Cancer co-opts differentiation of B-cell precursors into macrophage-like cells

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We have recently reported that some cancers induce accumulation of bone marrow (BM) B-cell precursors in the spleen to convert them into metastasis-promoting, immunosuppressive B cells. Here, using various murine tumor models and samples from humans with breast and ovarian cancers, we provide evidence that cancers also co-opt differentiation of these B-cell precursors to generate macrophage-like cells (termed B-MF). We link the transdifferentiation to a small subset of CSF1R+ Pax5Low cells within BM pre-B and immature B cells responding to cancer-secreted M-CSF with downregulation of the transcription factor Pax5 via CSF1R signaling. Although the primary source of tumor-associated macrophages is monocytes, B-MFs are phenotypically and functionally distinguishable. Compared to monocyte-derived macrophages, B-MFs more efficiently phagocytize apoptotic cells, suppress proliferation of T cells and induce FoxP3+ regulatory T cells. In mouse tumor models, B-MFs promote shrinkage of the tumor-infiltrating IFNγ+ CD4+ T cell pool and increase cancer progression and metastasis, suggesting that this cancer-induced transdifferentiation pathway is functionally relevant and hence could serve as an immunotherapeutic target.

The role of B cells in cancer remains poorly understood, as their presence is positively and negatively associated with the disease outcome. Even in the same murine tumor models, different types of B cells promote or retard cancer escape, thereby affecting the progression of B16-F10 melanoma in C57BL/6 mice1,2 and lung metastasis of orthotopic 4T1 breast cancer cells in BALB/c mice1,2. At least some cancer-promoting functions of B cells can be attributed to their regulatory subsets, such as TGFβ+ CD25+ Bregs (tBregs), which support lung metastasis by inducing FoxP3+ Tregs or educating MDSCs via targeting TGFβRII4,5. The generation and activation of B cells and Bregs, in turn, is regulated by cancer-secreted factors, such as B lymphocyte stimulator (BlyS/BAFF), thymic stromal lymphopoietin (TSLP), colony-stimulating factors (M-CSF, GM-CSF, and G-CSF), and lipid mediators such as 5-lipoxygenase (5-LO) metabolites6-11. For example, we recently reported that cancer remotely downregulates CXCR4 and α4β1 integrin on pro-B and pre-B cells in the bone marrow (BM) using TSLP to cause their premature emigration and accumulation in the spleen11. It is to convert these cells into tBregs by targeting PPARα signaling with 5-LO metabolites8. We also found that TSLP from cancers prepares the metastasis “soil”, such as inducing expression of CCL17 in the lungs to recruit CCR4+ cancer cells and their protector CCR4+FoxP3+ Tregs and Th2-skewed CD4+ T cells7,12. Cancer-secreted or induced M-CSF and GM-CSF promotes differentiation and survival of cancer-promoting myeloid suppressive cells and tumor-associated macrophages (TAM) from BM monocytes6,10. However, their role in the differentiation of lymphocytes remains poorly understood. Although bifurcation of myeloid and lymphoid lineage from multipotent progenitors occurs before specialization of B-cell progenitors in BM and monocytes give rise to macrophages, B-cell precursors appear to retain the macrophage-differentiation potential, as they can transdifferentiate into macrophages after forced expression or deletion of single transcription factors13-15. In

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naive mice, a small subset of biphenotypic pro-B cells (CD19+ B220+CD16/32−CD11b+) with non-rearranged B-cell receptor (BCR) genes is recently reported to acquire macrophage phenotypes, albeit at very low levels\(^\text{16}\). The biological consequence of this rare event and whether cancers affect the B-cell-to-macrophage transdifferentiation remain unknown.\(^\text{20,22}\)

Here, we report that cancers transdifferentiate the bona fide BM B-cell precursors, including Csf1r Pax5\(^{+}\) pre-B and immature IgM\(^{-}\) B cells, into TAM (termed B-MF) using M-CSF. Unlike monocyte-derived TAM\(^{\text{9,23}}\), cancers use B-MF to mediate escape and metastasis via suppressing antitumor IFN\(\gamma\) CD4\(^{+}\) T cells. This does not appear to be a mouse-specific phenomenon, as B-MF-like cells and their transcriptional signature can be detected in patients with breast and ovarian cancers and in published scRNA sequence data of human cancers.

### Results

#### TAM expresses B-cell markers

We previously reported that some cancers mobilize BM B-cell precursors in the spleen\(^{\text{16}}\) to convert them into TGFB\(^{+}\) Tregs\(^{\text{3,4,6}}\). Microarray transcription profiling of these B cells in the spleen of BALB/c mice with orthotopic 4T1.2 breast cancer (a model for human triple-negative breast cancer\(^\text{17}\)) surprisingly revealed significant upregulation of macrophage-associated genes, such as CD68, Csfir (encodes Csf1r), Cebp (CCAAT Enhancer binding protein beta), Ccsg (CCAAT Enhancer binding protein gamma), Ccl2 (CCL2), and Csf2 (M-CSF) (Fig. 1a). Given that CEPBP and Csfir play essential roles in defining macrophage fate\(^\text{18,19}\) and that the biphenotypic B-cell progenitors and BI B cells can generate macrophages in mice\(^\text{16}\), we tested whether cancer induces the macrophage-like cells from pre-B cells by FACS evaluating tumor-infiltrating CD19\(^+\) B cells (TIB) and macrophages (TAM, based on F4/80/CD11b\(^+\))\(^{\text{9,24}}\) in B-cell sufficient (WT) and deficient BALB/c mice (\(\mu\)MT, where B cells do not differentiate beyond pro-B cells\(^{\text{25}}\)) with 4T1.2 cancer. WT mice contained small numbers of F4/80/CD11b\(^+\) TIB and CD19\(^+\) and CD79a\(^+\) TAM, which were almost undetectable in \(\mu\)MT mice (Fig. 1b, gating strategy is in Supplementary Fig. 1a). Compared with CD79a\(^+\) TAM (presumably bona fide macrophages), the CD79a\(^+\) TAM expressed CD20, IgM, and IgD and significantly upregulated F4/80, CD11b, CD206, IL4Ra, and binding to Filipin (a fluorescent polyanionic dye that detects cellular free cholesterol\(^\text{26}\)) (Supplementary Fig. 1a, b). These cells (hereafter referred to as B-MF) were also found in primary tumors of C57BL/6 mice with s.c. MC38 colon cancer and in the tumor microenvironment (peritoneum) of mice with spontaneous ovarian Mogp cancers, but again were almost lost in \(\mu\)MT and JHT mice (Fig. 1c and Supplementary Fig. 1g, h). We also evaluated B-MF in Mb1-Cre/Rosa-EYFP crossed (Mb1-EYFP) mice (Fig. 1d, h). While both B-MF and Mo-MF upregulated F4/80 and CD11b but not DC and granulocyte markers (Supplementary Fig. 5a), adhered to plastic, showed similar cell size and peritoneal macrophages (Supplementary Fig. 5b), only B-MF expressed the B-cell-specific markers CD79a and IgM (Fig. 5a, b and Supplementary Fig. 5c). In mRNA microarray analysis, B-MF and Mo-MF shared expression of numerous macrophage-related genes regardless of the origin (Supplementary Fig. 5d–f and Supplementary Data file 1), although principal component analysis (PCA) clearly separated the two cell types (Fig. 5e and Supplementary Data file 2). B-MF expressed higher levels of genes involved in fatty acid metabolism, oxidative phosphorylation, cell cycle, steroid–cholesterol biosynthesis, and downregulated expression of fatty acid metabolism enzymes.\(^\text{27,28}\)

#### Cancer induces B-cell transdifferentiation

Because B-MF could be misinterpreted as trogocytosis or cell fusion\(^{27,28}\), we performed a series of B-cell differentiation experiments using highly FACS-purified CD19\(^+\) B cells (Lin\(^{-}\), >99% purity, Supplementary Fig. 3a) from BM of naive mice. The cells were cultured in 10% conditioned medium (CM) of 4T1.2 cancer cells (4T1.2-CM) to FACS-evaluate surface expression of B-cell and macrophage markers. B cells gradually became CD11b\(^+\) F4/80\(^+\) while downregulating CD19 and some CD79a expression by 7–8 days of incubation in 4T1-CM (Fig. 2a, b and Supplementary Fig. 3b). After 15-day culture, the cells remained IgM\(^{-}\)CD11b\(^{+}\)F4/80\(^{+}\) but further decreased CD19 and CD79a (Supplementary Fig. 3c). From here on, to capture these cells in "transition", we used 7-day incubation for experimental timepoints, unless specified otherwise. To further examine these cells, we performed single-cell Imagestream FACS analysis and confirmed the Mb1-EYFP/CD79a\(^+\) Mb1-EYFP+/CD79a\(^+\) Mb1-EYFP+/CD79a\(^+\) cells expressed CD20, F4/80, and CD11b and were larger in size than bona fide B cells (Fig. 2c and Supplementary Fig. 3d). B-MF also acquired additional macrophage features, such as the ability to adhere to plastic and phagocytize fluorochrome-labelled E. coli (Supplementary Fig. 3e). By culturing FACS-purified B-cell subsets in 4T1.2-CM, we linked the B-MF generation to BM B-cell precursors and immature IgM\(^{-}\) B cells (collectively termed as BMBP), but not to peripheral B cells in naive mice, including splenic transitional, follicular (FOL), or marginal zone (MZB) B cells (Fig. 2e, f; Supplementary Fig. 3f, g; gating strategy in Supplementary Fig. 3g). Similarly, CM from almost every type of cancer cells, except B16-F10 melanoma, induced the generation of B-MF from naive mouse BM and the immortalized 702/3 pre-B-cell line after 7 and 30 days of culture, respectively (Fig. 2g and Supplementary Fig. 3h–k). B cells cultured in the control eRPMI medium did not generate B-MF (Fig. 2d, g). To rule out trogocytosis/cell fusion, we performed in vitro and in vivo B-MF conversion assays using CD45.1 or CD45.2 allotransplantation procedures (Supplementary Fig. 4a, b). They were not derived from trogocytosis/cell fusion. To confirm this conclusion in vivo, we transferred FACS-purified EYFP\(^{+}\) BM cells from naive CD45.2\(^{+}\) mice into the peritoneum of CD45.1\(^{+}\) mice with a 21-day-old ID8 tumor (Fig. 2i). After 7 days, FACS analysis of the transferred cells revealed that only a very small fraction of them co-expressed CD45.3 and CD45.2/EYFP (presumably due to trogocytosis/cell fusion), while the majority of EYFP\(^{+}\) B-MF did not express CD45.3 (Fig. 2i, j and Supplementary Fig. 4c). Taken together, we concluded that cancer generates TAM by transdifferentiating BMBP in addition to their hitherto known source, monocytes\(^{\text{29}}\).

#### B-MF transcription profiles are distinct from Mo-MF

To understand the nature of these B-MF, we compared their phenotypes to that of Mo-MF (generated in 4T1.2-CM, as described above). While both B-MF and Mo-MF highly upregulated F4/80 and CD11b but not DC and granulocyte markers (Supplementary Fig. 5a), adhered to plastic, showed similar cell size as peritoneal macrophages (Supplementary Fig. 5b), only B-MF expressed the B-cell-specific markers CD79a and IgM (Fig. 2a, b and Supplementary Fig. 5c). B-MF expressed higher levels of genes involved in fatty acid metabolism, oxidative phosphorylation, cell cycle, steroid–cholesterol biosynthesis, and downregulated expression of fatty acid metabolism enzymes.\(^\text{27,28}\)
of pro-inflammatory and IFNγ response genes (Fig. 3d, e). While Mo-MF were enriched for a M1-like transcription profile, M2-skewing was more pronounced in B-MF (Fig. 3e and Supplementary Fig. 5g). The unique transcription profiles were also confirmed in single-cell RNA sequencing (scRNA-seq) of B-MF (10,563) and Mo-MF (10,235) cells, with UMAP clustering identifying mostly separate cell clusters of the two cell types (Fig. 3f and Supplementary Data file 3). We distinguished 12 cell clusters with the Leiden algorithm, using shared nearest neighbor (SNN) in PCA space and identified the key genes establishing the six clusters accounting for the majority of single cells (Fig. 3g, h and Supplementary Fig. 5i). B-MF appeared to be more phagocytic than Mo-MF, as they markedly upregulated Mrc1 (encodes CD206, Fig. 3i).
As in microarray analysis (Fig. 3d), expression of genes for oxidative phosphorylation were more upregulated in B-MF than Mo-MF (clusters 0, 4, and 6, Supplementary Fig. 5b), consistent with their M2-skewing29. Given the unique transcriptional signatures of the B-MF, we next examined scRNA profiles of TAM purified from four different mice with 4T1.2 cancer (Fig. 3j). To identify potential in vivo B-MF, we used signature genes identified from in vitro-generated macrophages (Fig. 3h) and noted three clusters (0, 6, and 8) with robust expression signature genes identified from in vivo-generated macrophages (Fig. 3j). To confirm potential in vivo B-MF, we used signature genes identified from in vitro-generated macrophages (Fig. 3h) and noted three clusters (0, 6, and 8) with robust expression signature genes identified from in vivo-generated macrophages (Fig. 3j). To confirm potential in vivo B-MF, we used signature genes identified from in vitro-generated macrophages (Fig. 3h) and noted three clusters (0, 6, and 8) with robust expression signature genes identified from in vivo-generated macrophages (Fig. 3j).

Cancer generates B-MF to suppress antitumor CD4+ T cells

To confirm the differences between the two macrophages at the functional levels, we quantified their proliferation, phosphorylation of red fluorescent protein (RFP)-tagged apoptotic ID8 cells, and intracellular cholesterol. Only B-MF readily incorporated BrDU (pulsed on day 6 and tested on day 7 of the culture) and expressed higher levels of Ki67 (Fig. 4a and Supplementary Fig. 6a, b). Although the two macrophages phagocytized fluorochrome-labeled apoptotic cancer cells (Fig. 4c, b) and contained elevated levels of cellular cholesterol (Fig. 4d), both these features were significantly upregulated in B-MF compared to Mo-MF per cell-to-cell comparisons (Fig. 4c, e). Similarly, CD79+ TAM exhibited markedly higher Filipin binding than CD79+ TAM (Supplementary Fig. 1a–f). Because B-MF and CD79+ TAM also significantly upregulated TGFβ/LAP and PD-L1 (Supplementary Fig. 1a–f and Supplementary Fig. 2c) and lipid accumulation in TAM associated with suppression of antitumor CD8+ T cells40, we wondered whether these cells promote tumor progression via regulating the activity of T cells.

B-MF significantly increased tumor weight in mice with melanoma (p < 0.01, Fig. 4b) and numbers of metastatic foci in the lungs of mice with 4T1.2 cancer (p < 0.05, Fig. 4i). FACS evaluation of their tumors surprisingly did not detect a difference in the presence of CD8+ and CD4+ T cells and FoxP3+ Tregs (Supplementary Fig. 6f, g). Instead, the B-MF transfer significantly decreased the frequency and numbers of IFNγ-expressing CD4+ T cells in both cancer models (Fig. 4j–m). In 4T1.2 tumors, B-MF also markedly decreased granzyme (Gr) B+ CD4+ T cells (Supplementary Fig. 6g), which were implicated in tumor cell killing. A separate transfer experiment with equal numbers of FACS-purified naive mouse follicular B cells (FOB) or B-MF in μMT mice with 4T1.2 cancer (3 × 10^5 cells/mouse, n = 5–7 mice per group) revealed that both cells comparably support lung metastasis (Supplementary Fig. 6h). Compared to B-MF, FOB upregulated numbers of CD4+ T cells but decreased frequency of IL10+ CD4+ T cells and GrB+ and Lamp1+ (cycloptic) CD8+ T cells in the tumor (Supplementary Fig. 6h), implying that the two cells support cancer independently and without reversal of B-MF to B cells. To confirm this, we performed a 3-day tracking experiment by i.v. transferring fluorochrome-labeled B-MF (500,000 cells/mouse) with 4T1.2 tumor with 14-day 4T1.2 tumor. The majority of transferred cells were in the spleen and tumor (and less in dLN, Supplementary Fig. 6i). Per gram tumor, numbers of transferred B-MF were slightly less (about 7-fold) than that of 4T1.2 tumor in BALB/c mice and MC38 tumor in BNT/PEF mice (Supplementary Fig. 6k). Consistent with in vitro stability of B-MF phenotype (Fig. 2a, b and Supplementary Fig. 3b, c), the transferred cells were exclusively CD11b+ T4/80+ (98%, Supplementary Fig. 6j). Taken together, we concluded that cancers generate B-MF mostly to downregulate antitumor IFNγ-expressing CD4+ T cells.

Cancer mobilizes BMBP in the spleen to convert them to B-MF

Because cancer can mobilize BM pre-B cells in the spleen and tumor to generate Bregs11, we tested whether this pool of BMBP is the source of B-MF. Compared with naive mice, the total number of CD93+ BMBP was markedly decreased in BM but increased in the spleen as well as present in the tumors of mice with 4T1.2 and Mgp cancers (Fig. 5a, Supplementary Fig. 7a, b and not depicted). To link them to the generation of B-MF, we FACS-purified CD93+ and CD93− BMBP from the spleen of mice with 4T1.2 cancer and naive mice and cultured these cells in 4T1.2-CM. Only splenic CD93−, but not CD93+ BMBP from tumor-bearing mice generated B-MF, while B cells from spleens of naive mice failed to do so regardless of CD93 expression (Supplementary Fig. 7c and Supplementary Fig. 3f), implying that cancer accumulates CD93− BMBP in the spleen and tumor to generate B-MF.

Cancer targets CSF1R+CD93− BMBP by secreting M-CSF

To understand the mechanism of the B-MF generation, we analyzed CM of cancer cells for secreted factors that could affect the differentiation of macrophages. M-CSF, a regulator of macrophage differentiation and survival15, was among the factors that were highly increased in the cancer cells that induce B-MF (Fig. 5b). Conversely,
M-CSF was almost absent in CM from B16-F10 cells (Fig. 5b and Supplementary Fig. 7d, e), which did not induce the generation of B-MF (Fig. 2g and Supplementary Fig. 3h, i). Compared to naïve mice, serum M-CSF was also significantly upregulated in mice with 4T1.2 cancer (Supplementary Fig. 7f). Importantly, CD93+CD19+ BMBP of naïve mice expressed its cognate receptor CSF1R (about 15% of immature B cells, 6% of pre-B cells, and 1.5% pro-B cells, Supplementary Fig. 7g). In mice with 4T1 cancer, CSF1R+CD93+CD19+ BMBP were markedly reduced in BM but increased in the spleen (Fig. 5a and Supplementary Fig. 7g), consistent with their cancer-induced emigration from BM, as discussed above. To link these CSF1R+CD93+CD19+ BMBP to the generation of B-MF, highly FACS-purified CSF1R+ and CSF1R− B cells from BM and...
spleen of naive mice were cultured in 4T1.2-CM. While CSF1R+ BM B cells readily generated B-MF, the BM CSF1R− subset failed to do so (Fig. 5c). Consistent with the inability of splenic CD93− B cells of naïve mice to generate B-MF (Supplementary Fig. 7c and Supplementary Table 1), we failed to convert naïve mouse splenic B cells into B-MF regardless of CSF1R expression (Fig. 5c). We also cultured primary BM BMBP or 70/2/3 cells with 4T1.2-CM in the presence or absence of neutralizing M-CSF antibody (Ab) or Ki20227, a specific inhibitor of c-Fms/CSF1R32. Both cells failed to generate B-MF upon M-CSF neutralization or CSF1R signaling inhibition (Fig. 5d, e and Supplementary Fig. 8a). Importantly, unlike WT littermates or monocytes from Mb1-CSF1RFlox/Flox mice, the loss of CSF1R in BM-MBP significantly impaired the cancer CM-induced B-MF differentiation (Fig. 5f, g). Of note, the residual macrophage differentiation seen in Fig. 5f is presumably due to CSF1R expression preceding Mst expression, while Mb1-CSF1Rlox/lox mice will have CSF1R deletion only in pro-B cells and onward.

Given that PAX5 is the key pro-B-cell factor that represses CSF1R and other myeloid lineage-specific genes14,33, we reasoned that cancer decreases levels of this transcription factor using M-CSF. FACS staining confirmed that PAX5 was markedly decreased in BM CD93− BMBP, particularly in CSF1R− but not CSF1R+ subsets, from mice with 4T1.2 or Mogg cancers (Fig. 6a and Supplementary Fig. 9a–c). Importantly, PAX5 was also significantly decreased in BM CSF1R− BMBP from naive mice and 70/2/3 cells upon treatment with 4T1.2-CM or M-CSF (Fig. 6b, c and Supplementary Fig. 9b). As PAX5 deficiency alone is sufficient to render pro-B cells susceptible to myeloid differentiation15, we concluded that cancer uses M-CSF to reduce expression of PAX5 in CSF1R− CD93− BMBP and thereby promote macrophage differentiation. To further understand the B-cell susceptibility towards macrophage conversion, we analyzed chromatin accessibility by performing ATAC-seq on CSF1R− and CSF1R+ BMBP isolated from both BM and spleen of naive mice. PCA clustering showed the most robust differences in chromatin profiles were driven by the location of the BMBP (BM vs spleen) regardless of CSF1R expression, driving the PC2 axis (blue and purple vs orange and green, Fig. 6d). The chromatin landscapes of the CSF1R− and CSF1R+ BMBP isolated from BM (orange and green, Fig. 6d) also significantly differed from each other, driving the PC3 axis. We then examined the differentially accessible regions (DAR) between CSF1R− and CSF1R+ cells isolated from the spleen or BM. Whereas comparisons between CSF1R− and CSF1R+ cells from the spleen did not show any differences reaching our threshold for significance, confirming their close clustering on the PC3 axis; the BM CSF1R− cells contained significantly more open chromatin than the BM CSF1R+ cells (Fig. 6d, e). These data suggest the BM CSF1R− BMBP may have a more permissive chromatin environment, susceptible to macrophage-differentiation signals. As the splenic B cells and BM CSF1R+ cells were refractory to macrophage conversion (Fig. 5c), we looked at DARs with less accessibility in CSF1R− compared to CSF1R+ BM cells (749 loci) in spleen cells to determine if these regions remain closed and potentially “lock in” the lymphoid lineage potential. Indeed, the overwhelming majority of regions with decreased accessibility in the BM CSF1R− cells remained closed in the cells from the spleen (679 of 749). Evaluation of these consensus open regions found in BM CSF1R+ cells for potential transcription factor binding sites permitting macrophage differentiation showed significantly increased accessibility of ERG and RUNX1 sites (Fig. 6f). ERG is known to be expressed both in myeloid and lymphoid progenitor cells36 and has particular importance in early hematopoietic progenitor cells as it binds to coregulators such as RUNX and GATA1. RUNX1 regulates the growth and survival of macrophages via binding to promoter and enhancer regions of CSF1R and upregulating its expression37. RUNX1 is also robustly expressed in early progenitor and myeloid-committed progenitor cells. Thus, the increased accessibility to binding sites of both ERG and RUNX1 suggests a potentially more primitive, permissive chromatin state allowing for myeloid lineage transformation of the BM CSF1R+ cells.

**B-MF-generating CSF1R− BMBP accumulate in humans with cancer**

We recently reported that peripheral mobilization of BMBP also occurs in humans with breast cancer (BC)38, suggesting the generation of B-MF. To test this possibility, we FACS evaluated peripheral blood (PB) of healthy donors (HD, n = 7) and patients with BC (n = 8). Compared with HD, PB of BC was markedly increased in CSF1R− BMBP (Supplementary Fig. 9d), as we described in mice with cancer. Moreover, microarray transcription profiling of sort-purified B cells from PB of BC patients revealed that they significantly upregulated macrophage-associated genes, such as Cebpα, Marco, and CSF1R, as compared with B cells from HD (Fig. 6g). We also FACS evaluated B cells from PB of patients with ovarian cancer (OC, n = 5). Compared with HD, OC patients significantly increased CSF1R− BMBP (Fig. 6h and Supplementary Fig. 9e) with upregulated expression of CD68 and LDLR (Fig. 6h), similar to mice with cancer. Using recently published scRNA-seq data of tumor-infiltrated immune cells from patients with breast cancer38, we also found a macrophage cluster with overlapping signatures of B-MF-like cells (cluster 3, Fig. 6i) by examining genes with differential expression defined in murine in vitro-generated B-MF (cluster 0, Fig. 3h). In particular, cluster 3 was enriched for expression of EGR1, IRF2, IER3, and SLCOA1, which were major drivers of identity for murine in vitro-generated B-MF (Fig. 6i). Similarly, in the single-cell transcriptome data from human high-grade serous OC38, we also detected the B-MF-like signature in macrophages (Cluster 0, Fig. 6j), although with a lesser overlap than in BC, further suggesting that human cancers can promote the B-cell transdifferentiation into macrophages.

**Discussion**

BMBP undergo a series of subsequent and tightly regulated differentiation steps after their bifurcation from multipotent cells to
Fig. 3 | Distinct gene expression profiles of B-MF and Mo-MF. a, b Representative FACS plots (a) and histograms (b) of BM-MF and Mo-MF generated from BM B cells or monocytes after 7-day treatment with 4T1.2-CM. Numbers are for proportion of gated (F4/80hiCD11bhi) cells (a).

b Shows expression of CD79a and IgM in B-MF (Red) and Mo-MF (Orange).

c PCA plot of mRNA expression profiles generated from microarray data of sort-purified B-MF (Blue), Mo-MF (Orange) and BM B cells (Green) (n = 3 mice). d, e Bar plots of GSEA predicted pathways enriched in B-MF (d) or Mo-MF cells (e) from the Molecular Signature Database.

f, g UMAP plots of scRNA sequencing (scRNA-seq) of B-MFs (10,563 cells) and Mo-MFs (10,235 cells) analyzed using Seurat with colors depicting clusters by cell type (f) or by gene expression (g).

h Heatmap of top differentially expressed genes (DEG) in 6 major clusters of in vitro-generated B-MF and Mo-MF. i Mrc1 expression in B-MF and Mo-MF single cells shown in f. j scRNA-seq UMAP plot of FACS-purified TAM (10,885 cells) from 4 mice with 4T1.2 cancer cells shows 13 unique cell clusters. Three clusters with overlapping signatures with B-MF are highlighted. k Violin plots of three DEG (Egr1, Ier3 and Slc40a1) upregulated in B-MF in vitro and in TAM from mice with 4T1.2 cancer.
committed lymphoid lineage cells. Despite this, experiments with forced expression or inhibition of a single transcription factor or mutations that drive leukemogenesis as well as recent findings of a small proportion biphenotypic CD19+B220+CD16/32+CD11b+ pro-B cells with non-rearranged BCR genes, which become peritoneal CD19+CD79b− macrophages in mice, suggest that BMBP retain plasticity and myeloid transdifferentiation potential. Unlike these artificial manipulations or rare events, here we report that the B-cell-to-macrophage transdifferentiation is commonly used by murine cancers to generate TAM/B-MF. In PB of humans with metastatic/recurrent triple-negative BC and high-grade serous OC, we also detect a significant increase of CSF1R+ CD68+LDLR+ BMBP, which also express the...
macrophage-specific genes Cebpα, Cebpβ, and Marco. Importantly, the B-MF signature is also identifiable within unique macrophage clusters using recently published scRNA-seq profiles of tumor-infiltrating cells in patients with BC38 and high-grade serous OC39. Our results suggest that human and murine cancers primarily transdifferentiate BMBP into macrophages, adding one more feature to the heterogeneity and complexity of this cell population.

In vitro-generated B-MF (3 × 10⁵) from C57BL/6 and BALB/c mice were i.v. transferred into μMT C57BL/6 and μMT BALB/c mice, respectively, at days 3 and 7 post-tumor challenge. Shown are quantifications of tumor weight in mice with B16-F10 melanoma (n = 10 for PBS and n = 12 for B-MF, P = 0.0053, h), metastatic foci in the lungs (P = 0.0137, i), and frequency and absolute numbers of IFNy+ CD4+ T cells per gram primary tumor in mice with B16-F10 melanoma (j, P = 0.0018 and k, P = 0.0353) and 4T1.2 cancer (l, P = 0.0083 and m, P = 0.0144). P-values in c, e, f, h–m were calculated using two-tailed unpaired t-test. Results were independently confirmed at least twice. Each symbol in h–m is for a single mouse.
CSF1R^+  |  CSF1R^-
---|---
BM  |  BM
60.3 |  0.19

CD11b  |  F4/80
---|---
Spleen  |  Spleen
0.81 |  0.84

C57BL/6 background mice were purchased from the Jackson Laboratory (Bar Harbor, ME); RAG2-GFP mice expressing bacterial artificial chromosome modified GFP instead of RAG2 were a gift of Dr. Michael Nussenzweig (Howard Hughes Medical Institute, NY, NY) and reported elsewhere.\(^46\), \(\mu\)MT mice in BALB/c background were a gift from Dr. Thomas Blankenstein (Max-Delbrück-Center for Molecular Medicine, Berlin, Germany)\(^47\). Mb1-Cre mice in C57BL/6 background (B6.C(Cg)-Cd79atm1(cre) Reth/EhobJ) were a gift from Dr. Richard Maraia (National Institute of Child Health and Human Development, Bethesda, MD)\(^48\). Mogp-tag mice (Mogp, spontaneous Article [https://doi.org/10.1038/s41467-022-33117-y](https://doi.org/10.1038/s41467-022-33117-y)
ovarian cancer model in C57BL/6 mice) were a gift from Dr. J. Miyoshi (Tohoku University Graduate School of Medicine, Miyagi, Japan). To create mice with B-cell-specific EYFP reporter (Mbi-EYFP) or CSF1R deletion (Mbi-CSF1RFlox/Flox), Mbi-cre mice were bred with R26R-EYFP and CSF1Rloxlox mice, respectively.

4T1.2 cells were a gift from Dr. Robin L. Anderson (Peter McCallum Cancer Center, Melbourne, Australia); MC38 colonic adenocarcinoma cells were a gift from Dr. Jeffrey Schlom (National Cancer Institute, Bethesda, MD); mammary carcinoma AT3 cells (University of Notre Dame, IN); and EMT6 cells and melanoma B16-F10 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were tested free of mycoplasma with Mycoplasma Detection Kits (Lonza Basel, Switzerland) and IDEXX BioAnalytics, Columbia, MO.

Tissues and blood processing
PBMC from healthy human donors were collected with written informed consent at the Clinical Core Laboratory, NIA, under Human Subjects Protocol # 2003054 and Tissue Procurement Protocol # 2003-071; and from patients with recurrent breast and ovarian cancer enrolled in Phase II clinical study of prexasertib (NCT02203513) at the Clinical Center, Center for Cancer Research, National Cancer Institute. All patients, including 13 participants in this research project, provided written informed consent before enrolment and on using their samples for research. The study has been conducted in accordance with ethical principles that have their origin in the Declaration of Helsinki and are consistent with the International Council on Harmonization guidelines on Good Clinical Practice, all applicable laws and regulatory requirements, and all conditions required by a regulatory authority and/or institutional review board. The study protocol was approved by the Institutional Review Board of the Center for Cancer Research, National Cancer Institute. All experiments were performed on PBMC, which were cryopreserved after collection. Mouse BM cells were flushed out of femurs and tibias with cold cRPMI. Single-cell suspension of BM, spleen, LN were prepared with 70 μm strainer (Falcon, Bedford, MA). BM, spleen, and blood cells were treated with ACK buffer to remove red blood cells. Mouse tumor tissues were cut into 3–5 mm pieces and digested with a mouse tumor dissociation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer’s instructions.

Flow cytometry (FACS)
For immune cell phenotyping, cells were pre-incubated with TruStain Fe(C)™ solution before immunostaining with different combinations of anti-mouse or anti-human Abs (1 μg per 10⁶ cells, Supplementary Table 1) and fixable viability dye, then fixed/permeabilized with eBioscience™ Intracellular Fixation & Permeabilization Buffer (Thermo Fisher, Waltham, MA). The samples were evaluated on FACSsymphony™ BD (Franklin Lakes, NJ), Amnis ImageStreamX MkII (Millipore, Burlington, MA), or CytoFLEX (Beckman Coulter, Brea, CA). The results were analyzed with FlowJo v10(BD), IDEAS (Millipore), or Cytoexpert 2.3 (Beckman).

Immunofluorescent staining
Dissected tumors from mice were fixed with 4% PFA in PBS for 24 h and then transferred to 30% sucrose in PBS for about 2 days until the tissue sank to the bottom of 15 ml Falcon tubes. Tumors were embedded in OCT compound, frozen on dry ice, and stored at −80 °C before cryosection. Ten micrometer thick sections were prepared and adhered to superfrost glass slides. After three washes with PBS, the tumor slices were incubated in 0.3 M glycine in PBS for 30 min and then blocked and perméabilized with IF buffer (5% donkey serum, 2% BSA, and 0.1% Triton X-100 in PBS) for 60 min at room temperature (RT). Tumors slices were incubated with anti-CSF1R (abcam, Cat # ab245235, dilution 1:100, final concentration 4.6 μg/ml) and anti-CD68 (abcam, Cat # ab53441, dilution 1:300, final concentration 3.3 μg/ml) antibodies for 24 h at 4 °C. After three washes with PBS, the slices were incubated with donkey anti-rabbit IgG H&L Alexa Fluor 488 (abcam, Cat # ab150073, dilution 1:500, final concentration 4 μg/ml) and donkey anti-rat IgG H&L Alexa Fluor 568 (abcam, Cat # ab175475, dilution 1:500, final concentration 4 μg/ml) at RT for 2 h. After washing with PBS three times, slides were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Invitrogen) and imaged using a Zeiss LSM 710 confocal microscope.

Cancer CM media preparation and cytokine quantification
Cells were cultured in RPMI1640 or DMEM (for ID8 cells) supplemented with 10% FBS, 1% HEPES, sodium pyruvate, nonessential amino acids solution, penicillin–streptomycin–glutamine (Gibco, Gaithersburg, MD), and 55 mMol/L β-mercaptethanol in T75 flask to 70–80% confluency. CM was collected after 5 min centrifugation at 1500 rpm, filtered with 0.2 μm filter, and stored at −80 °C as single-use aliquots. For cytokine tests, confluent cells were cultured with RPMI without FBS for 24 h. Mouse serum was collected using BD Microtainer™ Tubes following the manufacturer’s instruction. Cytokines and M-CSF in filtered CM or sera were evaluated with Quantikine ELISA kit (R&D, Minneapolis, MN) or with Proteome Profiler Mouse XL Cytokine Array (R&D). Images were captured and analyzed with Fiji software.

B-MF conversion assay
BM Lin− (TER119, CD11b, Gr-1, CD3e, NK1.1 or CD49b, Ly6C, Ly6G, CD11c−) CD19− B cells were isolated from C57BL/CJ or BALB/c mice using FACSaria™ Fusion sorter and 10⁶/ml B cells were cultured in 50% cancer CM in cRPMI for 7 days in Nunc™ Multidishes with UpCell™ Surface (Thermo Fisher) without changing media for 7 days. 70z/3 pre-B cells (10⁶/ml) were cultured in 50% cancer CM for up to 30 days with a replenishing culture medium every 3–4 days. Adherent cells (macrophages) were harvested by detaching them at 4 °C for 15 min in PBS. For Giemsa staining, B-MF was fixed with ethanol for 5 min, and Wright-Giemsa stained according to the manufacturer’s instructions. CSFIR receptor signaling was blocked with Ki20227 (R&D).

In vitro assays
For bacterial uptake assay, E. coli (Thermo Fisher) labeled with pHrodo™ red (0.1 mg/ml) were cultured with B-MF generated from RAG2-GFP for 7 days in cRPMI™ fusion medium and phagocytosed by BM cells. Cells were washed with PBS, fixed with 4% formaldehyde, and stained with DAPI. For phagocytosis of apoptotic cancer cells, ID8-RFP cells (10⁶/ml) were pretreated with 300 nM...
gemcitabine hydrochloride (Sigma, St. Louis, MO) for 24 h, then washed with PBS and cultured with macrophages for 2 h. Macrophages were stained with anti-F4/80-FITC Ab, and DAPI and phagocytosis was evaluated using Zeiss LSM 710 (Carl Zeiss AG, Jena, Germany) and analyzed with Fiji software. For macrophage proliferation test, BrdU (10 μM, BD) was added to macrophage cultures on day 5, and the BrdU incorporation was quantified on day 7 using FACSymphony™ and analyzed by FlowJo.

T-cell suppression assay was described elsewhere. Briefly, splenic T cells isolated with CD3⁺ T-cell enrichment column (R&D) were labeled with eFluor® 450 and cultured with macrophages at 1:10, 1:20, and 1:40 E:T ratios in 96-well flat-bottom plates coated with 5 μg/ml anti-mouse CD3ε antibody (clone 145-2C11, BD) and free anti-mouse CD28 antibody (2 μg/ml, clone 37.51, BD) for 4 days. The Treg conversion assay was described elsewhere. In brief, FACS-sorted splenic CD4⁺CD25⁺ T cells were cultured with macrophages at 1:5, 1:10, and

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cells were FACS evaluated. To evaluate the tumor-supporting role of discovered by GSEA tool with Molecular Signature Database v7.4. All cells (5 × 10⁶) were i.p. injected into C57BL/6 J mice. Spontaneous quanti in the fourth mammary gland and the lungs were analyzed with B16-F10 melanoma cells (day 0) or 4T1.2 breast cancer cells vitro-generated B-MF or PBS 3 and 7 days after subcutaneous challenge. PeC were i.p. injected with BM Lin e melanoma as measured days 11, 14, 16, 18, and 21, and on day 21, mice were euthanized to evaluate tumor weight and T cells. For B-MF tracking experiment, in vitro-generated B-MF from BALB/c mice were eFluor® 450 labeled and i.v. injected (5 × 10⁵ cells/mouse) into μMT BALB/c mice with 14-day s.c. 4T1.2 tumor. To compare B-MF to B cells, μMT BALB/c mice were i.v. injected with naive BALB/c mouse in vitro-generated B-MF or FACS-purified FOB (3 × 10⁵ cells/mouse) 3 and 7 days after s.c. challenge with 4T1.2 cells. Lungs and TILs were quantified at day 30 post-tumor challenge.

**Cellular cholesterol content quantification**

Macrophages were fixed with 4% formaldehyde solution in TBS for 5 min, then after TBS washes, they were incubated with Filipin III at 1:20 E:T ratios in plates coated with 5 μg/ml anti-mouse CD3ε antibody and free recombinant murine IL-2 (5 ng/ml, PepProTech, Rocky Hill, NJ) in for 5 days. Control T cells were cultured with recombinant mouse TGF-β1 (5 ng/ml, R&D) in cRPMS without macrophages.

**In vivo experiments**

For evaluation of macrophages in vivo, tumor cells were subcutaneously injected into congenic mice, such as 4T1.2 cells and EMT6 cells (1 × 10⁶) in BALB/c and μMT mice, and B16-F10, and AT3 and MC38 cells (1 × 10⁵) in C57BL/6, 3T3, and MBL-EYFP mice. Tumor size was measured in the fourth mammary gland and the lungs. All samples were analyzed by microarray analyses were performed using the R environment for statistical computing (version 3.6.2).

**scRNA-seq**

Sort-purified single-cell suspensions were loaded into a 10x Chromium controller (10x Genomics, Pleasanton, CA, USA) and converted to a barcoded single-cell RNA expression library according to the standard protocol of the Chromium Next GEM Single cell 3' kit (v3.1 chemistry) in Laboratory of Immunology and Molecular Biology, National Institute on Aging, and the single-cell 3' gene expression libraries were sequenced on NovaSeq 6000 (Illumina, San Diego, CA, USA) in the Genomics Core facility of the Johns Hopkins School of Medicine. Raw sequencing data were processed using the Cell Ranger version 5.0 (10x Genomics, Pleasanton, CA, USA) pipeline. The raw gene expression matrix was normalized and scaled using the SCImpute method in the Seurat R package (version 4.0). The minimum number of detected genes was set to 1000, and genes were chosen when they were detected in more than three cells. Dimension reduction was performed using principal component analysis (PCA). For visualizing the generated clusters, we used the Uniform Manifold Approximation and Projections (UMAP) plot. We defined clusters with a leiden algorithm using shared nearest neighbor (SNN) in PCA space. From in vitro B-MF and Mo-MF, we generated a total of 12 clusters for in vitro samples (0–11). Integration of in vivo samples with canonical correlation analysis (CCA) was performed, and we generated 13 clusters for in vivo tumor macrophage samples (0–12). Finally, we performed a nonparametric Wilcoxon rank-sum test to search for highly expressed genes in the clusters. In addition, human tumor single-cell transcriptions were downloaded from GEO (GSE14725, and GSE146026) and also processed with the same pipeline described. We used only macrophage clusters for downstream analysis. All single-cell analyses were performed using the R environment for statistical computing (version 4.0.5).

**ATAC-seq**

We utilized a Hi-Seq 2000 machine to sequence the ATAC-seq libraries (Illumina, San Diego, CA). We prepared 12 pair-end ATAC-seq libraries including BM CSFIR (±) and Spleen CSFIR (±) samples (n = 3 per group). In total, 369 M reads were sequenced, and average 31 M reads were sequenced per sample. We applied NIH TaRGETII ATAC-seq pipelines, which are available to the genomics community. All raw reads were trimmed using cutadapt package, and trimmed reads (>36 bp minimum alignment length) were mapped against the mm10 reference genome using BWA aligner. We used de-duplicated and uniquely mapped reads for peak calling analysis after excluding blacklist regions defined by ENCODE. The candidate peaks were predicted by MACS peak calling tool. In addition, we also applied the Deseq2 to determine differentially accessible regions (DARs); cutoff: Fold change > 1.5, log2CPM > 1.2, FDR < 0.05. The differentially accessible regions were submitted for the search of potential transcription factor binding sites using HOMER software. We used non-DARs as background regions in de novo motif analysis.

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**Fig. 6 | Cancer targets BM CSFIR/PAX5⁺ B-cell precursors.** a–c Pax5 MF1 (Mean ± SEM) in freshly isolated BM CSFIR vs CSFIR B-cell precursors (P = 0.0001, n = 5 mice, a); in pre-B 702/5 cells treated with indicated cancer CM (P = 0.0002 R/P) vs 4T1.2 CM, P = 0.001 R/P vs EMT6 CM, AT3 CM and MC38 CM, n = 3 independent cell cultures, b; and BM Lin CSFIR CD19+B220+CD93+IgD⁻ B-cell precursors after treatment RPMI vs M-CSF for 48 h (P = 0.0025, n = 3, e). P-values in a–c were calculated using two-tailed unpaired t-test. d 3D PCA plot of chromatin accessibility data of BMPP CSFIR and CSFIR from BM and spleen of naive mice (n = 3 per group). e Heatmap of differentially accessible regions (DARs) in BM CSFIR and CSFIR BMPP. No significant DARs with FDR < 0.05 and FC (fold change) >1.5 were detected in splenic cells. f Significant de novo motifs predicted from 678 sites that are more open in BM CSFIR compared to both BM and splenic CSFIR BMPP. g mRNA microarray heatmap of macrophage-related DEGs in B cells isolated from PB of patients with breast cancer (BC, n = 8) compared to healthy donors (HD, n = 7). Scale bar is for expression z-score. h Frequency ± SEM of CSFIR (P = 0.0002, left), CD68⁺ (P = 0.0004, middle), and LDLR⁺ (P = 0.0095, right) cells within CD9⁰ CD1⁰ B cells from PB of patients with ovarian cancer (OC) vs healthy donors (HD) (n = 5 for OC, n = 7 for HD). P-values in h were calculated using two-tailed unpaired t-test. i, j UMAP of scRNA-seq data of macrophages (left) and expression levels of the in vitro-generated B-MF genes (right) in published human BC (i) and OC (j) datasets. Highlighted regions show clusters with overlapping expression signatures of B-MF.
Statistical analysis
The results are presented as the mean with each individual data point or in bar graph ± SEM. GraphPad Prism (Prism 6; GraphPad Software, Inc) was used to perform statistical analysis. Data were analyzed using Welch t-test or one-way ANOVA. A P-value less than 0.05 was considered significant (**P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05).

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

Data availability
The authors declare that the data that support the findings of this study are available within the Article and its Supplementary Information file. RNA-seq and ATAC-seq data are deposited in https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE178716 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE180285. Source data are provided with this paper.

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Author contributions
C.C. performed the research, collected, and analyzed data; E.R., M.B., X.W., and L.Z. performed experiments; B.P. worked on bioinformatics analyses; J.M.L. provided clinical trial samples; I.B. supervised bioinformatics analysis; A.B. conceived, designed, and supervised the study and wrote the manuscript.

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