Neutrophil diversity and plasticity in tumour progression and therapy

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Abstract | Neutrophils play a key role in defence against infection and in the activation and regulation of innate and adaptive immunity. In cancer, tumour-associated neutrophils (TANs) have emerged as an important component of the tumour microenvironment. Here, they can exert dual functions. TANs can be part of tumour-promoting inflammation by driving angiogenesis, extracellular matrix remodelling, metastasis and immunosuppression. Conversely, neutrophils can also mediate antitumour responses by direct killing of tumour cells and by participating in cellular networks that mediate antitumour resistance. Neutrophil diversity and plasticity underlie the dual potential of TANs in the tumour microenvironment. Myeloid checkpoints as well as the tumour and tissue contexture shape neutrophil function in response to conventional therapies and immunotherapy. We surmise that neutrophils can provide tools to tailor current immunotherapy strategies and pave the way to myeloid cell-centred therapeutic strategies, which would be complementary to current approaches.

Neutrophils have long been known to serve as an essential line of resistance against infectious agents in innate immunity and downstream of polarized T helper 17 (T_{H17}) cell-driven adaptive immune responses1–3. Moreover, not only do neutrophils represent a hallmark of acute inflammation but they are also an important component of circuits that orchestrate the activation, orientation and regulation of adaptive immune responses and chronic inflammation. By expressing a wide repertoire of cytokines, and immunosuppressive and stimulatory molecules, neutrophils engage in complex bidirectional interactions with lymphoid cells and macrophages4,5.

The tumour microenvironment (TME) has emerged as an essential component of neoplasia6. Infiltrating tumour cells and components of the humoral arm of innate immunity are key players in cancer-related inflammation, and contribute to tumour progression, from initiation to seeding at distant anatomical sites6–9.

Although attention has long been focused on macrophages as a paradigm of cancer-related inflammation10, several lines of evidence in preclinical and clinical conditions point to a role for neutrophils11,12. Neutrophils infiltrate solid tumours to a variable extent, as assessed by conventional immunohistochemical staining for neutrophil markers (for example, CD66b in humans and lymphocyte antigen 6G (Ly6G) in mice) and expression of a neutrophil transcriptional signature13–17. In most, but not all, human tumours, high infiltration with tumour-associated neutrophils (TANs) has been associated with poor prognosis12. Accordingly, in tumour cell line transplantation models and genetically engineered mouse models (GEMMs) of cancer, TANs have been reported to be a component of tumour-promoting inflammation1,12,18. However, neutrophils can engage in pathways of antitumour resistance by killing tumour cells directly and/or by interacting with other components of immunity11,12,19. Thus, neutrophils have the potential to be both pro-tumorigenic and antitumorigenic within the TME, and this dual function is likely a reflection of their unexpected plasticity in response to environmental cues.

Here, we review current evidence for the role of neutrophils in tumour progression and metastasis in light of their diversity and plasticity. Previous reviews on the immunobiology of neutrophils and TANs11,12,18,19 provide a framework for the present Review, in which emphasis is placed on describing neutrophil diversity and their prognostic and therapeutic implications.

Homeostasis and recruitment

Development and mobilization. Neutrophils represent 50–70% and 10–25% of circulating leukocytes in humans and mice, respectively11,21. In peripheral blood, neutrophils are short-lived cells and require a constant replenishment from bone marrow precursors dependent on signalling through the granulocyte colony-stimulating factor receptor (G-CSFR)12,22. Indeed, mutations in colony-stimulating factor 3 receptor (CSF3R), which encodes G-CSFR, and in haematopoietic cell-specific
Lyn substrate 1-associated protein X1 (HAX1), whose protein product contributes to the G-CSFR signalling pathway, have been associated with severe neutropenia in humans\(^{20,24}\). Similarly, deficiency in granulocyte colony-stimulating factor (G-CSF) or G-CSFR leads to severe neutropenia in mice\(^{20,24}\). In addition to the essential role played by G-CSF, other mediators, such as granulocyte–macrophage colony-stimulating factor (GM-CSF) and the pro-inflammatory cytokine interleukin-6 (IL-6), are involved in the regulation of the development of neutrophils, in particular during an inflammatory response\(^{22,27,28}\).

The trafficking of neutrophils from bone marrow into peripheral blood is tightly regulated, in particular by signalling through the chemokine receptors CXC-chemokine receptor 2 (CXCR2) and CXCR4. Expression of CXC-chemokine ligand 12 (CXCL12) by bone marrow stromal cells mediates the retention of CXCR4\(^+\) immature neutrophils (NI); as an operational nomenclature based on the expression of selected molecules, we use \(N_0\), \(N_{AP}\), \(N_2\) and \(N_{BC}\) to refer to immature neutrophils, mature neutrophils, aged neutrophils and neutrophils with an interferon-stimulated gene signature, respectively (see below) [Box 2]. Decreased expression of CXCR4 in bone marrow-localized \(N_2\) coupled with activation of CXCR2 signalling triggers the entry of \(N_2\) into the circulation\(^{29}\). Ageing neutrophils upregulate the expression of CXCR4, driving their homing back to the bone marrow and their subsequent elimination by macrophages\(^{29,30}\). The cellular composition and molecular signature of the haematopoietic niche, including the number of CXCL12-abundant reticular mesenchymal stem cells (CAR MSCs), is regulated by the rhythmic clearance of neutrophils by macrophages\(^{30}\).

The process of neutrophil ageing in the circulation is regulated by gut microbiota and is controlled by neutrophils themselves through a cell-autonomous transcriptional programme\(^{30,32}\). Whether this applies to other myeloid cells, such as monocytes, remains unknown. Indeed, circadian expression of the transcription factor BMAL1 controls the production of CXCL2 by neutrophils in a cell-intrinsic manner. In turn, CXCL2 signals through CXCR2 to induce neutrophil ageing\(^{31}\). Elimination of apoptotic neutrophils and formation of

**Box 1 | Neutrophil differentiation**

The differentiation of neutrophils occurs in the bone marrow through a stepwise maturation process (see the figure). At steady state, \(1 \times 10^{11}–2 \times 10^{10}\) neutrophils are generated per day in humans and \(1 \times 10^{7}\) neutrophils in mice\(^{32,45}\). Haematopoietic stem cells (HSCs) differentiate into common myeloid progenitors (CMPs), which give rise to the granulocyte–monocyte progenitors (GMPs). The differentiation of neutrophils and monocytes is orchestrated by the expression of specific transcription factors with CCAAT/enhancer binding protein-ε (C/EBP\(\varepsilon\)) and growth factor independent 1 (GF1) driving neutrophil production and C/EBPα and PU.1 driving monocyte production\(^{12,26}\).

Differentiation of GMPs into neutrophils is regulated by mediators expressed by bone marrow stromal cells (that is, granulocyte colony-stimulating factor (G-CSF) and granulocyte–macrophage colony-stimulating factor (GM-CSF)) and begins with the formation of myeloblasts. The myeloblasts then differentiate into promyelocytes, which subsequently give rise to myelocytes, metamyelocytes, band neutrophils and, finally, mature neutrophils\(^\alpha\). Historically, this classification was based on histological staining and electron microscopy analysis\(^\beta\). Recent studies using single-cell RNA sequencing (scRNA-seq) and mass cytometry by time-of-flight (CyTOF) have further characterized neutrophil development and identified multiple discrete steps along their differentiation process\(^{13,49,247,248}\). In particular, two subsets of proliferative neutrophil-committed bone marrow-residing cells, named early neutrophil progenitors (NePs) and pre-neutrophils (preNeus), have been identified\(^{14,249}\). NePs might represent a precursor of preNeus. Importantly, human NePs are unipotent and produce only neutrophils after adoptive transfer into immunodeficient mice\(^{249}\). In cancer, distinct subsets of neutrophils composed of both immature and mature cells and potentially endowed with immunosuppressive and pro-tumour activities appear in the circulation and tumour sites [Box 2]. CXCR, CXC-chemokine receptor; FcεR, Fcε receptor I; IL-7R, interleukin-7 receptor; Ly6, lymphocyte antigen 6.
Neutrophilia
An increase in the absolute number of peripheral blood neutrophils compared with the control value.

**Recruitment in cancer.** In established neoplasia in mice and humans, altered haematopoiesis is usually observed, a reflection of the production of growth factors (G-CSF and GM-CSF) and inflammatory cytokines (for example, IL-6, IL-1β and IL-17) by tumour new cells are interconnected processes functioning as a homeostatic rheostat essential to prevent exacerbated inflammation and tissue damage.\(^{12-14}\).

**Box 2** | Nomenclature for neutrophil diversity in cancer: a guiding map

| Circulating neutrophils | TANs |
|-------------------------|------|
| Immature neutrophil (N\(_I\)) | IFN\(_{\beta}\) |
| Mature neutrophil (N\(_M\)) | GM-CSF |
| Aged neutrophil (N\(_A\)) | IFN\(_{\gamma}\) |
| Neutrophil with interferon-stimulated gene signature (N\(_{ISG}\)) | |

**Human**

- CD66\(_{b}\), CD11b\(_{b}\), CD101\(_{exd}\), CD10\(_{exd}\), CD16\(_{exd}\), CD62L\(_{high}\), CD117\(_{exd}\), CD49d\(_{exd}\), CD79a\(_{exd}\), CXCR4\(_{exd}\), CXCR2\(_{exd}\)

**Mouse**

- Ly6G\(_{high}\), CD11b\(_{b}\), CD101\(_{exd}\), CD62L\(_{high}\), CXCR4\(_{exd}\), CXCR2\(_{exd}\)

| Human | Mouse |
|-------|-------|
| CD66\(_{b}\), CD11b\(_{b}\), CD101\(_{exd}\), CD10\(_{exd}\), CD16\(_{exd}\), CD62L\(_{high}\), CD117\(_{exd}\), CD49d\(_{exd}\), CD79a\(_{exd}\), CXCR4\(_{exd}\), CXCR2\(_{exd}\) | Ly6G\(_{high}\), CD11b\(_{b}\), CD101\(_{exd}\), CD62L\(_{high}\), CXCR4\(_{exd}\), CXCR2\(_{exd}\) |

There is no consensus nomenclature for the emerging complexity of neutrophil differentiation and activation states, including tumour-associated neutrophils (TANs) and granulocytic myeloid-derived suppressor cells (G-MDSCs) in cancer. Reflecting the M1 and M2 dichotomy of tumour-associated macrophages (TAMs), the N1 and N2 nomenclature has been introduced to define neutrophils with antitumour and pro-tumour functions, respectively.\(^1\)\(^\text{-}^\text{4,6}\). Yet the definition of N1 and N2 does not parallel other more widely used type 1 and type 2 dichotomies of polarized immune responses (for example, T helper 1 (tH1) cell and tH2 cell; type 1 and type 2 immunity; m1 and m2 dichotomies of polarized immune responses (for example, t helper 1 (tH1) cell and tH2 cell; type 1 and type 2 immunity; m1 and m2 suppressor cells (G- mDSCs) in cancer. mirroring the m1 and m2 subsets but an unequivocal strategy to detect immunosuppressive neutrophils was also observed in the expected high-density neutrophil (HDN) fraction, highlighting the need for a more robust system to define the neutrophil immunosuppressive subsets.

Lastly, select molecules proposed to identify neutrophil subsets in cancer have also been reported and these include CD101 (REF.\(^{15}\)) and CD177 (REF.\(^{16}\)), which have been associated with tumour regression, and CD117 (REF.\(^{5,10,17}\), programmed cell death 1 ligand 1 (PDL1)\(^{18,19}\), CD170 (REF.\(^{20}\)), lectin-like oxidized LDL receptor 1 (LOX1)\(^{21,22}\), CD84 and junctional adhesion molecule-like (JAML)\(^{23}\), which have been associated with T cell immunosuppression and disease progression. CRC, colorectal cancer; CXCL2, CXC-chemokine ligand 2; CXCR, CXC-chemokine receptor; GM-CSF, granulocyte–macrophage colony-stimulating factor; HLA-DR, human leucocyte antigen-DR; IFIT1, interferon-induced protein with tetratricopeptide repeats 1; IFR7, interferon-regulatory factor 7; RSAD2, radical S-adenosyl methionine domain-containing protein 2; TGF\(_{\beta}\), transforming growth factor-\(\beta\). The expression of CD11b and CD66b on side-scatter (SSC\(_{high}\)CD45\(_{low}\)) indicates inflammatory neutrophils (NI), a guiding map for classifying neutrophil diversity found in tumours. As mDSCs are typically characterized based on function, we propose that G-MDSCs should refer to a neutrophil population with proven immunosuppressive activity.

Attempts have been made to determine specific markers of neutrophil subsets but an unequivocal strategy to detect immunosuppressive neutrophils and other neutrophil subsets by flow cytometry remains to be developed.\(^{16}\). In addition to molecular markers, neutrophils can also be identified by purifying them on a density gradient. This approach revealed a subset of circulating low-density neutrophils (lDNs) consisting of immature and mature neutrophils\(^{66}\). lDNs accumulate in cancer and are generally endowed with an immunosuppressive capacity.\(^{66,114,250}\). However, immunosuppressive neutrophils were also observed in the expected high-density neutrophil (HDN) fraction, highlighting the need for a more robust system to define the neutrophil immunosuppressive subsets.
their recruitment to the TME where CXC chemokine ligands (for example, CXCL1, CXCL2, CXCL5, CXCL6 and CXCL8 (also known as IL-8)) are expressed by tumour cells, tumour-infiltrating leukocytes, endothelial cells and fibroblasts\(^{[39,40]}\). In addition to chemokines, inflammatory cytokines (for example, IL-17, IL-1β and tumour necrosis factor (TNF)) have been implicated in neutrophil mobilization and recruitment in cancer. In particular, these cytokines are part of an inflammatory circuit that leads to the production of G-CSF and the subsequent formation and
Neutrophils have also been reported to accumulate in the metastatic niche, where the expression of G-CSF, CXCL1 and CXCL2 by cancer cells and stromal cells promoted their recruitment\(^{1,5,6,32}\) (Fig. 1b). In an orthotopic transplantation model of breast cancer and a GEMM of oncogene-driven mammary carcinogenesis, the mobilization of neutrophils into the metastatic lung was regulated by the atypical chemokine receptor 2 (ACKR2), a decoy and scavenger receptor for inflammatory CC chemokines, expressed in early haematopoietic progenitors\(^{48}\). Genetic deficiency of this molecule in mice increased the expression of inflammatory CC-chemokine receptor 1 (CCR1), CCR2 and CCR5 in haematopoietic progenitors, which accelerated the maturation rate, mobilization and activation of neutrophils, and thus restrained metastasis\(^{48}\). In patients with breast cancer, expression of ACKR2 was found to be inversely correlated with the stage of the disease\(^{41}\). However, ACKR2 was also expressed by non-tumoural and tumoural mammary epithelial cells and the relative importance of haematopoietic versus tumour cell expression in this neoplasm remains to be assessed.

Thus, results obtained in preclinical mouse models and in humans suggest that neutrophil recruitment to and survival in neoplastic tissues involves upstream regulation of myelopoiesis and a complex network of chemokines, cytokines, G-CSF and complement components.

**Neutrophil diversity**

*Neutrophil diversity in health.* Under homeostatic conditions, circulating and tissue neutrophils exhibit considerable diversity, with phenotypic and functional heterogeneity driven by maturation and ageing as well as cues from the tissue microenvironment\(^{17}\). Circadian oscillations and ageing affect the neutrophil proteome, including the repertoire of chemokine receptors, pattern recognition receptors and molecules involved in adhesion, the inflammasome and vesicular transport as well as the production of neutrophil extracellular traps (NETs) and the capacity to migrate\(^{30–32}\).

In the circulation, N\(_{\text{A}}\) newly released from the bone marrow are characterized by high expression of CD62L and CXCR2 and low expression of CXCR4 (BOX 2). N\(_{\text{A}}\) are released during the night and the early morning, and predominate at zeitgeber time (ZT) 13 (that is, 13 h after initiation of a 12-h light–12-h dark cycle)\(^{30}\). The process of neutrophil ageing is a bona fide circadian process. Over a period of 6–8 h, expression of CD62L is dramatically reduced and N\(_{\text{A}}\) characterized by high expression of CXCR4 and CD11b and a hypersegmented nucleus, predominate at ZT5 (that is, 5 h after lights on)\(^{30–32}\). These phenotypic variations favour neutrophil clearance and suggest that neutrophil-dependent immune and inflammatory responses are not stable over time and may fluctuate during the circadian cycle. In agreement with this hypothesis, N\(_{\text{A}}\) display reduced migration into inflamed tissues, compared with N\(_{\text{M}}\)\(^{31}\).

Divergent results have been reported with respect to the capacity of ageing neutrophils to produce NETs\(^{1,4,44}\). These apparently conflicting findings may reflect different methods used for enriching N\(_{\text{A}}\) (that is, prevention
of neutrophil extravasation by injection of antibodies to block P-selectin and E-selectin or isolation of neutrophils at ZT5 in untreated mice). Furthermore, in steady-state conditions, circulating neutrophils were shown to undergo homeostatic degranulation and to lose their capacity to form NETs before they penetrate tissues, limiting their tissue-damaging potential41. This process is driven by a cell-intrinsic mechanism controlling the circadian expression of CXCL2 induced by BMAL1, as observed for neutrophil ageing (see above)42. Therefore, neutrophil neutralization and ‘disarming’ and neutrophil ageing share molecular mechanisms and are integrated into a circadian programme, which protects the host from an excessive inflammatory response.

In addition to \(N_{\alpha}\) and \(N_{\nu}\), single-cell RNA sequencing (scRNA-seq) analysis of circulating neutrophils has identified \(N_{\nu,\nu}\), which are characterized by the expression of a set of interferon-stimulated genes (reported in a preprint43). This neutrophil subset is present in mice and humans and could represent a population of neutrophils primed to fight infections. Interestingly, a similar population has been observed in tumours44 (see below) (BOX 2).

Within tissues, neutrophils can undertake important homeostatic functions and acquire specific immunomodulatory properties, as occurs in the lymph nodes and spleen24,45. In particular, under homeostatic conditions, neutrophils expressing the major histocompatibility complex (MHC) class II molecule are present in lymph nodes in proximity to T cells, suggestive of a role as an antigen-presenting cell (APC)46. Neutrophils present in the marginal zone of the spleen promote immunoglobulin class switching and production of antibodies by activating B cells through the expression of B cell-activating factor (BAFF), a proliferation-inducing ligand (APRIL) and IL-21 (REF.47).

Thus, in homeostasis, neutrophils exhibit a previously unanticipated heterogeneity and are integrated into regulatory circuits of immunity24,45,46. Among mononuclear phagocytes, those cells originating from embryonic precursors perform mainly homeostatic functions, whereas the main function of macrophages derived from circulating monocytes in postnatal life is to respond to damage and inflammation, with plasticity of these cells being a major driver of diversity48. However, there is no evidence for ontogenetically distinct neutrophils or such strictly defined subsets. It can therefore be surmised that neutrophil diversity is the result of plasticity in response to differentiation and environmental signals.

**Neutrophil diversity in cancer.** Cancer has served as a paradigm for the plasticity and diversity of neutrophils, generated by the neutrophil maturation stage, response to tissue cues and cancer progression (BOX 2). Neutrophil differentiation and maturation trajectories are profoundly altered in tumour-bearing mice49,61. In mice with advanced neoplasia, immature myeloid cells endowed with immunosuppressive properties appear in the circulation, primary tumours and metastases52,62,63 (BOX 2). Similarly, early unipotent neutrophil progenitors (NePs) (BOX 1) were found to accumulate in both the bone marrow and periphery in a transplantation mouse model of melanoma and a comparable cell subset (CD66b+;CD117+;CD34−) was identified in the blood of patients with melanoma50. NePs represent approximately 1% and 0.02% of all circulating CD45+ cells in healthy humans and non-tumour-bearing mice, respectively, and these frequencies increase to 3–9% in patients with melanoma and 0.2% in tumour-bearing mice51. Although these progenitors do not correspond to a subset of \(N_{\alpha}\), they do contribute to the diversity of neutrophils found in patients with tumours and in tumour-bearing mice.

Transcriptomic analysis of neutrophils from the spleen and blood of mice bearing mammary carcinomas and tumour-free mice revealed a profound alteration of the transcriptional programme in neutrophils from tumour-bearing mice leading to an immunosuppressive phenotype, characterized by production of reactive oxygen species (ROS), nitric oxide (NO) and arginase 2 (ARG2) with potential to inhibit T cell proliferation ex vivo52,53,54 (FIG. 1c). In human and mouse lung cancers, scRNA-seq analysis of tumour-infiltrating myeloid cells revealed that TANs formed a continuum of phenotypic states, which can be resolved into five and six cell subsets, respectively, in humans and mice49. Three modules of gene expression within these cell subsets are conserved between humans and mice, including a module expressing canonical neutrophil markers (for example, matrix metalloproteinase 9 (MMP9), S100A8 and S100A9), a module expressing molecules involved in tumour inflammation and growth (for example, CC-chemokine ligand 3 (CCL3) and macrophage colony-stimulating factor 1 (CSF1)) and a module with a limited number of cells displaying strong expression of type I interferon-response genes (for example, interferon-regulatory factor 7 (IRF7) and interferon-induced protein with tetratricopeptide repeats 1 (IFIT1))55.

Analysis of TANs has revealed how signals present in the TME shape their function. In a GEMM of lung adenocarcinoma induced by oncogenic Kras and transplantation models of lung cancer and mesothelioma, transforming growth factor-β (TGFβ) was found to polarize neutrophil function in a pro-tumour direction characterized by TANs with high expression of ARG1, CCL17 and CXCL14 and low expression of CXCL10, CXCL13, CCL6, TNF and intercellular adhesion molecule 1 (ICAM1)56,57. Mirroring the M1–M2 nomenclature used for polarized macrophages58,59, N1 and N2 have been used to refer to antitumour and pro-tumour neutrophils, respectively56,60–62 (BOX 2). However, in 3-MCA-induced primary sarcomas, TANs presented as a hybrid phenotype between N1 and N2 (REF.55).

In contrast to TGFβ, interferon-β (IFNβ) or a combination of IFNγ and GM-CSF drives neutrophils towards an antitumour state63–65 (FIG. 2). In early, but not late, non-small-cell lung cancer (NSCLC), IFNγ and GM-CSF have been shown to drive the differentiation of APC-like MHCII+ neutrophils expressing the co-stimulatory molecules OX40 ligand (OX40L), CD86 and 4-1BB ligand (4-1BB; also known as TNFSF9)66,67 (FIG. 2a). A similar human leukocyte antigen-DR (HLA-DR)+ neutrophil population was observed in human head and neck cancer, spatially associated with activated T cells68.

In general, the evidence indicates that, early in carcinogenesis, TANs are part of cellular networks mediating...
Antitumour neutrophils (N1) and N2 neutrophils

N1 neutrophils are characterized by a normal density, a hypersegmented nucleus and a cytotoxic activity towards cancer cells, whereas N2 neutrophils have immunosuppressive activity. This classification may represent an oversimplification of neutrophil polarization, activation or maturation states.

Within the TME, TANs have been shown to affect epithelial genetic instability, tumour cell proliferation, angiogenesis, tissue remodelling and suppression of innate and adaptive lymphoid cell-mediated immunity (immunosuppression is discussed in detail in the next section). Production of a high quantity of ROS is a fundamental property of neutrophils. In cancer, neutrophil-derived ROS have been associated with DNA damage and genetic instability in epithelial cells. However, ROS-independent mechanisms for inducing DNA damage also exist and include neutrophil-derived microparticles, which deliver specific pro-inflammatory microRNAs (that is, miR-23A and miR-155) into intestinal epithelial cells, promoting the accumulation of DNA double-strand breaks via downregulation of the nuclear
Box 3 | Strategies to deplete neutrophils

Two monoclonal antibodies (mAbs), RB6-8C5 and 1A8, have been extensively used to deplete neutrophils in mice. The mAb RB6-8C5, characterized by its interaction with the granulocyte-differentiation antigen (Gr1), can interact with both the lymphocyte antigen 6C (Ly6G) and Ly6C molecules, promoting the depletion of neutrophils and other Ly6C-expressing cells, including monocytes.25,26 By contrast, the Ly6G-specific mAb 1A8 drives exclusive depletion of neutrophils25,27,28. It is important to note that monitoring the efficiency of depletion using the same mAb is not reliable as target antigens are ‘masked’ by the depleting antibody. Thus, depletion efficacy should be assessed by using alternative antibodies (for example, Ly6B). Several studies (including one described in a preprint)29,30 have reported that sustained administration of the Ly6G-specific antibody leads to effective depletion in naïve FVB/N and BALB/c mice but not in C57BL/6J mice.31,32,33 The mechanisms responsible for this difference have not been identified and may depend on differences in haematopoiesis or antibody-dependent neutrophil phagocytosis by macrophages.34,35 Data suggest that the combined use of 1A8 and a secondary rat antibody in C57BL/6J mice results in increased efficacy and duration of neutrophil depletion in vivo.36,37

Genetic strategies represent a valuable tool to overcome the limitations of mAb-induced neutrophil depletion. Models of genetic neutropenia leverage the importance of molecules involved in neutrophil development (for example, colony-stimulating factor 3 (CSF3)38, CSF3 receptor (CSF3R)39,40 and growth factor independent 1 (GFI1)41,42). However, these models also have limitations as the genetic deficiencies can affect the development of other cell types (that is, monocytes). Conditional gene deficiency can represent a more sophisticated tool to achieve specific and durable depletion of neutrophils. For instance, ablation of the anti-apoptotic protein MCL1 in the myeloid compartment (LysM-Cre mice) causes neutrophil deficiency43. Nevertheless, it is important to note that almost complete depletion of circulating and tissue neutrophils (98%–99%) using LysM-Cre;Mcl1fl/fl mice requires a high level of Cre-mediated deletion of Mcl1, for which biallelic expression of the Cre-recombinase may be required.44,45 The high specificity of Ly6G expression in neutrophils makes targeting this locus a promising strategy.45 However, results made available in a preprint showed that the combined use of Ly6G–Cre with Cre-inducible diphtheria toxin receptor (iDTR) (to make Ly6Gfl/fl mice) was insufficient to deplete neutrophils because these cells were resistant to diphtheria.46,47 By contrast, S100A8knockout mice display complete neutrophil depletion, suggesting that S100A8 expression during neutrophil development occurs when the progenitors are still sensitive to diphtheria.48,49

A conceptually different strategy to assess neutrophil functions in tumours is achieved by genetic ablation of CXC-chemokine receptor 2 (CXC2) (Cxcr2−/− mice). However, this approach presents significant limitations owing to the expression of CXC2 in other myeloid cells and non-immune cells, such as cancer cells.50,51,52,53

To unequivocally demonstrate the involvement of neutrophils in tumour progression, different approaches for neutrophil depletion should be used and the extent of neutrophil depletion in peripheral blood, distant organs as well as tumours should be assessed.54

Homologous recombination
Genetic recombination in which nucleotides are exchanged between two similar or identical molecules of DNA.

Neutrophil elastase (NE)
A serine protease stored in the neutrophil intracellular granules.

Circulating tumour cells (CTCs): Cancer cells that have detached from a primary tumour and are found in the bloodstream.

Transactivation of the EGF receptor (EGFR) and Toll-like receptor 4 (TLR4), and human prostate cancer cells through activation of the MAPK signalling pathway.

Neutrophils play an important role in promoting tumour angiogenesis through the production of pro-angiogenic factors, including BV8, MMP9 and vascular endothelial growth factor A (VEGFA)51,83,85–87. In the extracellular matrix, TAM-derived MMP9 induced the liberation and activation of VEGFA and consequent angiogenesis, whereas BV8 induced myeloid cell mobilization and acted as a mitogen for endothelial cells.52,53 Neutrophil-derived BV8 has been implicated in resistance to anti-VEGFA therapy and inhibition of G-CSF or IL-17 increased the therapeutic efficacy of anti-VEGF54–56. Collectively, these studies support a role for neutrophils in the initial angiogenic switch during tumorigenesis.57,58,59,60

NETs have been observed in different tumour types (that is, liver, breast, intestinal and gastric cancers) and their production has been shown to be driven by hypoxia, complement or fatty acids.61,62,63 NET-associated molecules such as high mobility group protein B1 (HMGB1), NE and MMP9 can induce the proliferation of cancer cells.64,65 Indeed, the proteolytic remodelling of the extracellular matrix component laminin 111 by NE and MMP9, contained within NETs, induced the generation of a new epitope that triggered the proliferation of dormant cancer cells through α3β1 integrin activation.66,67 In addition, entrapment of circulating tumour cells (CTCs) within NETs promoted the formation of metastases, and in vivo administration of DNase to eliminate NETs could reduce this effect.68,69,70,71,72 In mouse models of liver metastases induced by intrasplenic injection of lung and colon cancer cells, intravital microscopy revealed decreased adhesion of CTCs to liver sinusoids in mice treated with DNase. In vitro studies indicated that HMGB1 within NETs induced a TLR9-dependent activation of cancer cells, promoting their proliferation, migration and invasion capacity.73,74,75,76,77 In addition, myeloperoxidase (MPO) contained in NETs promoted a hydrogen peroxide (H2O2)-induced TLR4-dependent pro-angiogenic response in endothelial cells, characterized by proliferation and motility.78,79,80 Therefore, NETs can participate in tumour-promoting inflammation by driving angiogenesis, extracellular matrix remodelling and proliferation of tumour cells.

The tumour-promoting function of neutrophils occurs throughout the multistep process of dissemination and seeding at distant anatomical sites. Neutrophils have been reported to prepare the metastatic niche in organs as diverse as the lung and the liver.81,82,83,84,85,86,87 Moreover, neutrophils have been reported to engage with CTCs in the bloodstream and to favour extravasation and subsequent metastatic growth.88,89,90,91,92,93,94,95,96,97,98,99,100,101 In GEMMs of cancer, including mammary tumours induced in keratin 14 (K14)-Cre;E-cadherin (Cdht1) a/a;Trp53+/− (KEP) mice15,16 and mouse mammary tumour virus (MMTV)-polyoma middle T antigen
(PyMT) mice\textsuperscript{36,56}, and colorectal cancer (CRC) induced in villinCre\textsuperscript{villin};Kras\textsuperscript{G12D/+};Trp53\textsuperscript{fl/fl};Rosa26\textsuperscript{CreER} (KPN) mice\textsuperscript{35}, have provided insights into molecular mechanisms underpinning neutrophil-mediated promotion of metastasis. In KEP mice, systemic accumulation of neutrophils with immunosuppressive activity was associated with increased formation of spontaneous lung metastases\textsuperscript{35}. Mechanistically, the loss of p53 in cancer cells promoted the secretion of WNT ligands that stimulated the production of IL-1β by macrophages\textsuperscript{36}. In turn, IL-1β activated the production of IL-17 by γδ T cells that drives neutrophil accumulation in the circulation and in the lung and promoted formation of metastases\textsuperscript{46}. As IL-17 lies upstream of G-CSF in the signalling pathway, it increases the formation of neutrophils and their polarization into cells with immunosuppressive activity\textsuperscript{11,35,36}. Indeed, G-CSF can promote the generation of immunosuppressive neutrophils and in vivo neutralization of G-CSF in KEP mice reversed the immunosuppressive phenotype of neutrophils\textsuperscript{35,61}. Therefore, IL-17 derived from T\textsubscript{h}17 cells or γδ T cells participates in the neutrophilia observed in tumour-bearing individuals and drives the neutrophil-derived pro-tumour activities\textsuperscript{11,35}. In the pre-metastatic niche of the lung, neutrophils produced factors facilitating the extravasation and growth of metastasis-initiating cells, including the pro-angiogenic molecules BV8 and MMP9 (observed in MMTV-PyMT mice)\textsuperscript{37}, the chemoattractants S100A8 and S100A9 (observed in KEP mice)\textsuperscript{38}, the proteases NE and cathepsin G that mediate the cleavage of thrombospondin 1 (TSP1; the resultant outcome of which is degradation of the extracellular matrix and abrogation of the TSP1-mediated suppression of tumour cell growth)\textsuperscript{39,40} (observed in MMTV-PyMT mice)\textsuperscript{40} and the pro-inflammatory cytokine IL-1β and the neutrophil chemoattractant leukotriene B\textsubscript{4} (LTB\textsubscript{4}) (observed in MMTV-PyMT mice)\textsuperscript{40} ([Fig. 1c]).

**Immunosuppression**

N\textsubscript{a} and N\textsubscript{b} can express a host of mediators capable of suppressing innate and adaptive lymphoid cell function. These include ROS, reactive nitrogen intermediates (RNI), ARG1, prostaglandins and ligands of immune checkpoints.

Neutrophil-derived ROS have long been associated with suppression of T cell activation in cancer ([Fig. 1c]), in particular in advanced tumours\textsuperscript{11,17,18,26,50}. In a transplantation mouse model of breast cancer, glucose deprivation in the TME triggered a metabolic switch in neutrophils that resulted in enhanced mitochondrial fatty acid oxidation, increased ROS production and consequent T cell suppression\textsuperscript{107}. In addition to ROS, neutrophils can inhibit T cell activation through the inducible NO synthase (iNOS)-dependent production of NO, as observed in neutrophils from tumour-bearing KEP mice\textsuperscript{5,58} ([Fig. 1c]).

The production of ARG1 by TANs reduced the availability of L-arginine in the TME, which controls T cell metabolism and promotes T cell survival. In turn, this resulted in T cell dysfunction and alteration of T cell-mediated antitumour immunity\textsuperscript{63,64}. The expression of ARG1 by TANs can be driven by TGFβ\textsuperscript{3,64,66} ([Fig. 1c]). Importantly, production of ARG1 by neutrophils has been shown to hamper the T cell response in human cancer, including in renal cell carcinoma and advanced-stage NSCLC\textsuperscript{106,110}.

Endoplasmic reticulum (ER) stress has been associated with altered lipid metabolism, pathological activation and immunosuppressive activity of myeloid cells in cancer\textsuperscript{111–113}, including neutrophils from patients with NSCLC and head and neck cancer\textsuperscript{114–116}. In patients with NSCLC, a GEMM of pancreatic cancer induced by oncogenic Kras coupled with a Trp53 mutation and a transplantable mouse model of lymphoma, immunosuppressive neutrophils were characterized by their low density and increased expression of genes associated with the ER stress response (for example, C/EBP-homologous protein (CHOP; also known as DDIT3), X-box-binding protein 1 (XBP1), binding-immunoglobulin protein (BIP; also known as HSPA5) and AMP-dependent transcription factor (ATF4))\textsuperscript{116}. Induction of ER stress in neutrophils upregulated the expression of lectin-like oxidized LDL receptor 1 (LOX1), a scavenger receptor involved in lipid metabolism, together with the onset of potent immunosuppressive activity\textsuperscript{114}. In patients with NSCLC and head and neck cancer, LOX1+ neutrophils showed higher expression of ROS and ARG1 compared with LOX1− neutrophils and defined the neutrophil population as having immunosuppressive activity\textsuperscript{114}.

In addition to LOX1, immunosuppressive neutrophils present in tumour-bearing mice and patients with head and neck, breast and lung tumours exhibited an upregulation of other proteins involved in trafficking of lipids, such as CD36 and fatty acid transport protein 2 (FATP2)\textsuperscript{115}. Although the role of LOX1 in the immunosuppressive activity of neutrophils remains to be defined, increased uptake of arachidonic acid by FATP2-expressing neutrophils drives the biosynthesis of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) and subsequent immunosuppression\textsuperscript{115}. Therefore, administration of a FATP2 inhibitor in tumour-bearing mice reduced both the immunosuppressive activity of neutrophils and tumour growth\textsuperscript{115}. These results may pave the way for new strategies targeting neutrophil lipid metabolism.

Neutrophils can express ligands that activate immune checkpoints on T cells ([Fig. 5a]). Programmed cell death 1 ligand 1 (PD-L1) was shown to be induced by the hypoxia-inducible factor 1α (HIF1α) pathway in the mouse and by inflammatory cytokines (for example, IL-6, IFNγ and GM-CSF) in humans\textsuperscript{117–120}. PD-L1-expressing neutrophils have been identified in human hepatocellular carcinoma and gastric carcinoma and shown to have prognostic significance\textsuperscript{120,121}. Therefore, neutrophils are part of the myeloid and stromal cell network expressing PD-L1 that drives immune checkpoint engagement and T cell exhaustion. However, further studies are needed to evaluate the expression and function of PD-L1 in neutrophils in different cancer types.

In addition to PD-L1, V-domain immunoglobulin suppressor of T-cell activation (VISTA) is expressed on tumour-associated myeloid cells, including monocytes, macrophages, dendritic cells and neutrophils\textsuperscript{122,123}. In a transplantation mouse model of melanoma, blockade...
Myeloid checkpoints and ligands of lymphocyte checkpoints

- Neutrophils express a set of myeloid checkpoints, including signal regulatory protein-α (SIRPα), CD200 receptor (CD200R), leukocyte immunoglobulin-like receptor B2 (LILRB2), paired immunoglobulin-like type 2 receptor-α (PILRα), and Fcγ receptor-α (FcγRα).

Innate lymphoid cells (ILCs): A group of cells of the innate immune response that belong to the lymphoid lineage and are characterized by a lack of antigen-specific receptors.

Fig. 3 | Therapeutic targeting of neutrophils. a | Neutrophils express a set of myeloid checkpoints, including signal regulatory protein-α (SIRPα), CD200 receptor (CD200R), leukocyte immunoglobulin-like receptor B2 (LILRB2), paired immunoglobulin-like type 2 receptor-α (PILRα) and, expressed on neutrophil precursors, programmed cell death 1 (PD1) and atypical chemokine receptor 2 (ACKR2). The significance of targeting SIRPα, LILRB2 and ACKR2 on neutrophils has been established in preclinical models (see main text). However, the potential antitumour effects of blocking CD200R and PILRα and PD1 (Ref. 26) have not been similarly demonstrated. Neutrophils also express a set of ligands for lymphocyte checkpoints (that is, V-domain immunoglobulin suppressor of T cell activation (VISTA), PD1 ligand 1 (PDL1), CD86, 4-1BB ligand (4-1BBL) and OX40 ligand (OX40L)), representing potential targets to limit the process of neutrophil-mediated immunosuppression in cancer. The interaction with their cognate receptors expressed by T cells (P-selectin glycoprotein ligand 1 (PSGL1), PD1, cytotoxic T lymphocyte-associated antigen 4 (CTLA4), 4-1BB and OX40, respectively), delivers a positive (+) or negative (−) signal to T cells, dependent on the specific receptor–ligand pairing.

b | Inhibition of CXC-chemokine receptor 1 (CXCR1) or CXCR2 dampens the recruitment of immunosuppressive neutrophils in cancer. c | Blocking the transforming growth factor-β receptor (TGFβR), interferon-β (IFNβ) signalling or antagonism of angiotensin II type 1 receptor (AGTR1) can increase the cytotoxic activity of neutrophils towards cancer cells. CXCR4 blockade increases the production of interleukin-18 (IL-18) by neutrophils and the activation of natural killer (NK) cells. d | Neutrophils express the immunoglobulin G (IgG) Fc receptors (FcyR) and the IgA Fc receptor (FcαR). The innate immune response of targeting VISTA, and approaches combining VISTA inhibitors and neutrophil depletion or reprogramming should be assessed in preclinical models.

Although the existence of important crosstalk between neutrophils and innate lymphoid cells (ILCs), in particular natural killer (NK) cells, in an inflammatory context is well established, only limited findings have been reported on the bidirectional interaction between these two innate cells in the TME. Neutrophils have been shown to promote metastatic dissemination by preventing NK cell–mediated clearance of tumour cells from initial sites of dissemination. Evidence from in vitro experiments and in vivo transplantation models...
of tumour cell lines has shown that ligands of CXCR1 and CXCR2 produced by tumour cells can induce the formation of NETs in the TME, which in turn coat and protect tumour cells from the cytotoxic activity of NK cells and T cells by impairing their contact with tumour cells127. In humans, G-CSF-mobilized neutrophils inhibited the activation of NK cells128. Conversely, NK cells can control the tumour-promoting and angiogenic function of neutrophils in an IFNγ-dependent manner by inhibiting VEGFA expression129. However, significant antitumour NK cell-mediated activity attributable to enhanced NK cell activation and survival has also been reported following NK cell interactions with neutrophils in haematopoietic stem cell transplantation recipient mice transplanted with a syngeneic colon cancer cell line130. Thus, the interaction of neutrophils with NK cells in the TME can be context-dependent.

Neutrophils in antitumour resistance

The results discussed above and clinical correlative evidence suggest that neutrophils are an important component of tumour-promoting inflammation and immunosuppression in numerous mouse and human tumours. In apparent contrast to these observations, neutrophils have also been shown to mediate antitumour resistance in vitro and in vivo, suggesting a dual potential for these cells.

It has long been known that substantial recruitment and activation of neutrophils can result in antitumour activity131. Neutrophils can kill tumour cells through direct contact and via the generation of ROS132,133 (Fig. 2b). ROS-mediated killing involves the transient receptor potential cation channel, subfamily M, member 2 (TRPM2), an H2O2-dependent channel that induces a receptor potential cation channel, subfamily M, member 2 (TRPM2), an H2O2-dependent channel that induces a lethal influx of calcium (Ca2+) into target cells134 (Fig. 2b). Expression of TRPM2 was upregulated in cancer cells undergoing an epithelial-to-mesenchymal transition (EMT) and this was also associated with an increase in the secretion of CXCL2, suggesting that, in addition to triggering an apoptotic cascade, TRPM2 sustains the recruitment of neutrophils134,135.

The neutrophil killing armamentarium includes expression of NO, TNF-related apoptosis inducing ligand (TRAIL) and TNF136,137. The latter induced the expression of the hepatocyte growth factor receptor (HGFR; also known as MET) on neutrophils138. Studies performed in different tumour cell line transplantation mouse models (for example, Lewis lung carcinoma and fibrosarcoma) showed that HGFR present in the TME induced neutrophil recruitment and production of NO, which resulted in killing of tumour cells139. However, in a transplantation mouse model of melanoma, HGFR–MET signalling in neutrophils led to an immunosuppressive phenotype associated with a limited expansion of anti-tumour T cells and a reduced response to adoptive T cell transfer and immune checkpoint blockade therapies138. Thus, the impact of the expression of MET on neutrophils remains to be fully elucidated in different tumour contexts and therapeutic conditions136,138.

 Accumulation and activation of neutrophils in the metastatic niche can reduce the formation of metastases through the elimination of cancer cells140,141,142. In an orthotopic transplantation mouse model of breast cancer, expression of G-CSF and CCL2 by the primary tumour induced the mobilization and activation of neutrophils in the pre-metastatic lung, and consequent ROS-dependent killing of tumour cells143. Antibody-mediated neutrophil depletion did not affect the growth rate of the primary tumour or the number of CTCs but increased the metastatic burden in the lung, suggesting that neutrophils act in the metastatic niche (see also above)144. This result suggests interplay between primary tumours and neutrophils to activate their antitumour activity and control metastatic progression. In immunodeficient mice, the transplantation of human breast cancer cells with low spontaneous metastatic potential into the mammary fat pad resulted in the reprogramming of neutrophils in the pre-metastatic lung, with high cytotoxic activity associated with expression of transmembrane protein 173 (TMEM173; also known as STING)145. In this case, breast cancer cells with low spontaneous metastatic efficiency showed increased expression of CCL2 compared with breast cancer cells with high metastatic potential. Tumour-derived CCL2 induced the recruitment of IFNγ-producing CCR2+ monocytes. In turn, IFNγ upregulated TMEM173 and enhanced the cytotoxic activity of neutrophils146. These observations highlight the capacity of neutrophils to act as effector cells.

In addition to mediating direct killing, neutrophils engage in networks of T cell-dependent antitumour immunity. TANs have been shown to produce chemokines including CXCL10, CCL2, CCL3, CXCL1 and CXCL2, which recruit T cells as well as other leukocytes132,134. Neutrophils can acquire an APC phenotype, and in early-stage human lung cancer a population of immature CD11b+CD15hiCD10–CD16int/low monocytes. In turn, IFNγ upregulated TMEM173 and enhanced the cytotoxic activity of neutrophils145. In vitro experiments showed that in response to GM-CSF and IFNγ present in the TME, these N1 acquired APC features, characterized by the expression of HLA-DR and CD86 and the capacity to amplify the antitumour T cell response144 (Fig. 2a). In addition, neutrophils isolated from human CRC biopsy specimens amplified the activation of CD8+ T cells in response to T cell receptor (TCR) triggering in vitro144.

The intestinal microbiota play a role in inflammation and colorectal carcinogenesis143,144. Neutrophils were reported to have a tumour-suppressive effect in CRC via the response to IL-1 produced in the TME by monocytes and tumour cells, which enhanced the expression of antimicrobial peptides by neutrophils and their subsequent antibacterial activities144,145 (Fig. 2c). In addition to CRC, lung carcinogenesis in a GEMM induced by Kras mutation coupled with Trp53 loss has also been associated with dysregulation of the airway microbiota, which stimulates IL-17 production by resident γδ T cells resulting in neutrophilia and tumour growth146. Therefore, neutrophils can play a role in the control of microbiota-induced tumour-promoting inflammation144,145.

In 3-MCA-induced primary sarcomagenesis, a tripartite interaction between neutrophils, macrophages and a subset of unconventional T cells (UTCs), known

Unconventional T cells (UTCs). A group of T lymphocytes that express the T cell receptor (TCR) αβ or γδ chains and are characterized by a lack of recognition of classical peptide antigens. UTCs include the mucosal-associated invariant T (MAIT) cells and invariant natural killer T (iNKT) cells.
as CD4–CD8–TCRaβ+ double-negative UTCs (UTCαβ), was found to be essential for the establishment of effective antitumour immunity16 (Fig. 2d). During the early phase of sarcoma development, neutrophils amplified the production of IL-12 by macrophages, which in turn promoted polarization of the UTCαβ towards a type 1 immune response and IFNγ production14 (Fig. 2d). However, further investigation is needed to determine the mechanism(s) by which UTCαβ, as a subset of T cells, can act as antitumour effector cells, as well as their presence, significance and role in human tumours. Interestingly, in silico analyses suggest that this neutrophil-dependent antitumour axis is relevant in select human tumours13.

Thus, neutrophils can exert dual, seemingly opposite, functions in tumour immunity. The disease stage as well as the tumour type and tissue context are key determinants of the specific role of these cells in promoting or restraining cancer. The levels and nature of inflammatory mediators found in the context of different tumour types and at different tumour stages may dictate the phenotype of neutrophils7,66,13. The complexity of the regulatory pathways involved is underlined by the fact that the same growth factor, G-CSF, can drive the differentiation and activation of both antitumour and pro-tumour neutrophils, by stimulating their cytotoxic activity or the acquisition of immunosuppressive activity, depending on the conditions61,131,146. In an attempt to synthesize available data on the dual role of neutrophils in tumours, we surmise that at early stages of tumour development, myeloid cells are set in an antitumour state61,131,146, whereas progression to invasion and metastasis is associated with and driven by the acquisition of a pro-tumour, immunosuppressive phenotype61,66,13.

**Neutrophils in human cancer**

**Occurrence and significance.** As discussed above, increased myelopoiesis is a common feature of advanced neoplasia and neutrophil diversity has been also observed in patients with cancer, including lung cancer, head and neck cancer and melanoma11,12,13,141,146. Using mass cytometry by time-of-flight (CyTOF) on blood samples, a study identified distinct phenotypes of CD66b+ neutrophils at different stages of melanoma progression (reported in a preprint145). Notably, the abundance of the terminally differentiated N5, CD100–, CD1010–, and CD16+, gradually decreased during tumour progression, whereas N5+ characterized by expression of CD66b+, CD117+, CD49d+ and CD79b+, increased146. Nevertheless, it is important to note that the association between neutrophil immaturity and immunosuppressive activity remains a matter of debate. Indeed, immature human CD10 CD66b+ neutrophils have been described to promote T cell activation whereas an opposite effect has been reported for the CD10+ N5αβ124.

In peripheral blood, high neutrophil counts and a high neutrophil-to-lymphocyte ratio (NLR) are associated with bad prognosis for patients with a wide spectrum of solid tumours (for example, CRC, melanoma and breast, prostate and lung cancers)12. The prognostic significance of NLR was validated in a meta-analysis involving 100 studies with 40,559 patients and 22 solid tumours13. However, the relevance of NLR in the clinic remains to be proven13,138. For instance, in patients with metastatic breast cancer, NLR was found to be associated with the stage of the disease, involvement of the central nervous system and the presence of visceral metastases but its prognostic significance was lost in multivariate analysis132.

Neutrophils are present in variable numbers in human solid tumours, as assessed by conventional immunohistochemistry (for example, by staining for CD66b) and neutrophil transcriptional signatures13–17,154. In general, high TAN infiltration is associated with worse prognosis12. For instance, in a large study using the CIBERSORT (cell type identification by estimating relative subsets of known RNA transcripts) method to quantify 22 leukocyte populations in approximately 18,000 patients with 39 different tumour types, a neutrophil signature emerged as the most significant negative prognostic factor12. In early NSCLC, TANs were the most represented leukocyte population and were negatively correlated with T cell infiltration12. Thus, suppression of T cell-mediated immunity is likely one of the mechanisms underlying their adverse clinical significance. In addition, correlative analysis on hepatocellular carcinoma biopsy specimens revealed an association between the occurrence of neutrophils and angiogenesis146.

In apparent contrast to the above results, in select human tumours high levels of TANs as assessed by immunohistochemistry or neutrophil transcriptional signatures were associated with better prognosis. These include CRC13,141,156–158, endometrial cancer132, invasive ductal breast carcinoma133, low-grade glioma132 and undifferentiated pleomorphic sarcoma (UPS)13. In CRC, TANs co-localized with CD8+ T cells and combined infiltration of TANs and CD8+ T cells was associated with a better prognostic value compared with CD8+ T cells alone145. In UPS, but not in other sarcomas, neutrophil signatures were associated with a type I immune response and better clinical outcome146. As UPS is likely to be the human counterpart of 3-MCA-induced primary sarcomagenesis in mice, here neutrophils may engage in antitumour resistance mediated by UTCαβ15 (Fig. 2d). Thus, in select human tumours it would appear that TANs can mediate antitumour resistance by direct killing of tumours cells132,133 or by engaging in cooperative networks with innate and adaptive lymphoid cells132,145. Collectively, current data suggest that the significance of neutrophils and their functions, in the circulation and in the neoplastic setting, may be strongly influenced by the tissue and tumour context.

**In response to chemotherapy, radiotherapy and immunotherapy.** TAN infiltration affects response to different anticancer treatment modalities (TABLE 1). High neutrophil infiltration was generally reported to be associated with a worse response to chemotherapy and radiotherapy (TABLE 1). Notable exceptions were CRC, gastric cancer and high-grade ovarian cancer, where higher levels of TANs were associated with a better response to chemotherapy13,136,156. These discordant observations
| Tumour type | Parameter assessed | Prognostic parameter | Therapy | Predictive value of neutrophils | Refs |
|-------------|--------------------|----------------------|---------|-------------------------------|------|
| Peripheral blood neutrophils | | | | | |
| CRC (stage IV) | NLR | OS | Chemotherapy | – | 204 |
| CRC (stage III) | NLR | OS | Chemotherapy | No correlation | 205 |
| CRC (stage IV) | NLR | OS and PFS | Chemotherapy + bevacizumab | – | 206 |
| Breast cancer (stage II–III) | NLR | DFS | Chemotherapy | – | 207 |
| Breast cancer | NLR | Complete or partial response | Chemotherapy | – | 208 |
| Breast cancer (stage I–III) | NLR | Complete response | Chemotherapy | – | 209 |
| Melanoma (unresectable stage III, IV) | NLR | OS, PFS and clinical response | Immunotherapy (ipilimumab) | – | 210 |
| Melanoma (unresectable stage III, IV) | NLR | OS, PFS and clinical response | BRAF inhibitor | No correlation | 210 |
| Melanoma (stage IV) | NLR | OS | Immunotherapies (nivolumab or ipilimumab) | – | 211, 212 |
| Melanoma (stage IV) | ANC | OS | Chemotherapy ± IL-2 | – | 213 |
| Melanoma (stage III–IV) | NLR | OS | Immunotherapies (ipilimumab or nivolumab) | – | 214–217 |
| Ovarian cancer (stage I–IV) | NLR | OS, DFS | Chemotherapy | – | 218 |
| Cervical cancer | ANC | OS, MFS | Chemoradiotherapy | – | 219 |
| mRCC | ANC | OS, PFS | MVA-5T4 vaccination | – | 220 |
| mRCC | NLR | OS, PFS | Immunotherapy (nivolumab) | – | 221 |
| Oesophageal cancer (stage I–IV) | NLR | OS, DFS | Chemotherapy | – | 222 |
| NSCLC (stage III, IV) | NLR | OS, PFS | Immunotherapy (nivolumab) | – | 223–228 |
| NSCLC (stage III–IV) | ANC | OS, PFS | Immunotherapy (nivolumab) | – | 229 |
| NSCLC (stage IIIb–IV) | Peripheral CD15+ CD33+ cells (FC) | Clinical response | Bevacizumab | – | 230 |
| NSCLC (stage IV) | NLR | OS, DFS | Chemotherapy | – | 231 |
| Cervical cancer, anal cancer, oesophageal cancer, lung cancer, glioma and HNC | ANC | OS | Radiotherapy | – | 232 |
| Meta-analysis (melanoma, NSCLC and mRCC) | NLR | OS, PFS | Immunotherapies (ipilimumab, nivolumab or pembrolizumab) | – | 233–234 |

**TANs**

| Tumour type | Parameter assessed | Prognostic parameter | Therapy | Predictive value of neutrophils | Refs |
|-------------|--------------------|----------------------|---------|-------------------------------|------|
| CRC (stage III) | Density of CD66b+ TANs (IHC) | DFS | Chemotherapy | + | 13 |
| CRC (stage IV) | Density of CD177+ TANs (IHC) | OS | Bevacizumab | – | 235 |
| Gastric cancer (stage I–IV) | Density of CD66b+ TANs (IHC) | OS | Chemotherapy | + | 159 |
| Ovarian cancer (high grade) | Density of CD66b+ TANs (IHC) | PFS | Chemotherapy | + | 160 |
| Biliary cancer (stage I–IV) | Density of CD66b+ TANs (IHC) | OS | Chemotherapy | – | 236 |
| NSCLC | Ratio of CD8+ cells to CD66b+ TANs (IHC) | PFS | Immunotherapy (nivolumab or pembrolizumab) | – | 237 |
| DLBCL | ELANE mRNA expression (in silico) | OS | Immunotherapy (rituximab) + chemotherapy | – | 238 |
| HCC | Density of CD66b+ TANs (IHC) | OS | Sorafenib | – | 239 |
| Cervical cancer (stage IB–IVA) | Density of CD66b+ TANs (IHC) | PFS | Radiotherapy | – | 240 |

–, adverse prognosis; +, favourable prognosis. ANC, absolute neutrophil count; bevacizumab, monoclonal antibody against vascular endothelial growth factor (VEGF); CRC, colorectal cancer; DFS, disease-free survival; DLBCL, diffuse large B cell lymphoma; FC, flow cytometry; HCC, hepatocellular carcinoma; HNC, head and neck cancer; IHC, immunohistochemistry; IL-2, interleukin 2; ipilimumab, a cytotoxic T lymphocyte-associated antigen 4 (CTLA4) antibody; MFS, metastasis-free survival; mRCC, metastatic renal cell carcinoma; nivolumab, a programmed cell death 1 (PD1) antibody; NLR, neutrophil-to-lymphocyte ratio; NSCLC, non-small-cell lung cancer; OS, overall survival; pembrolizumab, a PD1 antibody; PFS, progression-free survival; rituximab, a monoclonal antibody against CD20; sorafenib, a tyrosine kinase inhibitor; TAN, tumour-associated neutrophil. *A novel immunotherapeutic vaccine that uses a modified vaccinia Ankara (MVA) to deliver the tumour-associated antigen ST4.
Neutrophil targeting and reprogramming

A better mechanistic understanding of the complex role of neutrophils in tumour progression will provide a basis to design therapeutic approaches. In particular, dissecting the diversity and plasticity of neutrophils in different cancer types and sites (that is, in the peripheral blood or the TME) represents a significant challenge for specifically targeting these cells and setting them in an antitumour state. As described above, chemokine receptors CXCR2 and CXCR1 expressed by neutrophils are important for their recruitment to the TME, modulation of their activation state and their circadian oscillations\(^\text{32,39,54}\). Based on results in preclinical models, inhibition of neutrophil recruitment by blocking CXCL8, CXCR1 and/or CXCR2 (\text{REFS}\text{39,40,52,168}) has now entered clinical evaluation (FIG. 5b). Recent studies showed that higher levels of CXCL8 in serum and tumours of patients with advanced solid cancers (that is, 1,344 patients with melanoma, NSCLC and renal cell carcinoma\(^\text{169}\), and 1,445 patients with urothelial carcinoma and renal cell carcinoma\(^\text{170}\)) were associated with increased tumour infiltration by neutrophils, shorter survival and decreased clinical response to ICIs (for example, the programmed cell death 1 (PD1) antibody nivolumab\(^\text{171}\), nivolumab plus the cytotoxic T lymphocyte-associated antigen 4 (CTLA4) antibody ipilimumab\(^\text{172}\) or the PD1 antibody atezolizumab\(^\text{173}\)) were associated with improved tumour infiltration by neutrophils, shorter survival and decreased clinical response to ICIs (for example, the programmed cell death 1 (PD1) antibody nivolumab\(^\text{171}\), nivolumab plus the cytotoxic T lymphocyte-associated antigen 4 (CTLA4) antibody ipilimumab\(^\text{172}\) or the PD1 antibody atezolizumab\(^\text{173}\)). The clinical benefit of a fully human CXCL8 mAb (BMS-986253) was under evaluation in patients with advanced solid tumours (NCT03400332 \text{REF}\text{174}; BMS-986253 in combination with nivolumab) or with hormone-sensitive prostate cancer (NCT03689699 \text{REF}\text{175}; BMS-986253 in combination with nivolumab) or with intermittent androgen deprivation. An initial phase 1 clinical trial of BMS-986253 in 15 patients with metastatic or unresectable advanced solid tumours showed that the treatment was safe and well tolerated\(^\text{176}\). CXCR2 inhibitors (that is, AZD5069, reparixin\(^\text{177}\), SX-682 and navarixin) are undergoing clinical evaluation in patients with metastatic castration-resistant prostate cancer (NCT03177187 \text{REF}\text{178}; AZD5069 in combination with the non-steroidal anti-androgen therapy enzalutamide), with early breast cancer (NCT01861054 \text{REF}\text{179}; reparixin), with metastatic breast cancer (NCT02001974 \text{REF}\text{175,177} and NCT02370238 \text{REF}\text{175}; reparixin in combination with the chemotherapy paclitaxel), with metastatic melanoma (NCT03161431 \text{REF}\text{180}; SX-682 in combination with the PD1 antibody pembrolizumab) and with NSCLC and CRC (NCT03473925 \text{REF}\text{181}; navarixin in combination with pembrolizumab). It will be important to assess whether these agents indeed affect TAN infiltration and/or the activation state given the disappointing results so far obtained with chemokine inhibitors in inflammatory conditions\(^\text{182}\).

Reprogramming neutrophil function in the TME presents a challenge for which different approaches have been proposed, including blocking TGF\(_\beta\)\(^\text{3}\) (FIG. 5c). Similarly, targeting angiotensin-converting enzyme (ACE) and the angiotensin II type 1 receptor (AGTR1), nicotinamide phosphoribosyltransferase (NAMPT) or CXCR4 in mouse models has been reported to switch neutrophils to an antitumour state\(^\text{183}\). Consistent with the role of HIF1\(\alpha\) in setting neutrophils in a pro-tumour state, hyperoxygenation and reversion of hypoxia activated the antitumour potential of neutrophils in a GEMM of uterine cancer induced by \text{Pten} loss\(^\text{184}\). Below, we focus on two other approaches, namely ADCC and myeloid checkpoints, because of their significance to neutrophil diversity and reprogramming.

Antibody-dependent cellular cytotoxicity. Neutrophils share with monocytes, macrophages and NK cells the expression of Fc receptors (Fc\(\gamma\)Rs) and mediate tumour cell elimination via ADCC\(^\text{4}\) (FIG. 5d). Highlighting the requirement for neutrophils in this process, depletion of neutrophils reduced the efficacy of treatment with mAbs directed against CD52 (alemtuzumab) and CD20 (rituximab) in mouse lymphomas\(^\text{185}\).

Human neutrophils also express the high-affinity receptor for immunoglobulin A (IgA), Fc\(\alpha\)RI (also known as CD89), a potent inducer of ADCC; expression of this receptor leads to increased killing of IgA-opsinized cancer cells compared with neutrophil-elicited Fc\(\gamma\)-mediated ADCC of IgG-opsinized target cells\(^\text{183,186}\). Furthermore, chimeric IgA anti-CD20 mAb containing the human constant region domain was found to be more efficient than chimeric IgG anti-CD20 mAb in transgenic mice expressing functional human Fc\(\alpha\)RI and protected against lymphoma development\(^\text{187}\). Interestingly, IgA-elicited neutrophil-mediated ADCC can be enhanced by concomitant blocking of the CD47–signal regulatory protein-\(\alpha\) (SIRP\(\alpha\)) myeloid checkpoint\(^\text{188}\) (see below). In a three-dimensional collagen culture model of human breast cancer cells, endothelial cells and neutrophils, triggering of neutrophil Fc\(\alpha\)RI through treatment with an Fc\(\alpha\)RI antibody promoted the release of LTB4, a potent chemoattractant for neutrophils, IL-1\(\beta\) and TNF, which in turn amplify the recruitment of neutrophils via the production of CXCL8 by endothelial cells\(^\text{189}\). In effect, antitumour IgA
treatment sustains the activation of neutrophils and creates an amplification loop for neutrophil recruitment. Thus, FcαRI-mediated ADCC may represent a valuable neutrophil-centred therapeutic strategy.

**Myeloid checkpoints.** The function of myeloid cells is under the control of numerous negative regulators (known as checkpoints), which are expressed by neutrophils, monocytes and macrophages. These include SIRPa, CD200 receptor (CD200R), leukocyte immunoglobulin-like receptor B2 (LILRB2), paired immunoglobulin-like type 2 receptor a (PILRα) and, expressed on neutrophil precursors, PD1 and ACKR2 (Fig. 5a). Preclinical evidence suggests that neutrophils contribute to the antitumour activity of agents that block the CD47–SIRPa signalling axis and LILRB2, whereas for the other molecules presented in Fig. 5a the significance of their expression on neutrophils is currently unknown.

SIRPa is highly expressed by neutrophils, monocytes and macrophages, and acts as a phagocytosis checkpoint via its interaction with the ‘do not eat me’ signal CD47 presented on target cells. CD47 is overexpressed on cancer cells, rendering them resistant to myeloid cells. Interestingly, CD47–SIRPa checkpoint blockade increased the elimination of cancer cells, including non-Hodgkin lymphoma cells, melanoma cells and breast cancer cells, during an antibody-based treatment and potentiated the cytotoxic activity of neutrophils in vitro against breast cancer cells opsonized by trastuzumab, an anti-human epidermal receptor 2 (HER2) mAb, through a process of trogocytosis. Furthermore, in transgenic mice expressing human SIRPa, the administration of a SIRPa mAb increased the elimination of tumour cells by macrophages and neutrophils when combined with antitumour mAbs (for example, anti-CD20, anti-HER2 or anti-EGFR mAbs). Importantly, in this case, complete antitumour activity was neutrophil-dependent. Anti-CD47, combined with anti-CD20, was reported to have remarkable antitumour activity in patients with non-Hodgkin lymphoma. However, the significance of neutrophils in this context and more generally in the activation and orientation of adaptive immunity downstream of blocking CD47–SIRPa remains to be defined.

LILRB2 is expressed by myeloid cells, including neutrophils, and acts as a negative regulator of cell activation. LILRB2 binds to classical and non-classical HLA class I molecules and contains immunoreceptor tyrosine-based inhibitory receptor motifs (ITIMs) in its cytoplasmic tail. Activation of LILRB2 on neutrophils by one of its ligands, HLA-G, inhibited their phagocytic activity and production of ROS. In a mouse model of lung cancer, LILRB2 blockade suppressed infiltration of immunosuppressive neutrophils and significantly promoted antitumour immunity when combined with anti-PDL1.

ACKR2 is expressed on haematopoietic precursors and is virtually absent on NKT cells. Genetic deletion of ACKR2 resulted in an increase in the mobilization of neutrophils endowed with antitumour properties, characterized by ROS-mediated cytotoxic activity towards cancer cells. Thus, although targeting ACKR2 may unleash CC-chemokine-mediated lymphocyte and monocyte mobilization in the periphery, it might also release neutrophil effector function.

Evidence obtained from dissecting the function of the CD47–SIRPa signalling axis has suggested that blocking myeloid checkpoints unleashes adaptive immune responses, by eliciting T cell-dependent immunity including CD8+ cytotoxic T cells. This indicates that targeting neutrophil checkpoints may represent a new frontier in cancer immunotherapy. However, not all myeloid checkpoints are expressed by neutrophils and, indeed, some negative regulators (for example, common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER1)) are present only on macrophages. Therefore, as these molecules enter the clinical arena as therapeutic targets, it will be important to carefully assess neutrophil numbers, diversity and function as candidate correlates of antitumour activity.

**Conclusions and perspectives**

The presence and significance of neutrophils in cancer have long been overlooked. More rigorous approaches to quantify their infiltration into the TME and analyse their diversity and plasticity have revealed new insights into TAN immunobiology. As a consequence, TANs have emerged as an important component of the niche of many mouse and human tumours. Current views on the opposing roles of neutrophils in cancer are based on neutrophil depletion studies using antibodies in mice or correlative analysis between neutrophil numbers found in the TME or peripheral blood and the survival of patients with cancer. However, a more systematic effort using gene-targeting approaches for neutrophil depletion and abrogation of select neutrophil functions in carcinogen-induced and GEMMs of cancer, rather than in tumour cell line transplantation mouse models, is needed to truly determine the specific roles of neutrophils in different neoplasias. Deconvoluting the diversity of TANs at the single-cell level and relating this complex information to neutrophil function as well as patient prognosis and response to therapy represent important challenges in the field.

The current nomenclature for the diversity of neutrophils and related myeloid-derived suppressor cell (MDSC) populations can be confusing for researchers both outside and within the field. Therefore, even imperfect nomenclatures can have some value as communication tools and hence are a heuristic process. Therefore, we call for a consensus effort to develop a provisional nomenclature for neutrophil plasticity and diversity, along the lines of previous exercises conducted for ILCs, macrophages, IL-1 and other cell types and molecules.

Myeloid cells at different stages of differentiation and activation represent a major pathway to immune suppression both at a systemic level and at the local TME level. Dissecting the relative importance and diversity of the monocytic versus the neutrophil differentiation pathway in different tumour contexts and integrating it with the general immunological landscape may pave the way for personalized therapeutic approaches.
TNαs can be part of antitumour resistance pathways. Neutrophils express myeloid checkpoints and there is now proof of principle that targeting the negative regulator CD47 and unleashing myeloid cell function can result in clinical therapeutic benefit184. We surmise that harnessing the antitumour potential of neutrophils in those tumour types for which there is evidence of their protective role (for example, sarcomas and CRC) and in patients currently resistant to immunotherapy may represent a strategy worth pursuing to complement the established T cell-centric therapeutic armamentarium.

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