7, Biolegend, cat # 121425), PD11-BV711 (1:100, clone M1H5, BD Biosciences, cat # 563369)
   - Myeloid cell phenotyping panel II: CD45-violetFluor450 (1:100, clone 30-F11, Tonbo, cat # 75-0451-U100), CD11b-APC (1:100, clone M1/70, Tonbo, cat # 20-0112-U100), Ly6G-PerCPcy5.5 (1:100, clone 1A8, Tonbo, cat # 65-1276-U100), Ly6C-BV711 (1:100,
Validation

All antibodies are from commercial sources (Tonbo Biosciences, BD Biosciences, Biolegend, R&D systems, ebioscience, Abcam, Santa Cruz, Dako, NeoMarkers, Cell Signaling, BioXcell) widely referenced in the field and were validated for the species specificity and intended applications according to the suppliers’ information. Antibodies against Ly6G, Ly6C, F4/80, CD3, CD4, CD8 have been validated by depletion studies and utilization of specific knockout animals for this manuscript as well as our previous publications (Welte, T. et al. Nat. Cell Biol. 18, 632–44 (2016), Tian, L. et al. Nature (2017). doi:10.1038/nature21724). All IHC antibodies have been routinely tested at BCM Breast Center Pathology Core.

Tonbo Biosciences: Tonbo Biosciences tests all antibodies by flow cytometry. Citations are provided as a resource for additional applications that have not been validated by Tonbo Biosciences. BD Biosciences: Our product development process includes testing on a combination of primary cells, cell lines and/or transfectant cell models with relevant controls using multiple immunassays to ensure biological accuracy. We also perform multiplexing with additional antibodies to interrogate antibody staining in multiple cell populations. Biolegend: All newly developed clones at BioLegend undergo validation testing for multiple applications. This serves as a cross-check for specificity and provides clarity for research uses. Each lot of this antibody is quality control tested by immunofluorescent staining with flow cytometric analysis. R&D systems: R&D Systems uses a rigorous process to design, test and validate its monoclonal antibody products. Thermo Fisher Scientific: To help ensure superior antibody results, we’ve expanded our specificity testing methodology using a 2-part approach for advanced verification. Abcam: Activity, stability and performance are important checks carried out by our laboratories in the US, Europe and China to ensure we produce high quality products. Our stringent quality control and validation processes use a variety of techniques, including western blot, ICC/IF, IHC, flow cytometry, ELISA, ChIP, IP and peptide array. Santa Cruz: Santa Cruz Biotechnology has focused on the ongoing development of research monoclonal antibodies, biochemicals, labware and CRISPR products. Santa Cruz Biotechnology has the highest commitment to quality and customer service. CST: Each Cell Signaling Technology (CST) antibody is validated in-house using rigorous standards including multiple experimental controls and, when available, multiple cell types. An antibody lot is only released when CST scientists are convinced of its specificity and sensitivity in the recommended applications. BioXcell: Our InVivoMab™ antibodies are specifically formulated for in vivo use. They feature greater than 95% purity, ultra-low endotoxin levels, and are preservative, stabilizer, and carrier protein-free. Many of our InVivoMab™ antibodies can also be used for in vitro applications including Western blotting, ELISA, flow cytometric analysis, immunofluorescence, immunohistochrometry, and immunoprecipitation. All InVivoMab™ products are screened for purity and integrity via SDS-PAGE and guaranteed to contain less than 2 endotoxin units per milligram.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) 4T1, 4T07 and 67NR cell lines were obtained from Dr. Fred Miller in Karmanos Cancer Institute. AT3 cell line was a kind gift of S.I. Abrams at Roswell Park Cancer Institute. T11 and MDA-MB-231 cell lines transduced with doxycycline inducible miR-200c overexpression vector were a kind gift of Dr. Jeffrey M. Rosen at Baylor College of Medicine. E0771 cell line was purchased from CH3 Biosystems. All other cell lines including p53-null tumor models (2208L and T11), MMTV-PyMT sublines (PyMT-M and -N), and ICBR (E0771 and PyMT-M) were derived in our laboratory.

Authentication No cell line authentication was performed.

Mycoplasma contamination All cell lines except PyMT-M-ICBR were tested for mycoplasma contamination and were confirmed negative.

Commonly misidentified lines (See ICLAC register) No cell lines used in this study were found in the Register of Misidentified Cell Lines maintained by ICLAC (version 9).

Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals Female animals of 6 – 8 weeks were used as the recipients of tumor tissue or cell line transplantation. Age-matched mice (10 – 12 weeks) were subjected to euthanization for immune profiling in all experiments. BALB/cAnN-Hsd (BALB/c), C57BL/6N-Hsd (C57BL/6 or B6), FVB, C.B-17/SkHsd-Ppckdcsd/Lystbg-j (SCID-beige) were purchased from Envigo and either directly used for experiments or bred in our facilities. B6.129S4-Ccr2tm1Ifc/J (CCR2 KO), B6.FVB-Tg(MMTV-PyVT)634Mju/LellJ (MMTV-PyMT), B6.Cg-Foxp3tm32Cct/J (BALB/c. FOXP3-GFP), and C57BL/6J (wild-type) were purchased from The Jackson Laboratory and bred in our facilities. To generate CCR2 KO mice in BALB/c background, CCR2 KO mice were crossed with wild-type BALB/c mice for five generation. The P53-PTEN DKO tumor tissue subsequently used for transplantation was derived from MMTV-cre;Trp53F/F;PtenF/F animal which was directly bred by Dr. Jianming Xu’s lab at Baylor College of Medicine.

Wild animals No wild animals were used in this study.

Field-collected samples No field-collected samples were used in this study.

Ethics oversight All animal experiments were conducted in accordance with a protocol approved by Institutional Animal Care and Use Committee at Baylor College of Medicine.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics The age at presentation varied from 26 to 81 years (mean age: 58 years), all women, the stage at presentation was Stage I: 32 (44.4%), Stage IIA: 20 (27.8%), Stage IIIA: 7 (9.7%), Stage IIIB: 8 (11.1%), Stage IIIA: 7 (9.7%); Stage IV: 3 (4.2%). The histologic grade on the excision specimen, grade 1: 2 (2.8%), grade 2: 9 (12.5%), grade 3: 59 (81.9%). All cases were triple negative (ER-PR-HER2-), Lymph node status at the time of presentation, involved 22 (30.6%) and not involved: 50 (69.4%). 9 underwent neoadjuvant treatment (12.5%) and 63 underwent adjuvant therapy (87.5%).

Recruitment All specimens from above patients were included. Bias may exist as race identification was self-identified. No other bias was identified.

Ethics oversight IRB approved at Baylor Scott and White Research Institute. This study is found to be exempt from Institutional Review Board review based on 45 CFR 46.101(b)(4) - Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

Confirm that:
☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Tumor
Tumor was resected when it reached approximately the size of 1 gram. For all immune cell analysis including RNA-seq as well as immune cell profiling, orthotopic breast tumors were collected in ice-cold PBS and subjected to dissociation using Tumor Dissociation Kit, mouse (Miltenyi Biotec) following supplier’s protocol. Tumors (0.2 – 0.4 g) were cut into small pieces (around 1mm3) and transferred to gentleMACS C tubes (Miltenyi) containing 2.35 ml of RPMI 1640, 100 μl of enzyme D, 50 μl of enzyme R, and 25 μl of enzyme A. Tissues were mechanically dissociated on a gentle MACS Dissociator (Miltenyi). Three consecutive ‘m_Lung_02’ program was briefly run on the dissociator, spaced with 10 min shaking incubation at 37°C in between. The dissociation reaction was stopped with ice-cold RPMI-1640 and a single cell suspension was obtained by filtering through 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).

Blood, bone marrow, and spleen
Blood was drawn and collected in 0.5 M EDTA-coated tubes. To separate plasma from blood cells, a 15 min centrifugation at 1,500g, 4°C was performed. Whole bone marrow and splenic immune cells were isolated by crushing the respective organ, and single cell suspension was obtained by filtering through 70-μm cell strainer. Erythrocytes were lysed with RBC lysis buffer (Tonbo, cat # TMB-4300-L100) by incubating on ice for 10 min, after which cells were washed with FACS buffer.

Instrument

All data were acquired using BD LSR Fortessa or LSRII Analyzer. FACS sorting was performed using Aria Cell Sorter (BD Biosciences) to purify tumor-infiltrating neutrophils and macrophages.

Software

All data were analyzed with Flow Jo v10.0.

Cell population abundance

Post-sort purity was > 85% for downstream applications.

Gating strategy

Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between “positive” and “negative” staining cell populations are defined.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.