Dysregulated hematopoiesis caused by mammary cancer is associated with epigenetic changes and Hox gene expression in hematopoietic cells

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Abstract (207 words)

Cancer is associated with immune dysfunction characterized by the presence of pro-inflammatory and immunosuppressive cells and factors that contribute to tumor growth and progression. Here we show that mammary tumor growth is associated with defects in hematopoiesis leading to myeloproliferative-like disease (leukemoid reaction), anemia and disruption of the bone marrow stem/progenitor compartment. The defects we characterized included impaired erythropoiesis, leukocytosis, loss of early progenitor cells in the bone marrow and splenic extramedullary hematopoiesis. We established an in vitro model to dissect interactions between mammary cancers and the hematopoietic system. Investigations in this model revealed that G-CSF produced by mammary tumors can synergize with FLT3L and GM-CSF to expand myeloid progenitors and their progeny in culture. Mammary tumor growth was associated with histone methylation changes within lineage-negative c-Kit-positive (LK) hematopoietic cells within the bone marrow of tumor-bearing mice. Similarly, parallel histone methylation patterns occurred in cultured bone marrow cells exposed to mammary tumor-conditioned cell culture media. Notably, changes in histone methylation in these cell populations correlated with dysregulated expression of genes controlling hematopoietic lineage commitment and differentiation, including Hox family genes and members of the PRC2 chromatin-remodeling complex. Together, our results demonstrate that mammary tumor-secreted factors induce profound perturbations in hematopoiesis and expression of key hematopoietic regulatory genes.
Introduction

The link between cancer and inflammation has been recognized for almost 150 years (1). However, the complexity of the interactions between tumors and the immune system is only beginning to be appreciated. Solid tumor development is often associated with cytokine and metabolite upregulation, and immunosuppressive cell expansion (including myeloid-derived suppressor cells (MDSCs), T regulatory cells, tumor-associated macrophages (TAMs), and tumor-associated dendritic cells (tDCs)). These cells facilitate tumor growth, metastasis and immunological escape (2). Cancer is also frequently associated with perturbations in hematopoiesis manifested as disturbed myelopoiesis, leukocytosis (leukemoid reaction), cytopenia and/or anemia, which may predict adverse patient outcome (3, 4). Since the nature of anti-tumor immune responses may hold better prognostic value than standard scoring regimes, a better understanding of tumor-induced changes to the immune response and hematopoietic system may lead to improved cancer diagnosis and treatment (5). Indeed, recent studies show that manipulation of tumor-induced stromal cells can impair tumour growth and elicit anti-tumor immunity (6).

Development of blood cell lineages is controlled by sequential differentiation of progressively restricted progenitor populations derived from hematopoietic stem cells (HSCs). Gene expression patterns governing blood cell production and lineage commitment are regulated by transcription factors and epigenetic control of chromosomal organization involving histone modifications and DNA methylation (7). Epigenetic changes are linked to control of cell fate, stem/progenitor cell function and tumorigenesis. In leukemia and myeloid neoplasia, epigenetic dysregulation induced by mutations in DNA- or histone-modifying genes is a significant step in disease progression. Mutations in genes encoding Trithorax group (TrxG) and Polycomb group (PcG) proteins regulating histone H3 lysine-4 (H3K4) and lysine-27 (H3K27) methylation, respectively, are frequently observed in hematological
malignancies and myeloproliferative/dysplastic neoplasms (8, 9). H3K4 trimethylation (H3K4me^{3}) correlates with enhanced gene expression, while H3K27 trimethylation (H3K27me^{3}) is generally linked to suppression of gene expression. EZH2, the H3K27 methyltransferase component of Polycomb repressive complex2 (PRC2), plays complex roles in leukocyte neoplasms with both loss- and gain-of-function mutations in Ezh2 associated with disease. EZH2 catalyzes progressive H3K27 methylation, resulting in suppression of genes including Hoxa9 (10, 11), with recent work revealing a role for PRC2 in HSC physiology (12, 13). Hoxa genes regulate steady state hematopoiesis governing stem/progenitor cell function. As blood cells differentiate, expression of Hoxa genes decreases, a process involving changes in H3K27 methylation catalyzed by enzymes like JMJD3 (14, 15). Hoxa gene family upregulation, particularly Hoxa7 and Hoxa9, promote hematopoietic stem/progenitor cell (HSPC) expansion and contribute to leukemogenic transformation (16, 17).

Here, we assessed the HSPC compartments in tumor-bearing mice and found that mammary tumors (MTs) induced greater changes in hematopoiesis than previously appreciated. All MTs examined affected primitive HSPC compartments leading to myeloproliferative-like disease (leukocytosis) and anemia. MT growth was associated with diminished Ezh2 expression, reduced H3K27me^{3}, and Hoxa9 upregulation in lineage^{-} (Lin^{-}) c-Kit^{+} (LK) hematopoietic progenitors in the bone marrow (BM).

We established an in vitro model to decipher the tumour-secreted and endogenous factors that might contribute to dysregulation of hematopoiesis, and found that the hematopoietic regulatory cytokines FLT3L and GM-CSF synergized with MT-produced G-CSF to enhance HSPC production. Additionally, MT-secreted factors led to global and gene-specific changes in histone methylation patterns associated with enhanced Hoxa9 gene expression. Taken together, our data suggests that MT-
secreted factors such as G-CSF lead to epigenetic changes and altered expression of key hematopoietic regulatory genes, ultimately resulting in leukocytosis, anemia and HSPC defects.

Materials and methods

Mice. MMTV-neu<sup>OTI/OTII</sup> mice were previously described (18, 19). Mice were C57BL/6 genetic background (>10 generations). Animal experiments were performed according to Canadian Council for Animal Care and the University of British Columbia Animal Care Committee guidelines. Mice were age/sex-matched and analyzed between 9-14 wks of age.

Tumor cell lines. NOP cell lines express Neu linked to OVA<sup>OTI/OTII</sup> peptide sequences and a dominant-negative p53 transgene (MMTV-neu<sup>OTI/OTII</sup>)(18, 19). NOP cells were maintained in RPMI-1640 (Invitrogen), 10% heat-inactivated (HI)-FBS, 100U/mL penicillin-G, 100μg/mL streptomycin, 2mM glutaMAX, 10μM 2-mercaptoethanol and insulin/transferrin/selenium (Lonza). NOP12,18,21,23 lines are referred to as MT-1,2,3,4. CMT-93 (ATCC-CLL-223), 4T1 (ATCC-CRL-6323), and B16-F1 (ATCC-CRL-6323) cells were maintained in DMEM, 10% HI-FBS. Tumor-conditioned media (T-CM) was produced by culturing cells at 5×10<sup>6</sup>cells/25mL for 4 days. All cell lines were screened by PCR and determined to be free of 20 viral pathogens (Impact-I PCR profile, RAADIL).

In vitro BM analysis. BM was cultured in NOP medium without insulin/transferrin/selenium in the presence of cytokines at 1×10<sup>6</sup>cells/mL for 3-8d. For GM-CSF experiments, BM was cultured at 2×10<sup>5</sup>cells/mL unless otherwise indicated. For T-CM treatments, BM was supplemented with 25% T-CM unless otherwise indicated.

Tumor injection and tissue processing. Mice were injected with 1-5×10<sup>6</sup> MT cells subcutaneously (SQ) or mammary fat-pad (MFP), 1×10<sup>5</sup> 4T1 cells (SQ) or 7.5×10<sup>5</sup> B16 cells (SQ). Spleens, femurs
and blood were analyzed once tumors reached 1cm³. Blood was analyzed on the scil Vet abc hematology analyzer (scil animal care company) or treated with NH₄Cl before flow cytometry.

**Flow cytometry and FACS.** Cells were incubated with 2.4G2 mAb (Fc block), before Ab addition. Data was acquired on an LSRII (BD Biosciences) and analyzed with FlowJo (TreeStar). Dead cells were excluded using PI or DAPI, RBCs were excluded by lysis/size.

**Menin inhibitor treatment of BM cultures.** BM was treated with menin inhibitors, MI-2/MI-3, on d0 of culture and analyzed on d3 for CFCs. Alternatively, inhibitors were added on d0 and cells were analyzed on d8 by flow/qPCR. For Hoxa9 qPCR, cells were treated for 24hrs with MI-2/MI-3.

**Statistical analysis.** Statistical comparisons were performed using unpaired *t*-test. Error bars and ± symbols represent SEM. *, *P*<0.05, **, *P*<0.01, ***, *P*<0.001, ****, *P*<0.0001.

**Reagents, antibodies, competitive-reconstitution, CFU-S₁₂ assay, chromatin immunoprecipitation (ChIP), qPCR, methylcellulose assay and Western blot.** See supplemental materials and methods.

**Results**

**Mammary tumors induce widespread changes in hematopoiesis.** Cancer is frequently associated with immune system perturbations, with the tumor microenvironment known to reprogram myeloid cells into MDSCs, TAMs and/or tDCs. We employed previously described MMTV-*neu<sup>OTI/OTII</sup></sup> MT lines (MT1-4) that are syngeneic to C57BL/6-*neu<sup>OTI/OTII</sup></sup> transgenic mice (18, 19). Tumor development was associated with a 3-7 fold increase in white blood cells (WBC) due to a 7-10 fold increase in granulocytes. We also observed decreased hematocrit, red blood cells (RBC), hemoglobin (HGB) and platelets (PLT), in MT-bearing mice (Table 1). Changes in monocyte and granulocyte frequency/numbers were confirmed by flow cytometry of blood, BM and spleen (Figure S1A). We observed increased splenic cellularity, monocytes (CD115<sup>hi</sup>CD11b<sup>Gr-1<sup>low/neg</sup></sup>) and granulocytes...
(CD115\textsubscript{low/neg}CD11b\textsuperscript{+}Gr-1\textsuperscript{hi}) (Figure S1B). By contrast, MT-bearing BM was hypocellular, with fewer monocytes (Figure S1B). Increased proportions and numbers of granulocytes and WBCs were observed in immunocompetent (syngeneic MMTV\textit{neu} or C57BL/6, both denoted as B6) or immunocompromised (RAG1\textsuperscript{−/−}) MT-bearing mice independent of injection site (MFP or SQ) (Figure S1, Table 1, data not shown). Mice with size-matched B16 tumors did not induce similar perturbations in monocytes/granulocytes in BM, spleen or blood, but did induce mild anemia (Figure S1A, data not shown).

To determine whether MTs in general perturbed myelopoiesis and erythropoiesis, we compared the well-characterized 4T1 mammary tumor cell line to MT-1. 4T1 tumor development resulted in splenomegaly (increased granulocytes and monocytes), WBC expansion and a reduction in RBCs, HGB, and PLTs (Figure S2A). BM was hypocellular with a corresponding reduction in monocytes, but a modest granulocyte increase, confirming observations in MT-1 bearing mice (Figure S2B, C).

Histological investigation of BM revealed a diminution in erythroid cells and megakaryocytes, and replacement with granulocytes and blast-like cells (Figure 1A). Erythroid cell maturation can be assessed by CD71 and Ter119 expression (20). The least mature, CD71\textsuperscript{+}Ter119\textsuperscript{−} cells differentiate into CD71\textsuperscript{+}Ter119\textsuperscript{−} cells, which then give rise to CD71\textsuperscript{−}Ter119\textsuperscript{+} erythroblasts. Analyses of erythroid populations revealed a reduction in BM CD71\textsuperscript{+}Ter119\textsuperscript{+} and CD71\textsuperscript{−}Ter119\textsuperscript{−} cells (Figure 1B, D, E), and a corresponding increase of these cell types and a CD71\textsuperscript{+}Ter119\textsuperscript{−} population in spleens of C57BL/6 and RAG1\textsuperscript{−/−} MT-bearing mice regardless of tumor site (Figure 1B-F). Analyses of 4T1 tumour-bearing mice showed a similar phenotype in the erythroid compartment with the exception that CD71\textsuperscript{+}Ter119\textsuperscript{−} cells in the BM were increased (Figure S2B). By contrast, the erythroid lineage in the BM of B16 tumor-bearing mice was unchanged, whereas a variable increase was observed in the splenic erythroid lineage (Figure 1F, S3A, data not shown).
Mammary tumor growth changes the hematopoietic stem/progenitor compartment. The widespread changes in blood, BM and spleens in tumor-bearing mice prompted an investigation of the HSPC compartment. An increase in frequency and number of LSKs (Lin⁻Sca-1⁻c-kit⁺FcεR1⁻) was observed in both BM and spleen of MT-bearing mice (Figure 2A-C). BM and spleen exhibited increased numbers of ST-HSCs (LSK, CD34⁺Flt3⁻) and MPPs (LSK, CD34⁺Flt3⁺), whereas spleen also possessed more LT-HSCs (LSK, CD34⁺Flt3⁻) (Figure 2B,C). BM exhibited a modest reduction or unchanged numbers of LT-HSCs (Figure 2B). By contrast, LKs (Lin⁻Sca-1⁻c-kit⁺) were increased in spleens, but reduced in BM of MT-bearing mice (Figure 2B,C). Diminished LKs in BM correlated with reduced megakaryocyte/erythroid progenitors (MEP) and their erythroid progeny but not common myeloid progenitors (CMP), or granulocyte/macrophage progenitors (GMP) (Figures 2B,1B,D,E). By contrast, spleens contained abundant MEPs correlating with increased erythroblasts (Figures 2C,1C,F). Changes in HSPCs were observed in mice bearing each of the four MTs and 4T1s, irrespective of injection site or genetic background (C57BL/6 or RAG1⁻/-) (Figure S2C,data not shown). B16 tumor-bearing mice did not exhibit changes in HSPC frequency/numbers (Figures 2A,S3B,data not shown).

To further investigate the impact of MT growth on HSPCs we assessed progenitor numbers using the methylcellulose colony-forming and CFU-S₁₂ assays, and stem cell functionality by competitive repopulation in radiation chimeric mice. Methylcellulose assays revealed increased splenic myeloid progenitors in MT-bearing mice, correlating with increased LKs (Figures 3A,2C). By contrast, BM CFU-G/M/GM numbers were similar in control and MT-bearing mice (Figure 3A). The CFU-S₁₂ assay identifies primitive multi-potent progenitor populations (21). Surprisingly, BM from MT-bearing mice exhibited a 5-10 fold loss in primitive progenitor activity (Figure 3B), whereas spleens from MT-bearing mice possessed large numbers of progenitors, correlating with increased splenic HSPCs (Figure 3B,2C). Competitive repopulation experiments with mixtures of control and MT-bearing mouse BM or
splenocytes, revealed that the BM LT-HSC population was intact in MT-bearing mice, with equal contributions from cells of control or MT-bearing mice observed 3-20 weeks following reconstitution (Figure 3C). By contrast, whereas control splenocytes provided only short-term repopulation, MT-bearing mouse splenocytes contained multi-potent stem cells capable of myeloid/lymphoid long-term reconstitution (Figure 3C). Therefore, MT growth leads to perturbations in the BM HSPC compartment with accompanying extramedullary hematopoiesis.

**Conditioned media from MTs, but not other tumor types, induces BM proliferation and expansion of hematopoietic progenitors in vitro.** Our results suggested that either MTs impacted HSPCs at sites distant from the tumor or that MTs had metastasized to BM. Histological examination of BM, and PCR for the tumor-specific neuOTU transgene in RAG1−/− recipients, failed to reveal BM metastasis (Figure 1A, data not shown) suggesting that MTs influenced stem/progenitor numbers and functionality from a distance, i.e. via tumor-derived soluble factors. Indeed, recent research suggests that breast cancers can induce changes in HSPC gene expression in a systemic manner (22). We therefore investigated whether culturing BM with tumor cells, or in medium supplemented with MT-conditioned media (MT-CM), would impact stem/progenitor numbers. Culture of BM with MT cells or MT-CM in the absence of supportive cytokines led to a <2-3 fold increase in cellularity but did not increase LSK/LKs or progenitor activity (Figure 4A, data not shown).

We next investigated whether MT-CM could synergize with cytokines known to facilitate HSPC survival and/or expansion, i.e. FLT3L, GM-CSF, IL-3, IL-6, TPO, M-CSF, G-CSF or a combination of IL-3/IL-6/SCF. Analysis of BM cultures revealed that FLT3L synergized with MT-CM, dramatically increasing culture cellularity and LSK/LK numbers (Figure 4A,E). Increased cellularity, LSKs, LKs, CMPs and GMPs in cultures supplemented with FLT3L and MT-CM was detected by day 3 (Figure 4E). GM-CSF synergized with MT-CM less effectively than FLT3L, where total cellularity
increased from day 3-9, while LSKs peaked at day 3 (Figure S4A). The ability of MT-CM to increase cellularity, LSKs and LKs in FLT3L BM cultures was shared with T-CM from all analyzed MT cell lines (MT-1-4, 4T1), while melanoma (B16) and colon cancer (CMT93) T-CM was ineffective (Figure 4B, data not shown).

To determine whether the appearance of BM cells with stem/progenitor cell markers correlated with progenitor activity, we cultured BM in MT-CM and GM-CSF or FLT3L for 3 or 8 days and enumerated methylcellulose CFU-G/M/GM colonies. FLT3L BM cultures containing MT-CM had a ≥400 fold increase in CFCs compared to FLT3L alone cultures, and a >20 fold increase in CFCs compared to an equivalent number of freshly harvested and plated BM cells, demonstrating expansion of progenitors upon exposure to MT-CM (Figure 4D). CFCs in GM-CSF and MT-CM BM cultures were increased 3-4 fold (Figure 4D). To investigate whether MT-CM was regulating progenitor survival, apoptosis and/or proliferation, we analyzed Ki67 expression, or Annexin V and DAPI staining in day 3 MT-CM-treated FLT3L and GM-CSF cultures. MT-CM treatment led to increased Ki67-expressing LSKs and LKs (FLT3L cultures) without affecting HSPC apoptosis (Figure S5A,B). However, a small but significant reduction in DAPI⁺ LSKs was evident in MT-CM GM-CSF and FLT3L cultures (Figure S5C).

Multi-lineage hematopoietic system reconstitution is the defining characteristic of HSCs, therefore LSKs or unfractionated BM cells from day 8 FLT3L MT-CM-treated cultures were injected into lethally irradiated C57BL/6 or sub-lethally irradiated RAG1⁻/⁻ recipients. Although MT-CM-treated FLT3L BM cultures contained large numbers of LSK/LKs and functional myeloid progenitors, no multi-lineage HSC activity was detected (data not shown).

Mammary/breast cancers can produce and enhance stromal cell production of cytokines including M-CSF, IL-6, VEGF, and G-CSF, which may contribute to tumor growth/spread (23, 24).
We tested whether mammary cancer-produced factors could synergize with FLT3L in cultured BM by assessing cytokine-induced changes in cellularity and LSK/LK content. Alone, no tested factors enhanced LSK numbers, however, IL-6 and G-CSF synergized with FLT3L, increasing total cellularity and LSK/LKs comparable to MT-CM (Figure 4A,C, data not shown). Other factors tested did not increase LSKs in combination with FLT3L, although some increased cell numbers (Figure 4C). We used a cytokine array to identify MT-1-secreted factors and found abundant G-CSF (of 40 cytokines/chemokines tested), and low IL-6 (Figure S4B). We confirmed that only MT cell lines MT1-4 and 4T1 secreted G-CSF (Figure S4C). Additionally, G-CSF was elevated in the serum of MT-1 bearing mice (Figure S4C). We assessed the contribution of G-CSF, IL-6, M-CSF and GM-CSF to the biological activity of MT-CM by adding neutralizing antibodies against each cytokine to FLT3L BM cultures containing a reduced dose of MT-CM (1.6%) to facilitate neutralization of cytokine. Only anti-G-CSF Abs significantly reduced the MT-CM-dependent increase in cellularity, LSK/LKs and CFCs (Figure 4F,G,S4D).

**Mammary tumors alter histone methylation patterns associated with hematopoietic regulatory genes.** The homeobox gene cluster plays key roles in hematopoiesis and leukemic transformation. Tumors have been shown to alter gene expression in BM myeloid cells (25). The similarities between MT-bearing mice and mice with leukemia/myeloproliferative disease led us to examine tumor induction of Hox genes. Hoxa7, Hoxa9 and Hoxa10 mRNAs were overexpressed in MT-CM-treated FLT3L and GM-CSF BM cultures (Figure 5A). Importantly, Hoxa9 expression in Lin− BM cells from MT-bearing mice was also greatly elevated in vivo (Figure 5B).

Activating and inhibitory epigenetic regulation controls Hoxa9 expression. Interestingly, expression of Ezh2, the methyl-transferase component of PRC2, was decreased in MT-CM-treated FLT3L and GM-CSF BM cultures (Figure S6A). Furthermore, expression of the PRC2 complex
components Ezh1, Suz12, and Eed were reduced in MT-CM-treated FLT3L cultures (Figure S6A). Levels of the H3K27 demethylase, Jmjd3, and H3K4 methyltransferases, Mll1-5 and the Mll complex component Rbbp5 were decreased in MT-CM-treated FLT3L BM cultures (Figure 5A,S6A). Conversely, in GM-CSF cultures, all other epigenetic regulators, excluding Mll1 and Mll3, were unchanged when treated with MT-CM (Figure S6A). We also detected reduced Ezh2 mRNA in Lin− BM cells from tumor-bearing mice, and a corresponding reduction in the EZH2 product H3K27me3 in BM LKs (MFI±SD, control: 5526±864; MT-1 bearing mice: 2996±706, p=0.018, Figure 5B). By contrast, H3K4me3 levels were indistinguishable in LKs from control and tumor-bearing mice (Figure 5B). Neutralization of G-CSF, but not GM-CSF or M-CSF, in BM cultures containing 1.6% MT-CM reduced Hoxa9 mRNA levels and partially restored Ezh2 mRNA to control levels, suggesting that G-CSF in MT-CM regulates Hoxa9 expression (Figure 5C,S4D). Importantly, analysis of total H3K27me3 in MT-CM-treated GM-CSF BM cultures revealed a dose-dependent reduction in H3K27me3, correlating with reduced H3K27me3 levels in tumor-bearing mouse BM LK cells (Figure 5D). Similarly, H3K27me3 was reduced in FLT3L BM cells cultured in MT-CM, which correlated with reduced EZH2/1 protein (Figure S6B,C).

Loss of the PRC2 (EZH2)-dependent epigenetic mark H3K27me3, and/or gain of H3K4me3 associated with the Hoxa9 locus correlate with HOXA9 expression (26, 27). Consistent with diminished Ezh2 expression in BM cultured with FLT3L or GM-CSF and MT-CM, H3K27me3 associated with the Hoxa9 locus (11) was decreased at amplicon A in FLT3L and GM-CSF cultures (Figure 5E,F) and variably decreased at the other amplicons (Figure S7A). Conversely, we observed increased H3K4me3 associated with multiple regions of the Hoxa9 locus (amplicons A-E) (28, 29) correlating with enhanced Hoxa9 expression (Figure 5E,F,S7A). Partial neutralization of G-CSF in FLT3L BM cultures treated with 1.6% MT-CM (Figure S4D) reduced H3K4me3 at amplicons A and C.
(Figure S7B). However, 1.6% MT-CM did not attenuate H3K27me3 at amplicon A, nor was neutralization of G-CSF able to restore H3K27me3 at amplicon C (Figure S7B). Addition of G-CSF to FLT3L and GM-CSF BM cultures decreased H3K27me3, enhanced Hoxa9 expression, and increased CFC progenitor content, suggesting that MT-secreted G-CSF is responsible for the majority of MT-CM bioactivity (Figure 6A,B, data not shown).

To determine whether histone methylation changes were required for MT-CM bioactivity, we employed recently described menin inhibitors MI-2 and MI-3, which attenuate H3K4me3 and suppress Hoxa9 expression and leukemic transformation (30). Menin is a key protein required to assemble SET1-like histone methyl-transferase complexes required for H3K4me3 epigenetic remodelling of the Hoxa9 locus (31). We confirmed that MI-3 treatment of BM cultures blocked H3K4me3 (Figure 6C inset). We detected a dose-dependent reduction in total cellularity and LSK/LKs in MT-CM-treated FLT3L and GM-CSF BM cultures upon treatment with MI-2/MI-3 compared to control inhibitor (MI-nc) (Figure 6C-E, data not shown). Additionally, MT-CM-treated BM cultures revealed a diminution of myeloid progenitors, and a reduction in Hoxa9 expression, following inhibitor addition (Figure 6F,G). Collectively, these results show that G-CSF secreted by MT cells can synergize with FLT3L and GM-CSF to induce expansion of hematopoietic progenitors with the effects of G-CSF correlating with changes in histone methylation patterns required for Hox gene expression and tumor-induced HSPC expansion.

**Discussion**

Tumors harness elements of both the innate and adaptive immune systems to facilitate their growth and metastasis. Perturbations in myelopoiesis are common in neoplastic and chronic inflammatory diseases, and may be critically important in disease aetiology. Many cancers induce
tumor-promoting TAMs, MDSCs and/or tDCs, and recent reports have revealed how significant these tumor-induced perturbations in the immune system are and have highlighted the need to better understand these perturbations to improve strategies to initiate or re-invigorate anti-tumor immune responses (32). In the case of breast cancer, the immune status of the tumor may predict treatment outcome (33). As different cancers affect the immune system uniquely, understanding these interactions will help predict patient responses to therapy and to advance immunotherapy strategies.

Here we show that mammary cancer affects the primitive HSPC compartments of the hematopoietic system leading to a myeloproliferative-like disease characterized by neutrophilia, anemia and HSPC defects. Importantly, tumor-induced changes were observed using five different MT-lines in mice of immune-deficient and -sufficient backgrounds. We established an in vitro system to investigate regulation of HSPCs by MTs and found that MT-CM synergized with FLT3L and GM-CSF to enhance BM proliferation and progenitor cell expansion, an ability shared with every MT line analyzed. By contrast, melanoma and colon cancer cells lacked this activity in vitro and melanoma cells did not significantly perturb myelopoiesis in vivo. Our data indicates that different tumor types interact with the immune system in unique ways, and that disruption of hematopoiesis is a common MT characteristic.

Identification of HSPCs requires functional and phenotypic assessment. We ensured that LSK/LKs were not contaminated with differentiated cells such as DCs or mast cells, however it was possible that LSK/LKs might express HSPC markers but lack HSPC activity. HSPC competitive reconstitution in chimeric mice showed that the BM LT-HSC compartment was intact in tumor-bearing mice, consistent with LT-HSC numbers in the BM. However, restricted primitive BM progenitors from the CFU-S12 assay were greatly reduced by MT growth. By contrast, spleens of MT-bearing mice contained large numbers of LSK/LKs that could reconstitute the myeloid and lymphoid compartments.
of lethally-irradiated recipients, and contained abundant primitive CFU-S_{12} colony-forming progenitors and more restricted myeloid progenitors. Therefore, LSK/LKs in tumor-bearing mice possessed HSPC activity. Although cells propagated for 8 days in vitro in MT-CM and FLT3L expressed LSK/LK markers and included large numbers of myeloid CFCs, they could not reconstitute lethally or sub-lethally irradiated mice. Notably, actively cycling HSCs and HSCs in inflammatory environments may be functionally defective (34, 35).

Our results suggest that tumor development induces mobilization and proliferation of the stem cell compartment with inappropriate seeding of spleen. This deviation in hematopoiesis by tumor-secreted factors, and compensatory splenic erythroid output, may partially explain the HSPC irregularities (36). Similar to our findings, an investigation of murine lung adenocarcinoma revealed tumor-induced extramedullary hematopoiesis in the spleen through migration of HSCs from the BM to the spleen, resulting in production of TAMs and neutrophils (37). However, tumor-induced effects on erythropoiesis were not examined.

An alternative explanation for HSPC mobilization to spleen may correspond to tumor-induced loss of erythropoietic potential in the BM, as BM MEPs and erythroblasts at various stages of development were dramatically reduced in MT-bearing mice. Interestingly, patients with pre-existing anemia before therapy exhibit reduced survival following treatment for breast, lung, prostate, and other cancers (4, 38). Our results support the hypothesis that breast cancer patients may develop anemia concomitant with disease severity or progression, a characteristic that may impact treatment options.

Tumors can express cytokines and factors important for tumor growth, survival or spread, e.g. the 4T1 MT line expresses GM-CSF, G-CSF and M-CSF associated with a leukemoid reaction (39). We showed that G-CSF was an abundant factor secreted by MTs, and used loss- and gain-of-function experiments to confirm that G-CSF present in MT-CM synergized with FLT3L and GM-CSF to
stimulate increases in LSKs, LKs, CFCs, and *Hoxa9* expression, *in vitro*. Interestingly, Waight *et al.* used loss- and gain-of-function experiments to show that tumour-secreted G-CSF increases tumour growth, splenomegaly and MDSC production in mice, although the underlying mechanisms leading to these changes were not explored (40). G-CSF can also impair erythropoiesis and synergize with FLT3L to mobilize stem cells (41).

G-CSF is produced by many human tumours, including pancreatic, cervical, bladder and breast (42, 43). G-CSF is elevated in breast carcinomas compared to healthy breast tissue (44). Similarly, G-CSF mRNA is elevated in breast cancer tissues compared to surrounding healthy tissues and G-CSF levels were higher in the serum of breast cancer patients compared to healthy controls (45). The association between increased G-CSF and breast cancer clinical grade and lymph node infiltration has been established, further supporting its role in tumor progression and disease severity (46, 47). Therefore, G-CSF may be useful in the diagnosis of breast cancer (45). Breast carcinoma activation of BM cells and progenitors of hematopoietic origin can instigate the growth of indolent tumors and metastases, therefore our results suggest that the effect of G-CSF on HSPCs and myeloid cells may enhance metastases (48). Additionally, MT-CM-derived G-CSF may be important in regulating BM and spleen stromal elements controlling HSPC release or receptivity, a possibility we are exploring.

G-CSF is a secreted glycoprotein that mobilizes hematopoietic stem cells, progenitors, mature cells, and stimulates granulocyte differentiation. G-CSF has been used to treat radiation- and chemotherapy-induced neutropenia. However, G-CSF promotes the proliferation and invasion/angiogenesis of various human tumours (45) and G-CSF-producing malignant tumors are associated with poor clinical outcome (49). The use of G-CSF to treat cancer patients underscores the need to better understand its effect on hematopoiesis, metastasis and anti-tumour immune responses.
The phenotypic similarities between MT-bearing mice and mice with myeloproliferative/myelodysplastic syndrome (leukemoid reaction, HSPC defects) suggested that MTs modulate key hematopoietic regulatory genes. Ezh2 loss-of-function mutations are associated with myeloid disorders (8) and Hox genes are critical regulators of HSC renewal, HSPC expansion (50), and differentiation repression (16). HOXA9 down-regulation promotes hematopoietic cell differentiation while its overexpression is linked to acute myeloid leukemia (16, 26). We showed that MT growth was associated with enhanced Hoxa9 expression in BM Lin− cells. Moreover, Hox genes were overexpressed, while Ezh2 was reduced, in MT-CM-treated BM cultures. Diminished Ezh2 expression correlated with reduced H3K27me3 in vitro and in vivo. In addition, H3K27me3 associated with the Hoxa9 locus (11) was diminished correlating with enhanced Hoxa9 expression.

H3K4me3 is an activating epigenetic histone modification catalyzed by MLL family members and coordinated by menin. Hoxa9 expression correlated with increased H3K4me3 at multiple regions of the Hoxa9 locus in MT-CM-treated cells, suggesting that increased H3K4me3 and diminished H3K27me3 are associated with tumor-induced changes in Hox expression. Addition of H3K4me3 inhibitors (30) to MT-CM-treated BM cultures reduced culture cellularity and progenitor content. Taken together, our data links H3K4me3 regulation of the Hoxa9 locus with the induction of HSPC expansion by tumor-secreted factors such as G-CSF. Our results suggest that therapeutic strategies for treating myeloid neoplasia may also be effective at attenuating tumor induced hematological defects.

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Authorship

Contribution: AS/MKC/KT/DLK/XF and MER performed experiments; KWH/AS/MKC/KT/DLK analyzed data/made figures; KWH/MKC/AS and DLK designed the study/wrote the paper; BHN/TC and JG provided reagents/advice.
References

Table 1. Hematologic analyses of MT-1 bearing mice.

<table>
<thead>
<tr>
<th>RAG1⁻⁻</th>
<th>B6</th>
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<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>WBC, 10³/µL</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td>RBC, 10⁶/µL</td>
<td>9.9 ± 0.1</td>
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<tr>
<td>HGB, g/dL</td>
<td>14.9 ± 0.1</td>
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<tr>
<td>HCT, %</td>
<td>46.4 ± 0.6</td>
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<tr>
<td>PLT, 10³/µL</td>
<td>1103 ± 59</td>
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<tr>
<td>LYM, 10³/µL</td>
<td>0.8 ± 0.2</td>
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<tr>
<td>MON, 10³/µL</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td>GRA, 10³/µL</td>
<td>2.5 ± 0.3</td>
</tr>
</tbody>
</table>

RAG1⁻⁻: Control n=13, MT-1 n=12; B6: Control n=4, MT-1 n=4. Blood was obtained from cardiac puncture and analyzed on a scil Vet abc hematology analyzer, scil animal care company, Gurnee, IL. *, P < .05; **, P < .01; ***, P < .001; ****, P < .0001. WBC indicates white blood cell; RBC, red blood cell; HGB, hemoglobin; HCT, hematocrit; PLT, platelet; LYM, lymphocyte; MON, monocyte; GRA, granulocyte.

Figure Legends

Figure 1. Mammary tumor development is associated with impaired BM erythropoiesis and compensatory splenic erythropoiesis. (A) Femurs from control and MT-1-bearing mice, H&E stained BM sections imaged at ×10 and ×40 original magnification. (B) Frequencies of CD71⁺Ter119⁻, CD71⁺Ter119⁺ and CD71⁻Ter119⁺ (erythroid populations) in BM of control and MT-1-bearing mice. SQ, subcutaneous; MFP, mammary fat-pad. (C) Frequencies of splenic erythroid populations in control and MT-1-bearing RAG1⁻⁻ and MMTV-neuOTI/OTII mice. (D) Numbers of erythroid populations in the BM of RAG1⁻⁻ mice, or (E) MMTV-neuOTI/OTII mice. (F) Numbers of erythroid populations in spleens of control and MT-1 (MMTV-neuOTI/OTII, RAG1⁻⁻), or control and B16 tumor-bearing (C57BL/6, RAG1⁻⁻) mice. B6=MMTV-neuOTI/OTII and C57BL/6. Data represent three experiments, n=3-4.

Figure 2. Perturbations in hematopoietic stem/progenitor cell numbers and location in mammary tumor-bearing mice. (A) Classification of HSPCs. BM from control and MT-1 or B16 tumor-bearing RAG1⁻⁻ mice was stained with Lin-specific antigen Abs (Sca-1, c-kit, CD34, FcγRII/III, and Flt3), and
Lin-DAPI cells were analyzed by flow cytometry. (B) Total numbers of BM and HSPC subsets; LSK (Lin\(^-\)Sca-1\(^+\)c-kit\(^+\)), LK (Lin\(^-\)Sca-1\(^-\)c-kit\(^+\)), LT-HSC (LSK,Flt3\(^+\)CD34\(^+\)), ST-HSC (LSK,Flt3\(^-\)CD34\(^+\)), MPP (LSK,Flt3\(^+\)CD34\(^+\)), CMP (LK,FcyRII/III\(^{hi}\)CD34\(^+\)), GMP (LK,FcyRII/III\(^{hi}\)CD34\(^+\)), and MEP (LK,FcyRII/III\(^{lo}\)CD34\(^-\)). (C) Splenocytes and HSPCs were analyzed as in B. B6=MTTV-\textit{neu}\textsuperscript{OTII/OTII}. Data represent \(\geq\) three experiments, \(n=3\).

**Figure 3. Mammary tumor growth is associated with functional changes in BM and spleen stem/progenitor compartments.** (A) Total numbers of colony-forming cells (CFU-G/M or CFU-GM) in spleen (left) and BM (right) of control and MT-1-bearing MMTV-\textit{neu}\textsuperscript{OTII/OTII} mice were determined by methylcellulose assay. (B) Numbers of primitive multi-potent colony forming cells in the BM and spleen of control and MT-1-bearing RAG1\(^{-/-}\) mice were determined by CFU-S\(_{12}\) assay. (C) CD45.2\(^+\) splenocytes or BM cells from MMTV-\textit{neu}\textsuperscript{OTII/OTII} control or MT-1-bearing mice, transplanted into lethally-irradiated CD45.1\(^+\) recipients at a 1:1 ratio with CD45.1\(^+\) carrier cells. Engraftment efficiency was assessed by the relative frequency of CD45.2\(^+\) versus CD45.1\(^+\) cells in blood at 3, 8 and 20 weeks. Percentages of CD45.2\(^+\) nucleated PI\(^-\) cells are shown. † euthanized due to lack of reconstitution. Data represent \(\geq\) two experiments, \(n=3-5\).

**Figure 4. Mammary tumor conditioned media expands hematopoietic progenitors in BM cultures supplemented with FLT3L or GM-CSF.** (A) Total cells and LSKs in BM cultured in media containing the indicated cytokines, plated at \(1\times10^6\)cells/mL, supplemented ± MT-1-conditioned media (MT-1-CM). (B) BM was cultured in FLT3L and CM from the indicated tumor cell lines and total cellularity and LSKs were enumerated. (C) As in A, except BM was cultured with FLT3L and the indicated cytokines or MT-1-CM. (D) Total CFCs in FLT3L or GM-CSF BM cultures ± MT-1-CM. BM was plated in methylcellulose after 8d (FLT3L) or 3d (GM-CSF) of culture. Freshly isolated BM was also plated to show the relative increase in CFCs in cultures compared to equivalent numbers of.
plated uncultured BM. (E) Total cells, HSPCs (LSK, LK, CMP, GMP), CD11b<sup>+</sup>CD115<sup>hi</sup> and CD11b<sup>+</sup>Gr-1<sup>hi</sup> cells in FLT3L BM cultures ± MT-1-CM over 9d. (F) Total cells, LSKs and LKs in FLT3L BM cultures ± a reduced dose (1.6%) of MT-1-CM, treated with G-CSF or IL-6 neutralizing Abs (or isotype-control Abs, iso). (G) CFCs were enumerated in control and G-CSF or IL-6 neutralizing Ab treated cultures as in D, except with 1.6% MT-1-CM. Data represent ≥ three experiments. MT-1-CM is designated as MT-1.

**Figure 5.** Mammary tumor growth is associated with enhanced *Hoxa* gene expression and changes in histone methylation at the *Hoxa9* locus. (A) Ezh2, Jmjd3, Hoxa7, Hoxa9 and Hoxa10 expression in FLT3L and GM-CSF BM cultures ± MT-1-CM. (B) Hoxa9 and Ezh2 expression in sorted Lin<sup>-</sup> BM cells (upper), and H3K4me<sup>3</sup> and H3K27me<sup>3</sup> levels in BM LKs from control and MT-1 bearing mice analyzed by intracellular flow cytometry (lower). (C) Hoxa9 and Ezh2 expression in FLT3L BM cultures treated with 1.6% MT-1-CM and isotype control (iso) or neutralizing Abs against G-CSF. (D) GM-CSF BM cell lysates treated with an increasing dose of MT-1-CM for 8d were Western blotted for H3K27me<sup>3</sup>, histone H3 and β-actin. (E) Depiction of amplicons (A-E) in the *Hoxa9* locus for ChIP analysis. (F) Repressive (H3K27me<sup>3</sup>-amplicon A) and activating (H3K4me<sup>3</sup>-amplicons B-E) histone modifications in FLT3L or GM-CSF BM cultures ± MT-1-CM were investigated by ChIP. Amplicons A-E were analyzed by qPCR. MT-1-CM is designated as MT-1. Data represent ≥ three experiments.

**Figure 6.** G-CSF enhances *Hoxa9* expression and CFC numbers, while menin inhibitors reverse mammary tumor conditioned media induced expansion of LSKs, LKs and CFCs in BM cultures. (A-B) Hoxa9 mRNA expression in FLT3L and GM-CSF BM cultures ± MT-1-CM or G-CSF (1, 5 or 20ng/mL), and CFC numbers in FLT3L and GM-CSF BM cultures ± MT-1-CM or 20ng/mL G-CSF. (C-E) Total cells, LSKs and LKs in BM cultures supplemented with FLT3L and MT-1-CM, and treated with menin inhibitors MI-2 or MI-3, or control drug (MI-nc) (3.1µM, 6.3µM, or 12.5µM). (C inset)
H3K4me$^3$ levels in GM-CSF BM cells treated with MI-3 (6μM and 3μM) were analyzed by Western blot. (F) Total CFCs in BM cultures supplemented with GM-CSF and MT-1-CM and treated with 3.1μM or 6.3μM of MI-nc or MI-2/MI-3. (G) *Hoxa9* mRNA expression in FLT3L BM cultures supplemented with MT-1-CM, treated with MI-2/MI-3 or MI-nc (6μM and 12.5μM). MT-1-CM is designated as MT-1. Data represent ≥ three experiments.
Figure 1

A

B

C

D

E

F

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Figure 5

A. Expression of Ezh2, Jmjd3, Hoxa7, Hoxa9, and Hoxa10 in cells treated with FLT3L and GM-CSF.

B. Expression of Hoxa9 and Ezh2 in lineage^ BM cells in the presence of FLT3L and GM-CSF.

C. Immunoblot analysis of Hoxa9 and Ezh2 expression in cells treated with FLT3L and GM-CSF.

D. ChIP analysis of H3K27me3 and H3K4me3 in cells treated with FLT3L and GM-CSF.

E. Amplicon analysis of cells treated with FLT3L and GM-CSF.

F. Amplicon analysis of cells treated with FLT3L and GM-CSF.
Figure 6

A) FLT3L

B) GM-CSF

C) total cells

D) LSK

E) LK

F) CFC

G) Hoxa9

** Figures showing data on colony-forming cells (CFCs) and total cells under different conditions.**

- **A** and **B**: Comparison of Hoxa9 expression and CFCs under FLT3L and GM-CSF conditions.
- **C**: Comparison of total cells under different conditions.
- **D**: Comparison of LSK cell counts.
- **E**: Comparison of LK cell counts.
- **F**: Count of CFCs.
- **G**: Normalized expression of Hoxa9.

*Significant differences are indicated with asterisks.*
Dysregulated hematopoiesis caused by mammary cancer is associated with epigenetic changes and Hox gene expression in hematopoietic cells

Alexander Sio, Manreet K. Chehal, Kevin Tsai, et al.

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