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Neutrophils, G-CSF and their contribution to breast cancer metastasis

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Abstract

Evidence is mounting for a role for neutrophils in breast cancer progression to metastasis. However, the role of G-CSF in neutrophil biology in a cancer setting remains to be defined. Herein we discuss the most recent clinical and experimental evidence for neutrophils and G-CSF in the promotion of metastasis, demonstrating a potential mechanistic link between them. Understanding this link is imperative both for the development of diagnostic tests and for therapies targeting neutrophils to improve the treatment of breast cancer patients with, or at risk of developing metastatic disease. Since a high neutrophil to lymphocyte ratio in patients predicts poor outcome, while mild neutropenia predicts an improved outcome, we urge caution in the use of G-CSF in neutrophil recovery following chemotherapy as there is increasing evidence in preclinical models that G-CSF can promote metastasis.

Abbreviations

G-CSF	granulocyte colony stimulating factor
NLR	neutrophil to lymphocyte ratio
OS	overall survival
DFS	disease free survival
TNBC	triple negative breast cancer
MDSC	myeloid derived suppressor cell
G-CSFR	granulocyte colony stimulating factor receptor
TAMs	tumour associated macrophages
LDN	low density neutrophil
HDN	high density neutrophil
TAN	tumour associated neutrophil
TGFβ	transforming growth factor-β
CSF-1	colony stimulating factor-1
CSF-1R	colony stimulating factor-1 receptor

iNOS	inducible nitric oxide synthase
IL-1β	interleukin 1 beta
IL-17	interleukin 17
INFγ	interferon gamma
NET	Neutrophil extracellular traps
MPO	Myeloperoxidase

1. Introduction

The number of papers reporting a role for neutrophils in breast cancer progression has increased markedly in recent years. These white blood cells that were once described purely as an indicator of chemotherapeutic side effects, and where the major priority was in avoiding their depletion, are now being recognized as having the capacity to enhance progression to metastasis in many cancer types. Here, we have summarized the most recent studies describing the pro-metastatic role of neutrophils and their major growth factor, granulocyte colony stimulating factor (G-CSF), in breast cancer. Recent reviews have discussed the role of neutrophils in cancer in great detail [1, 2], so we have endeavoured here to focus whereever possible on the relationship between neutrophils and G-CSF in metastatic disease.

While knowledge of neutrophil function is growing, the known effects of G-CSF itself on progression to metastasis are scarce in terms of patient data, which is of particular concern as it is used routinely for recovery from neutropenia in myelosuppressed patients undergoing chemotherapy [3, 4]. Better characterization of the function of neutrophils in driving breast cancer metastasis will allow for more targeted therapies to complement existing therapies, where their success is hampered by neutrophil-mediated immunosuppression. There may also be potential in harnessing the myelosuppressive side-effects of chemotherapy to reduce neutrophil load to a therapeutically beneficial level. Indeed, multiple studies and meta-analyses have reported improved survival for breast and other cancer patients experiencing mild neutropenia during chemotherapy [5-8]. As others have postulated,

perhaps the better outcome of breast cancer patients with chemotherapy-associated mild neutropenia is due to a reduction in pro-metastatic neutrophils, and is not just an indicator of therapy efficacy [9].

2. Neutrophils as prognostic indicators

One of the first reports describing neutrophils as a marker of poor patient prognosis was published in 1996 for metastatic renal cell carcinoma. Elevated blood neutrophils prior to the commencement of therapy were identified as an independent risk factor for shorter overall survival [10, 11]. Since then, elevated neutrophil numbers or a high neutrophil to lymphocyte ratio (NLR) have been associated with poor prognosis in many human cancers, including lung, esophageal, pancreatic, cervical and ovarian. The relevance of a high NLR is well established, as neutrophils have been found to suppress lymphocyte activity in cancer through production of arginase-1 and hydrogen peroxide [12, 13] (Fig. 1(2)). Experiments employing *ex vivo* co-cultures, as well as the observations in patients with activated granulocytes and metastatic cancer, indicate that neutrophils inhibit re-expression of the ζ-chain of CD3 on T cells after TCR internalisation, thereby reducing T cell function and cytokine production [12, 14].

Prognostic significance of NLR in breast cancer

In breast cancer, information on the prognostic significance of neutrophils remained scarce until 2015. Since then, a multitude of studies has emerged that have investigated the association between a high NLR and outcome. Most studies were undertaken on samples from patients prior to treatment and across a range of breast cancer subtypes.

More recently, a large meta-analysis of 15 published studies incorporating more than 8,500 breast cancer patients investigated the association of a high NLR ratio with overall survival (OS) and disease-free survival (DFS), superseding previous meta-analyses [15, 16]. It was found that a high NLR (between the range of 1.9-5.0) was associated with worse OS and DFS, with a high NLR being particularly prognostic for reduced DFS in patients with ER-negative and HER2-negative breast cancer [17]. This study acknowledged that the disease stage of individual reports, when combined, could be a possible source of heterogeneity. However, the association of NLR with OS was consistent across studies on patients with early disease, as well as

those with combined early and metastatic disease [17]. Importantly, only studies based on pre-treatment NLR were included in this study, as treatment could heavily influence peripheral blood counts.

Studies on the association of NLR and outcome in triple negative breast cancer (TNBC) patients are difficult, as TNBC sample sizes are usually small. However we noted one publication reporting that TNBC patients with a pre-treatment NLR greater than 2 had significantly lower OS and DFS, yet it was acknowledged that a larger prospective study was required [18]. Others have similarly reported a significant association between elevated NLR and increased mortality in ER-negative and PR-negative breast cancer that is not affected by HER2 status [19].

NLR – predictive of metastasis?

Outside of predicting OS and DFS, the potential usefulness of NLR is still under investigation. In terms of predicting metastasis, despite having low numbers of women presenting with distant metastasis at the time of pre-treatment NLR analysis (13.9%), there was a clear association of increasing NLR with metastasis [19]. Of these patients, 32.4% had a NLR greater than 4.

Data on the association between NLR and response to chemotherapy are conflicting. One study examining the association between pre-operative NLR and response to pre-operative systemic therapy in breast cancer patients found a trend to a pathological complete response (pCR) in those with higher NLR but this was not statistically significant [20]. Conversely, another study found in breast cancer patients receiving neoadjuvant chemotherapy that a lower NLR (<2.06) was associated with close to double the pCR rate compared to patients with a high NLR. Again, this study confirmed the association of a high NLR with worse survival, but importantly, also with recurrence-free survival after neoadjuvant chemotherapy [21]. Yet another study evaluating the response to neoadjuvant chemotherapy found that patients with low pre-treatment NLR had better responses, although their cut-off for what was considered a 'low' NLR varied from the aforementioned report [22]. This highlights one of the issues associated with the classification of NLR, in that there is not a strictly defined cut-off value for its analysis.

Interestingly, we found only one study comparing NLR measurements between two different time points: pre-treatment and at recurrence. The study by Iwase et al. found an increase in NLR by an average of 0.59 at recurrence compared to the initial

measurement in a small study on patients with recurrent breast cancer [23]. It was also reported that TNBC patients in this study demonstrated the highest NLR at recurrence (4.59) as well as the greatest increase in NLR (increase of 2.0) at recurrence.

Limitations of NLR

In summary, high pre-treatment NLR is increasingly being reported as associated with decreased survival, both overall and disease-free, as well as with decreased response to therapy across multiple breast cancer subtypes. The limitations of such a measurement include the fact that it is systemic and may not reflect the immune landscape of the tumour, where the ratio of immunosuppressive neutrophils to tumour infiltrating lymphocytes (TILs) may be most important, especially for subtypes such as TNBC. Contrary to the association of high NLR with poor prognosis in breast cancer, there are examples in other cancer types of neutrophil infiltration of tumours being associated with better prognosis. For example, Galdiero et al. demonstrated that increased tumour-associated neutrophil (TAN) presence by IHC staining was associated with better clinical outcome for colorectal cancer [24]. In addition, using The Cancer Genome Atlas (TCGA) data, a polymorphonuclear neutrophil (PMN) signature that is representative of capacity for neutrophil infiltration, correlated with a trend towards (not statistically significant) improved survival for the endometrioid subtype of endometrial adenocarcinoma (EC). However, when their signature was applied across all solid cancers, it was significantly associated with decreased survival [25].

The NLR may also be sensitive to fluctuation caused by tumour-associated inflammation or caused by tumour-independent factors, including inflammation, infection and ethnicity [16]. Without further stratification of neutrophil polarisation, it may be considered unclear as to whether a high NLR is truly representative of pro-metastatic neutrophils, or is simply an inflammatory marker/byproduct of the presence of tumour. Indeed, the NLR has been reported to increase with increasing tumour stage [21]. In our opinion, monitoring of NLR after surgical removal of the primary tumour may be the best application of NLR, as we see continued elevation of neutrophil numbers in the blood of mice that develop lung metastasis following surgery to remove the primary tumour in our preclinical models of breast cancer (unpublished data).

3. G-CSF as an alternative prognostic indicator

The simple presence of neutrophils alone in the blood or tumour of patients may not be sufficient to predict outcome. The functional classification of neutrophil subtypes into N1-like and N2-like is not as well established as for macrophages, and this binary classification represents an oversimplification of what is actually a phenotypic spectrum. It is therefore important to identify other markers associated with neutrophils that may predict outcome or provide guidance for suitable treatments. Attention is now also turning to the prognostic significance of the neutrophil growth factor, G-CSF. Most of the published clinical data relate to G-CSF administration to patients who are neutropenic upon chemotherapy. Although there are emerging studies on its presence in various cancers, very few indicate its prognostic significance in breast cancer.

A number of studies have reported the expression of G-CSF in solid cancers, most notably for non small cell lung cancer (NSCLC). However, the majority of these studies only report significant increases in G-CSF in the serum of cancer patients compared to healthy controls [26, 27], providing no association with tumour stage or histopathological subtype. Other tumour types found to express G-CSF include glioma, which has also been demonstrated to express the G-CSF receptor, therefore possibly promoting its own progression through autocrine/paracrine signaling [28]. G-CSF expression was also found to be significantly associated with poorer overall survival in cervical cancer, as it was associated with increased myeloid derived suppressor cell (MDSC) frequency, proposed to potentiate chemoresistance [29].

Circulating G-CSF levels

A small study of 110 treatment-naïve patients with ductal breast cancer compared plasma levels of G-CSF to levels in patients with benign lesions or in healthy controls [30]. Significantly higher plasma levels of G-CSF were observed in patients with stage III or IV disease, compared to those with stage I or II or healthy controls. However, G-CSF levels in patients with benign lesions were also significantly higher compared to healthy controls, indicating that G-CSF was not a factor whose levels specifically correlated with advanced disease. Another small study of 136 breast cancer patients showed an increase in serum G-CSF in patients with advanced stages of disease similar to the study above (an increase from stages I and II to III

and IV) [31]. Regardless of this increase with increasing stage, only 7.35% of all breast cancer patients had detectable levels of G-CSF in serum. Also, contrary to the previous study, the difference between the overall level of G-CSF across all breast cancer patients and healthy controls was not significant, possibly attributable to the failure to detect G-CSF in any of the healthy controls examined. Other studies have similarly demonstrated an increase in G-CSF secretion in breast tumour samples compared to undetectable levels in healthy samples [32]. Additional reports of an association between plasma/serum G-CSF and outcome or disease stage for cancer are rare or non-existent, the exception being chronic myeloid leukemia [33]. This is likely due to the normal levels of G-CSF in healthy subjects being extremely low to undetectable, therefore rendering statistical comparisons difficult [34].

G-CSF levels in tissues

A recent study by Hollmen et al. examined G-CSF by measuring the staining intensity in tumour sections from a large number of breast cancer patients [35]. G-CSF was reported to be localized at the invasive front of tumours and was expressed at higher levels in TNBC patients compared to ER+, HER2+ or ER+HER2+ patients ($p<0.001$). Of particular interest, high G-CSF levels within these TNBC tumours was a significant predictor of poorer overall survival compared to those with low G-CSF levels ($p=0.021$). Though comprehensive, a major limitation of this study was the failure to report treatment history, as addition of G-CSF to support chemotherapy regimens could dramatically affect the immune composition of these tumours, and possibly patient survival. Further implicating G-CSF in poor prognosis in breast cancer was a study by Welte at al [36]. TCGA data were used to examine the genomic landscape of human breast cancers against a G-CSF signature that was based on human haematopoietic cells treated with G-CSF [37]. This G-CSF signature was found to be expressed at higher levels in basal and luminal B subtypes of breast cancer, and, importantly, higher expression correlated with the risk of distant metastasis [36]. It is important to note that this signature encompasses genes affected by the presence of G-CSF, and is not necessarily representative of G-CSF expression itself.

G-CSF receptor (G-CSFR)

Similar to G-CSF, very little information exists on the expression of G-CSFR in breast cancer, although its expression has been reported across other cancer types. In

addition to demonstrating high expression of G-CSFR in ~90% of human gastric and colorectal tumours examined, Morris et al. also found that treatment of relevant gastric and colorectal carcinoma cell lines *in vitro* with G-CSF increased their proliferation and migration [38]. Similarly, significantly elevated G-CSFR expression was demonstrated in ~60% of ovarian cancer samples examined by Kumar et al., while treatment of G-CSFR positive ovarian cancer cell lines with G-CSF increased their migration *in vitro* [39]. These studies highlight a concern that treatment with G-CSF for neutrophil recovery may inadvertently increase the metastatic capacity of G-CSFR-expressing tumours in patients.

In terms of breast cancer, Wojtukiewicz et al. were able to demonstrate G-CSFR positivity by IHC in 20/28 breast cancer tissue samples [40]. Though present in 71% of samples examined, staining intensity was only classified as high in 21% of cases. These high levels were evident in small focal infiltrations as well as in vascular endothelial cells infiltrating the tumour and should be interpreted with caution as staining in the remaining positive cases was defined as 'weak and irregular' and would more likely be attributed to infiltrating monocytes and neutrophils. No correlation with clinical data was made, even though samples were defined as being all from T1-2N0M0 (Stage I-IIA) invasive ductal adenocarcinomas.

4. Neutrophil polarisation

As reviewed above, circulating immune markers such as NLR may not provide sufficient predictive information to guide treatment regimens, as they may not be truly representative of the tumour microenvironment. In terms of neutrophil markers in mice, researchers have already moved away from the classical and non-specific Gr-1 that was originally used to classify MDSCs [41]. The term MDSC applies to a heterogeneous population of cells with functional characteristics similar to mature and immature macrophages, granulocytes, dendritic cells and other myeloid cells at early stages of differentiation [41]. MDSCs are classified by their ability to promote tumour growth primarily via suppression of anti-tumour $CD8^+$ T cell responses [42]. However, this classification is responsible for much confusion as 1) similar functions of murine MDSCs have already been described for tumour associated macrophages (TAMs) and neutrophils, and 2) MDSC are defined as $CD11b^+$ $Gr\text{-}1^+$ cells, where Gr-1 does not differentiate between monocytes, macrophages and neutrophils. To elucidate which cells are involved in metastasis, it is necessary to discriminate

populations based on Ly6C and Ly6G, which are subtypes within Gr-1. $Ly6C^{lo}$ expression is thought to represent "non-classical" monocytes, while $Ly6C^{hi}$ are the "classical", less mature monocytes that are thought to arrive first at sites of inflammation [43]. Neutrophils are distinguished by their Ly6G expression.

Delineation of neutrophil subtypes is becoming especially relevant, as other pro-metastatic functions for neutrophils are uncovered outside of the classical MDSC characteristic of T cell suppression. For example, neutrophils have been recognized as facilitating angiogenesis and vascular remodelling [44-46], tumour cell migration [47] and escape via protease-mediated breakdown of the extracellular matrix (ECM) and basement membrane [48]. However, a role for G-CSF associated neutrophils in these processes in specifically breast cancer metastasis is less well established.

In contrast to these roles, neutrophils also have the capacity to reduce tumour growth and metastasis via anti-tumour functions. The majority of the literature describing an anti-tumour or 'N1' role for neutrophils centres around their production of reactive oxygen species (ROS), causing tumour cell apoptosis [49]. This anti-tumour cytotoxic activity has also been described as inhibiting metastatic seeding of the lung, although the protective effect was only transient [50]. Recent studies have demonstrated type 1 interferons [51] and the *MET* proto-oncogene [52] as requirements for driving anti-tumour functions and recruitment of neutrophils, respectively. However, the involvement of G-CSF in generating anti-tumour neutrophils appears limited. One recent study demonstrated that administration of G-CSF could enhance radiotherapy-mediated anti-tumour responses through activation of tumour infiltrating neutrophils in mice, although there was no therapeutic benefit to G-CSF treatment alone [53].

Morphological classification

Further stratification of neutrophil subtypes is still required, as their simple presence is not indicative of their activity. Instead of surface markers that can be examined by flow cytometry, some groups have attempted to classify neutrophils based on physical characteristics. Sagiv et al. reported in various tumour models, including 4T1, a separation of neutrophil subtypes based on density gradient purification [54]. The dominant population, termed low density neutrophils (LDNs), was found to increase with cancer progression, as opposed to neutrophils in naïve mice, where the majority were classified as high density neutrophils (HDNs). HDNs were proposed to be a mature population, comprising segmented neutrophils, whereas

LDNs were characterized as heterogeneous and more immature, with banded and ring-shaped nuclei. These results were confirmed in circulating neutrophils in cancer patients compared to healthy volunteers, with the additional observation of increased surface activation markers (CD11b and CD66b [55, 56]) on LDNs in cancer patients (Fig. 1(5)). Morphological classification may represent a simpler approach to identify the role of neutrophils in cancer as their polarisation has until now been classified into 'N1' or 'N2' on the basis of gene expression or *ex vivo* functional assays. One factor responsible for polarisation towards a pro-tumour 'N2' TAN phenotype is transforming growth factor-β (TGFβ) (Fig. 1 (5)). Inhibition of TGFβ in mouse models of cancer suppresses tumour growth by generating TANs with anti-tumour cytotoxic properties [50, 57]. Indeed, the study mentioned above found that TGFβ was sufficient to drive the accumulation of LDNs in the blood of tumour-bearing mice, thus suggesting that this LDN subset was 'N2' associated [54].

N2 associated factors – a role for G-CSF?

Aside from TGFβ, there is little information on other factors that may drive neutrophil polarisation towards a pro-tumour phenotype. Interferon-β (IFNβ) may be in part responsible for generating anti-tumour TANs, as inhibition of IFNβ in a mouse model of melanoma increased the expression of pro-tumour genes associated with angiogenesis, such as VEGF and MMP-9 [58]. Interestingly, a role for G-CSF in driving formation of N2 neutrophils has not been reported (Fig. 1 (5)), despite most tumour models that produce high levels of the cytokine being associated with tumour-promoting neutrophils. Perhaps this is because the potential roles of G-CSF are so broad, including driving the proliferation of neutrophils, their mobilisation from bone marrow, maturation of immature myeloid progenitor cells and chemoattraction of neutrophils into primary tumours. We have shown that treatment of weakly metastatic 66cl4 mammary tumours (that do not secrete G-CSF) with exogenous G-CSF increases metastasis to lung [59]. Neutrophils taken from mice treated with G-CSF were more able to suppress the proliferation of naïve $CD8^+$ T-cells than neutrophils from naïve mice [59], a characteristic associated with 'N2' pro-tumour neutrophils (Fig. 1 (5)).

Although similar immunosuppressive populations of circulating neutrophils have been identified in the blood of G-CSF treated stem cell donors, other populations of proinflammatory neutrophils were also present. Marini et al. investigated surface expression and functional activity of the proposed 'N2' like LDNs in G-CSF treated

donors compared to healthy donors [60]. Within the CD66b positive LDN and Normal Density Neutrophil (NDN) populations, it was the more mature CD10 positive neutrophils which were responsible for the decreased CD3/CD28-induced proliferation of T cells, where previously it was attributed simply to LDN cells [61]. Interestingly, CD10 positive NDNs from healthy donors were not immunosuppressive and CD10 negative LDNs from G-CSF treated donors instead enhanced CD3/CD28-induced T cell proliferation [60]. This difference in function within the CD66b positive LDN population highlights the heterogeneity of these cells and the issue of G-MDSC nomenclature, as it contains neutrophils that are not suppressive at all.

Interplay between G-CSF and CSF-1

So far, neutrophils have been discussed in isolation or with respect to the adaptive anti-tumour arm of the immune system. Evidence is now emerging to indicate that neutrophils may also have a complex role in innate immunity, particularly regarding their interaction with macrophages. We have demonstrated that targeting the colony stimulating factor-1 receptor (CSF-1R) on macrophages either with an antibody or with a small molecule kinase inhibitor in mice bearing 4T1.2 mammary tumours did not reduce TAM accumulation in tumours nor metastasis to lung. Instead, CSF-1R inhibition *increased* metastasis to lung, accompanied by an increase in circulating neutrophils and TANs, and an increase in plasma levels of G-CSF (Fig. 1(6)) [62]. Treatment with an anti-G-CSFR antibody overcame the increase in metastasis and neutrophil accumulation that was observed with CSF-1R inhibition [62]. Similarly, Hollmen et al. later found that CSF-1R inhibition in the 4T1 model also increased metastasis to lung and lymph nodes with the accompanying increase in blood neutrophils [35]. In both papers, the authors went on to urge caution with the use of CSF-1R inhibition for tumour-associated macrophage (TAM) depletion in breast cancer, suggesting that the levels of G-CSF in the primary tumour be considered prior to therapies targeting CSF-1R [35] [62]. Cautionary warnings regarding anti-CSF-1 therapies are especially relevant given that a small molecule inhibitor of the CSF-1R (PLX3397) is in clinical trials for metastatic breast cancer, although in combination with chemotherapy [63].

It has been reported that macrophages may be required for the clearance of apoptotic neutrophils [64] thus maintaining a certain level of control over inflammation, but the proposed likely reason for increased metastasis in the report by Hollmen et al [35] is more simple. The authors suggest that MHCII^{lo} monocytes and

TAMs abnormally express the G-CSFR under tumourigenic conditions – therefore when CSF-1R signaling is blocked, their differentiation is skewed by high levels of circulating G-CSF, resulting in M2-like, metastasis-promoting macrophages [35]. However, there was no explanation for the initial increases in G-CSF in the mice following inhibition of CSF-1R.

5. Sources of G-CSF in breast cancer

The cell lineage responsible for the systemic increases in G-CSF in patients and in murine tumour models is another issue of contention. Is the tumour solely responsible, or are sources such as host immune cells or the stroma also releasing G-CSF in response to signals from the tumour cells (Fig. 1(1))? Indeed, the 4T1 mammary carcinoma model is widely recognised as being a potent producer of G-CSF [65, 66] and for this reason has been used frequently for the study of neutrophils in breast cancer and for its ability to elicit the accumulation of MDSCs in blood, tumour and metastasis-involved lungs.

Tumour-derived G-CSF

One technique to explore the role of tumour-derived G-CSF is by specific interference with the gene. This was utilised in the previously mentioned study by Welte et al [36], who also showed the association of a G-CSF gene signature with the risk of metastasis in breast cancer patients. G-CSF expression was reduced by shRNA-mediated knock down in 4T1 tumour cells, resulting in a significant reduction in metastasis in mice that could be rescued by the reintroduction of MDSCs (the cells thought to be attracted by G-CSF) [36]. This finding supported the earlier study by Waight et al. [66] who used a gain or loss of function approach to show that tumour-derived G-CSF was responsible for granulocytic MDSC accumulation (ie. neutrophils) and for tumour growth, but their study did not assess metastasis.

Hollmen et al [35] reported high secretion of G-CSF from the MDA-MB-231 cell line, but in contrast to our current focus on neutrophils they reported that the major effects of G-CSF production were on the induction of immunosuppressive TAMs both in patients and in mice. Furthermore, Kowanetz et al. [65] used multiple cell lines within the 4T1 model to demonstrate a correlation between the increasing metastatic

potential of tumours, increased $CD11b^+Gr\text{-}1^+$ cells (particularly $Ly6G^+$ neutrophils) mobilised to lung and increased levels of the proangiogenic factor, Bv8, in lungs. G-CSF was the only mobilisation factor that was found to correlate with metastatic ability of these tumours [65].

Stroma-derived G-CSF

In contrast to the other two studies described above, Kowanetz et al. did not ignore the contribution of host cells to G-CSF production. The high levels of mouse G-CSF found within the tumour and circulation of mice bearing human MDA-MB-231 tumours indicated that host cells infiltrating the tumour were also major contributors. Other potential sources of G-CSF include stromal cells, endothelial cells, fibroblasts and monocytes/macrophages (Fig. 1(1)) [67].

A G-CSF knock-out mouse exists, characterized by reduced neutrophil numbers and a failure to undergo a neutrophilic response to infectious challenge, resulting in high mortality rates [68]. .Wculek and Malanchi [69] made use of the G-CSF null mouse crossed with the MMTV-PyMT transgenic model of metastatic breast cancer to demonstrate that neutrophils, while low in frequency in the primary tumour, were the major immune component in the pre- and metastatic lungs [69]. MMTV-PyMT mice on a background null for G-CSF failed to show this neutrophil accumulation in lung and had markedly reduced metastasis, whereas depletion of G-CSF from the tumour cells had no impact on neutrophil accumulation in the lungs or on the extent of metastasis in *Rag1*-null mice. Together, these results implicate the host as being an important source of G-CSF. Conversely, another study by Casbon et al. indicated that tumour-derived G-CSF was responsible for driving the production of immunosuppressive neutrophils in the PyMT mouse model [70]. Not only was G-CSF detected in the serum in increasing amounts with disease development, it was also found to be abundant in culture supernatants of PyMT tumour cells.

Coffelt et al. [71] utilised the 'KEP' transgenic mouse [72] to generate tumours that were transplanted into recipient mice, resulting in spontaneous lung metastases. These tumours elicit the production of circulating neutrophils that display high expression of the T-cell suppression-associated gene, iNOS [71]. The increase in pro-metastatic neutrophils was attributed to induction of an IL-1β- and IL-17-mediated inflammatory cascade, resulting in systemic increases in G-CSF (Fig. 1 (1)). Delineation of this cascade revealed that primary tumour-secreted IL-1β

stimulates the more recently identified gamma delta (γδ) T cell population [73] to produce IL-17. Addressing the tumour as a possible source, G-CSF was not upregulated in KEP tumours compared to normal mammary glands. Therefore, it remains unclear whether tumour cells contribute directly or indirectly to G-CSF production in this model [74].

6. Neutrophils and NK cell activity

Although it has long been established that neutrophils are potent suppressors of T-cell activation, characterization of their interaction with other anti-tumour effectors has only recently broadened to include NK cells. Compared to NK cells in normal human tissue, those in breast tumours have been found to exhibit a $CD56^{bright}$ $perforin^{low}$ phenotype [75] indicating decreased cytotoxic activity. Further to this, another study demonstrated that the decreased expression of activation receptors on NK cells (such as NKp30 and NKG2D) and the increased expression of an inhibitory receptor (NKG2A) was associated with the progression of human breast cancer and was indicative of decreased cytotoxicity of tumour-infiltrating NK cells [76]. However, neither study linked these decreases in NK function to neutrophil-mediated immunosuppression, with the latter study implicating tumour-secreted factors such as TGFβ [76].

The link between neutrophils and suppression of NK cell activity was hinted at in hepatocellular carcinoma (HCC), where the *ex vivo* co-culture of peripheral blood MDSCs from HCC patients with NK cells resulted in significant suppression of NK cell cytotoxicity as determined by ^{51}Cr-release assays and reduced INFγ secretion. However, this study did not characterize the generic population of 'MDSCs' further than being $CD14^{+}HLA\text{-}DR^{-/low}$; therefore it is not clear whether this profile specifically represents neutrophils. Also, suppression of NK cells was not deemed to be due to the classic MDSC property of arginase-1 production that is responsible for CD8 T-cell suppression [14]. Instead it was attributed to interaction with the activation receptor, NKp30, on NK cells.

NK cell suppression was confirmed more recently by Spiegel et al. [77] who demonstrated that neutrophils were responsible for reduced NK cell function, which

in turn increased intraluminal tumour cell survival time and facilitated metastasis (Fig. 1 (2-3)) [77]. When YAC-1 cells (well known targets of NK cell clearance) were injected intravenously into mice bearing 4T1 tumours, the number of YAC-1 cells retained four hours later in the lungs of these mice was 8-fold higher than those injected into naïve controls [77]. Conversely, when 4T1 tumour-bearing mice were depleted of neutrophils prior to YAC-1 cell injection by use of anti-Ly6G antibody, the number of YAC-1 cells retained in the lungs was reduced to the same levels as in naïve mice, indicating that neutrophils were facilitating this retention by disrupting NK cell-mediated clearance (Fig. 1 (3)). Confirmation was obtained by examining the *ex vivo* stimulation of NK cells with NKG2D or NKp46 antibodies. The CD107a and INFγ status of NK cells taken from the spleens and lungs of 4T1 tumour-bearing mice was significantly reduced after NKG2D or NKp46 antibody stimulation compared to naïve controls, and was attributed to the presence of neutrophils. This study again highlights the importance of understanding the effects of pro-metastatic neutrophils on other immune effectors to enable emerging immunotherapies, such as adoptive NK cell therapy, the best chance of success.

7. Neutrophil extracellular traps

An exciting recent development in delineating the role of neutrophils in the progression of cancer is the discovery of neutrophil extracellular traps (NETs). NETs were first reported by Brinkmann et al. in 2004 [78], when they were imaged by electron microscopy on activated neutrophils engaging in antimicrobial processes. *In vitro* examination of neutrophils activated with IL-8, phorbol myristate acetate (PMA) or LPS showed the production of distinctive extracellular fibres, a concept that was further consolidated *in vivo* where these structures were identified in infection settings in both preclinical models and in humans. NETs have since been implicated as a major mechanism by which neutrophils capture and kill bacteria and other pathogens. It was not until 2012 that NETs were first implicated in promoting cancer progression. Demers et al. [79] demonstrated that 4T1 mammary tumours, in addition to murine models of CML, generate neutrophils that are predisposed to the formation of NETs that escalate with increasing tumour stage. NETs were identified by increases in plasma DNA, as well as by immunofluorescence co-staining of extracellular histone H3 and DNA around neutrophils from tumour-bearing mice (Fig. 1 (4)). These markers are representative of the process of NETosis, whereby

neutrophils release decondensed chromatin-containing enzymatic granules into the extracellular space [80], usually resulting in non-apoptotic cell death.

G-CSF – a major player in NET formation

Numerous studies have since implicated G-CSF in exacerbating NET production (Fig. 1 (4)). The demonstration of NETs in 4T1 tumour-bearing mice by Demers et al. [79] was attributed to the tumour-derived G-CSF priming of neutrophils, that could be reversed by treatment of the mice with anti-G-CSF antibody. Similarly, neutrophils from naïve mice treated with recombinant G-CSF were predisposed to NET formation when stimulated with platelet activating factor *ex vivo* [79]. Although this study mechanistically recognized NETs as driving a pro-thrombotic state in the lungs of tumour-bearing mice, the authors later demonstrated that NETs have a role in tumour growth [81]. Interestingly, the latter study indicated that tumour-derived levels of G-CSF dictated the ability of tumour-infiltrating neutrophils to form NETs, linking their formation to a specific neutrophil phenotype ($CD11b^{high}$) that was found in high G-CSF-secreting tumours [81].

Other factors driving NET formation and consequences for patients

It was not until later that NETs were implicated in facilitating metastasis. Cools-Lartigue et al. [82] demonstrated in a mouse model of post-surgical infection that NETs could trap circulating tumour cells and increase metastasis. Their mouse model of caecal ligation and puncture (CLP) mimics the systemic sepsis often associated with gastrointestinal surgery in patients and was shown to cause NET formation [82]. Introduction of H59 Lewis lung carcinoma cells into the spleens of mice also undergoing CLP resulted in a significant increase in hepatic metastases. This was explained by the observation in two different tumour models undergoing CLP that tumour cells injected intravenously were trapped in microvessels by NET positive neutrophils, thus promoting their outgrowth into metastases (Fig. 1 (4)) [82]. However, no link was made to G-CSF in this study. Given that surgical resection of the primary tumour is a mainstream treatment for breast cancer, it would be interesting to know if this phenomenon is also occurring in breast cancer patients. Indeed, a recent study by Tohme et al. [83] demonstrated an increase in NET formation in the plasma of patients who had undergone major liver resection for metastatic colorectal cancer compared to those with minor resection or healthy volunteers. As major resection is associated with ischemia and reperfusion injury, the

result is inflammation, an effect that the authors had previously shown to exist in mice as well and to result in NET formation [84]. In addition, patients undergoing major liver resection who had higher levels of NETs in serum (in terms of MPO-DNA complexes) had a 4.2-fold greater risk of recurrence than patients with low levels of NETs [83]. Although evidence is strong for factors such as G-CSF, infection and surgical stress to be able to induce NETs that in turn promote metastasis, this area of research is new and extensive characterisation in other cancers, such as breast, does not yet exist.

One exception, however, is a recent study by Park et al. [9]. In addition to confirming that 4T1 mammary tumours stimulate NET formation (via G-CSF) as previously shown (Fig. 1 (4)) [79], the authors examined NET formation in breast cancer patients [9]. Immunofluorescence staining was undertaken on primary and matched lung metastases for DNA, MPO and citrullinated histone H3, revealing higher levels of NETs in lung metastases compared to that of primary tumours. This was not subdivided based on the subtype of breast cancer, but they did report higher numbers of NETs in TNBC samples, albeit with very small numbers of TNBC and HER2+ tumours. It would be interesting to expand this analysis and to measure G-CSF expression levels of these tumours and matched lung metastases, since treatment of human neutrophils *ex vivo* with recombinant G-CSF induces NET formation [9] and other studies have indicated that tumour-microenvironmental cues encourage this neutrophil response [81].

8. Neutrophils and G-CSF as therapeutic targets

Neutrophil depletion

There are a number of studies using antibody-mediated depletion of neutrophils to reduce metastasis in mice. However, this approach is not undertaken in patients. Also, the depletion of neutrophils with agents such as anti-Ly6G in mice (Fig. 1 (6)) can reduce neutrophils to a level considered neutropenic. In addition, the therapeutic output for depletion with anti-Ly6G is conflicting and seems to vary depending on the preclinical tumour model examined and the time administered, particularly in the studies we have reviewed herein. For example, treatment of P53N-C tumours pre-surgery with anti-Ly6G decreased tumour growth, and significantly decreased lung metastasis post-surgery [36]. Rag1-null mice bearing MMTV-PyMT tumours that

were depleted of neutrophils in the 'pre-metastatic' phase also showed a decrease in spontaneous lung metastasis [69]. Alternatively, anti-Ly6G depletion of neutrophils in the KEP model did not affect primary tumour growth, and only reduced lung metastasis when administered early in tumour growth and not post-surgery [71]. Pre-treatment with anti-Ly6G antibody prior to tumour cell inoculation also reduced experimental lung metastasis of D2A1 tumour cells [77]. Early intervention to deplete neutrophils may therefore be required to reduce metastasis. However, an exception to this, where neutrophil depletion resulted in increased metastasis, was a study by Granot et al [50]. Early treatment of 4T1 tumour bearing mice with either anti-Ly6G or anti-Gr-1 increased lung metastasis compared to controls, and was attributed to the eradication of tumour entrained neutrophils (TENs) that would normally inhibit metastatic seeding in the lung [50]. This detrimental effect of neutrophil depletion led the authors to report an anti-tumour role for neutrophils, which differs from their pro-tumour role reported in all other studies described in this section.

Therapies targeting G-CSF signaling – a more specific approach?

If G-CSF underlies or perhaps enhances the pro-metastatic functions of neutrophils, then interrupting G-CSF signaling may be an attractive strategy (Fig. 1 (6)). This can be achieved by treatment with anti-G-CSF or anti-G-CSF receptor antibodies. Anti-G-CSF treatment of 4T1 tumours resulted in decreased primary tumour growth [35], reduced metastasis [35, 65] and normalisation of monocyte and neutrophil mobilisation to blood. However, Hollmen et al. [35] did not attribute the anti-metastatic effects of anti-G-CSF therapy to neutrophils, as depletion of neutrophils specifically with anti-Ly6G treatment alone did not affect metastasis or 4T1 tumour growth compared to IgG treated controls in their study. Treatment of mice bearing highly metastatic 4T1.2 mammary tumours with an antibody targeting the G-CSF receptor was able to inhibit metastasis to lung and bone [62]. Other studies on the involvement of G-CSF in metastasis are scarce, involving gene deletion or overexpression, or are limited to an *ex vivo* approach such as that used by Park et al [9]. Anti-G-CSF treatment of mouse neutrophils co-cultured with 4T1 cells significantly reduced the size of NETs, a potentially important pro-metastatic feature of neutrophils that we have discussed above [9]. As reports of the G-CSF priming of neutrophils to form NETs are numerous, anti-G-CSF may be useful to prevent NETs in patients. This is likely to be more successful than the administration of an agent such as DNase to disrupt other NET components, which is currently in use for cystic fibrosis [85]. Thus, strategies disrupting G-CSF signaling require further investigation *in vivo*, especially with regard to effects on non-tumour induced granulopoiesis that is

required to fight infection. Hence anti-G-CSF or anti-G-CSF- receptor therapy [62] appears to be an attractive option to reduce pro-metastatic neutrophils, but remains somewhat counter-intuitive given the administration of G-CSF to neutropenic patients.

9. Concluding remarks

The number of studies describing the role of neutrophils as pro-metastatic in breast cancer is increasing. In fact, it has proven difficult to find recent information that implicates them as having an anti-tumour role. This is especially evident in the clear association of worse prognosis for patients with a high NLR, with ramifications for survival, recurrence and perhaps even metastasis [17, 19, 23]. The field is now widening to recognize the fact that neutrophils are also suppressive to the innate arm of the immune system, such as NK cells (Fig. 1 (2,3)) [77]. G-CSF is also beginning to gain attention as a pro-metastatic factor, a long way from its earlier characterization as the growth factor stimulating neutrophil release from bone marrow. This is in part due to the common use of the 4T1 mammary tumour model, that produces extremely high levels of the cytokine. The emergence of other models of metastatic breast cancer may prove more reliable for predicting G-CSF activity in patients, with levels of G-CSF possibly being more physiologically relevant.

The relationship between G-CSF, neutrophils and breast cancer metastasis is well substantiated in murine tumour models. We now foresee the next steps in any clinical application of this relationship as being two-fold. First, better characterization of treatments to reduce neutrophil number and function through targeting G-CSF in patients will likely benefit the rapidly accelerating field of immunotherapy, wherein response rates for solid cancers such as breast are as low as 18.5% [86]. The mechanisms underlying these failures to respond are not well understood, although neutrophils may be partially responsible for hindering their success through immunosuppression of T-cells (Fig. 1 (2)) as discussed throughout this review. Indeed, granulocytic-MDSC (ie. neutrophil) depletion via anti-Ly6G, HDAC or PI3K inhibitors enhances anti-PD-1 and anti-CTLA-4 combination treatment in the 4T1 model, in terms of eradicating primary tumours and metastases [87]. Checkpoint inhibitors alone are ineffective at evoking durable anti-tumour responses in this

poorly immunogenic model. However, to our knowledge, the combination of neutrophil depletion and checkpoint inhibition has not yet been trialled in breast cancer patients who fail to respond to immunotherapy.

Second, we strongly recommend that G-CSF mediated support of neutrophils during chemotherapy in breast cancer patients be monitored carefully and investigated for long-term outcome, given the evidence that we have presented herein on the major role for G-CSF in promoting metastasis.

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Figure Legend 1: Recently proposed mechanisms by which G-CSF facilitates metastasis via tumour-promoting neutrophils (adapted from Swierczak et al. [1]). Potential sources of G-CSF in mouse models are numerous and include tumour cells, macrophages, stromal cells such as fibroblasts and endothelial cells (**1**). In some systems, the source of G-CSF remains unclear, for example, the IL-1β/IL-17/G-CSF inflammatory cascade. A high neutrophil to lymphocyte ratio (NLR) is associated with worse prognosis in breast cancer patients due to suppression of anti-tumour T-cell responses (**2**). Neutrophils have also been recognized recently as driving suppression of NK cell responses by inhibiting clearance of extravasated tumour cells in the lungs of 4T1 tumour-bearing mice (**3**). G-CSF predisposes neutrophils to release neutrophil extracellular traps (NETs), containing

myeloperoxidase (MPO), DNA and Histones. NETs aid metastasis by trapping tumour cells in vessels, thereby enabling their outgrowth into overt metastases (**4**). Recent classification of neutrophil subtypes based on density, morphology and extracellular markers in patients (**5**). Possible antibody-based therapies to interrupt G-CSF signaling and reduce neutrophil number are indicated by crosses. The relationship between macrophages and neutrophils in cancer remains unclear, as targeting of the CSF-1R drives increases in G-CSF in mouse tumour models (**6**). Cell types are indicated in the figure key; dashed lines indicate our hypotheses and blunt arrowheads indicate inhibition.