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Caught in a Web: Emerging Roles of Neutrophil Extracellular Traps in Cancer

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Abstract

Neutrophil extracellular traps (NETs) are meshes of DNA decorated with granular proteins that are extruded from neutrophils during immune responses to pathogens. However, excessive NET formation is negatively associated with many diseases, including cancer. NETs contain, for example, proteases, danger-associated molecular patterns (DAMPs), and DNA. These components can act directly on the cancer cells but also affect the surrounding microenvironment, including altering the extracellular matrix and the immune response to tumors. Here, we discuss the emerging roles of NETs in cancer progression, from their ability to promote primary tumor growth and immune escape to their prometastatic effects. The potential clinical implication of targeting NETs as novel therapeutic strategies in cancer is also discussed.

1. INTRODUCTION

1.1. Neutrophil Extracellular Traps: A Normal Host Defense Mechanism Dysregulated in Cancer

Neutrophils (also known as polymorphonuclear granulocytes) are the most prevalent type of leukocytes in human blood. They are often considered first responder immune cells, as they rapidly (within minutes) are recruited to sites of infection. Neutrophils' ability to eradicate pathogens has classically been ascribed to their ability to (*a*) phagocytose the pathogens or (*b*) expel, or degranulate, the contents of their lytic granules into the extracellular space (Mayadas et al. 2014). However, in 2004, a previously overlooked mechanism was discovered: The neutrophil can extrude its chromatin into the extracellular space to capture pathogens (Brinkmann et al. 2004). The extracellular chromatin fibers are named neutrophil extracellular traps (NETs). The process by which neutrophils form NETs is sometimes called NETosis and it is distinct from other cell death processes, such as apoptosis and necrosis (Brinkmann et al. 2004, Brinkmann & Zychlinsky 2012, Kolaczkowska & Kubes 2013, Mantovani et al. 2011). NETs are decorated with histones and granular proteins, including proteases [such as cathepsin G (CG), neutrophil elastase (NE), proteinase 3 (PR3), and matrix metalloproteinase-9 (MMP-9)] (Pham 2006), myeloperoxidase (MPO) (Pham 2006), lactotransferrin, LL-37, calprotectin, bactericidal/permeability-increasing protein, and pentraxin 3 (Brinkmann et al. 2004, Jaillon et al. 2007, Kessenbrock et al. 2009, Lauth et al. 2009, Urban et al. 2009). Many of these granular proteins contribute critically to NET-mediated pathogen-killing and are sources of the microbicidal activity of NETs (Pham 2006). Although NETs were discovered as a pathogen-eradication mechanism, it quickly became apparent that excess NET formation is negatively associated with several diseases, including thrombosis, atherosclerosis, rheumatoid arthritis, cystic fibrosis, asthma, chronic inflammatory diseases, ischemia-reperfusion injury after myocardial infarction, acute respiratory distress syndrome (ARDS), and, as discussed here, cancer (Barnes et al. 2020, Cools-Lartigue et al. 2014, Mutua & Gershwin 2021, Papayannopoulos 2018).

The tumor microenvironment (TME) influences cancer progression, primarily aiding the cancer (Hanahan & Weinberg 2011). The TME contains several immune cells including neutrophils, which promote several steps of cancer progression (Smith & Kang 2013). NETs are one mechanism by which neutrophils promote cancer progression. Independent of their associations with NETs, many NET components (e.g., the proteases and the DNA scaffold) have been shown to promote cancer progression with specific effects on, for example, angiogenesis, cancer cell motility/invasion, metastasis, and immune surveillance of tumors (also see Cristinziano et al. 2022). Here, we discuss the emerging data linking NET formation with cancer progression, and the ability of NETs to regulate the function and activities of other immune and stromal cells. Additionally, we discuss the potential for therapeutic targeting of NETs in cancer and other diseases.

1.2. Signaling Pathways Leading to NET Formation

The factors that induce NETs range from pathogens, such as bacteria (Brinkmann et al. 2004), fungi (Guiducci et al. 2018), viruses (Raftery et al. 2014), and parasites (Maksimov et al. 2016), to pathogen-derived products such as lipopolysaccharides (LPS) (Landoni et al. 2012) and artificial inducers such as ionomycin (Parker et al. 2012), calcium ionophores (Neeli et al. 2008), and phorbol myristate acetate. NETs can also form in response to activated complement and coagulation system factors (de Bont et al. 2019); chemokines, including CXCL1, -2, -5, -6, and -8 (Teijeira et al. 2020); granulocyte colony-stimulating factor (G-CSF) (Demers et al. 2012, Park et al. 2016); and

cathepsin C (Xiao et al. 2020). Some of these factors have been shown to be released by cancer cells and induce NETs in animal models of cancer, as discussed below.

Toll-like receptors (TLRs) are essential for the innate immune system and can sense pathogen- and danger-associated molecular patterns (PAMPs and DAMPs, respectively) to elicit an immune response. LPS is a PAMP and a ligand for TLR4, as well as for TLR2 (Kawai & Akira 2010). High-mobility group box 1 (HMGB1), released from damaged cells, is a DAMP and can induce NETs as a ligand for TLR2 and -4 (Ma et al. 2016). Consistently, TLR2 and TLR4 activation can trigger NET formation (Tadie et al. 2013, Wang et al. 2020).

The downstream signaling processes leading to NET formation are still incompletely understood, and it is likely that there are several signaling pathways that lead to NET formation (**Figure 1**). Some of the critical cellular events occurring during NET formation include the

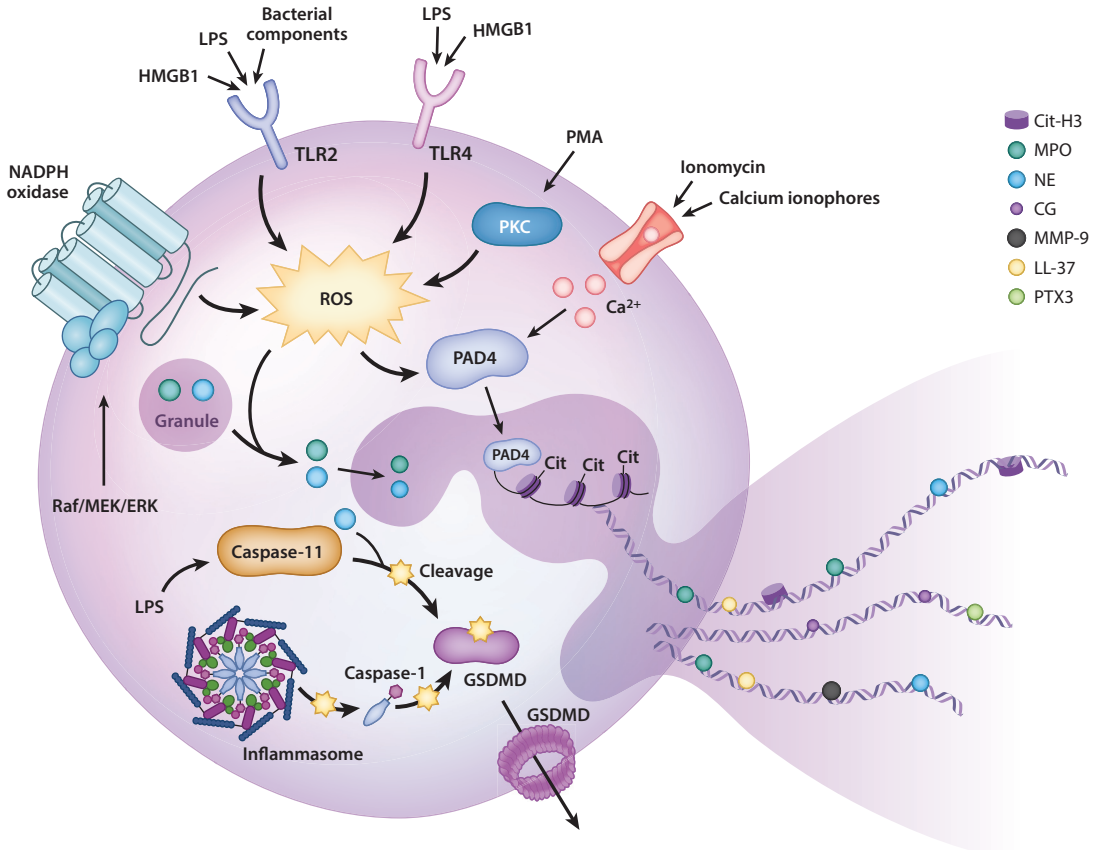

Figure 1

Signaling mechanism of NET formation. NET stimulators such as LPS, HMGB1, bacterial components, or PMA can induce the production of ROS, which can then activate PAD4 to modify histones and decondense the chromatin, allowing it to be expelled. ROS also allow NE and MPO to translocate from granules to the nucleus where these enzymes contribute to decondensation of the chromatin. NETs can also be released after cleavage and activation of GSDMD by caspase-1, caspase-11, or NE. Some figure elements were adapted from Servier Medical Art image library (**https://smart.servier.com**) (CC BY 3.0). Abbreviations: CG, cathepsin G; Cit-H3, citrullinated histone H3; GSDMD, gasdermin D; HMGB1, high-mobility group box 1; LPS, lipopolysaccharide; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; NE, neutrophil elastase; PAD4, peptidyl arginine deiminase 4; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTX3, pentraxin 3; ROS, reactive oxygen species; TLR, Toll-like receptor.

production of reactive oxygen species (ROS), translocation of NE and MPO to the nucleus, and histone citrullination and chromatin decondensation (Grayson & Kaplan 2016). NET formation is impaired in patients with chronic granulomatous disease, which is caused by mutations in NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (Grayson & Kaplan 2016). Consistently, NADPH oxidase activation occurs early after many NET-triggering stimuli and leads to the generation of ROS. Chromatin decondensation following peptidylarginine deiminase 4 (PAD4)-mediated histone citrullination is another important step during NET formation, and this generally happens downstream of ROS production. Inhibition of PAD4 reduces NET formation after most stimuli, and *Pad4*-null mice do not form NETs in vivo in response to bacteria in a necrotizing fasciitis mouse model (Li et al. 2010). Nevertheless, NETs can form independently of PAD4 in response to, for example, the yeast *Candida albicans* (Guiducci et al. 2018). Following chromatin decondensation, nuclear structure is lost and the DNA is extruded to the extracellular space (Brinkmann & Zychlinsky 2012). Translocation of NE and later MPO to the nucleus (Papayannopoulos et al. 2010) is required for DNA extrusion, and blocking NE activity, including by serum leukocyte protease inhibitor, blocks NET formation (Papayannopoulos et al. 2010). In addition, the Raf-MEK-ERK pathway is activated during NET formation, triggered by the production of ROS (Hakkim et al. 2011). One intriguing finding is that activation of cyclin-dependent kinase 4 and 6, which normally regulate proliferation, is an important step in NET formation, downstream of MAPK and ROS (Amulic et al. 2017). Finally, the pore-forming protein gasdermin D (GSDMD) is a critical signaling molecule in NET formation (Chen et al. 2018, Sollberger et al. 2018). Canonical inflammasome-activated caspase-1 can cleave GSDMD to release cytokines including interleukin (IL)-1β (Shi et al. 2015), and this pathway has recently been shown to also be able to drive NET formation (Munzer et al. 2021). However, noncanonical inflammasome activation by caspase-4/-11 was originally shown to cleave GSDMD during NET formation following, for example, cytosolic LPS transfection. Deleting either *Caspase-11* or *Gasdermin D* in vitro or in vivo disrupts cytosolic LPS-induced NET formation (Chen et al. 2018). Moreover, GSDMD can be activated by NE and upon activation can mediate NE release from the neutrophil granules to the cytoplasm, potentially generating a feed-forward loop to drive NET formation (Sollberger et al. 2018).

2. NETs AND THE PROMOTION OF PRIMARY TUMOR GROWTH

2.1. NETs Are Present in Human Cancer and High Levels of NETs Correlate with Poor Prognosis

The first demonstration of NETs in human tumors was in 2013 in Ewing sarcoma samples (Berger-Achituv et al. 2013). Since then, NETs have been found in a variety of cancers in samples from mouse models and patients, including breast (Park et al. 2016, Snoderly et al. 2019), colorectal (Arelaki et al. 2016, Rayes et al. 2019, Richardson et al. 2017), ovarian (Lee et al. 2019), pancreatic (Abdol Razak et al. 2017), lung (Li et al. 2019), liver (van der Windt et al. 2018) and gastric (Yang et al. 2015) cancers. Elevated levels of NETs in circulation, as measured by MPO/DNA conjugates (Rayes et al. 2019), NE/DNA conjugates (Zhang et al. 2020b), citrullinated histone H3 (Mauracher et al. 2018, Thalin et al. 2018), or DNA-histone complexes (Seo et al. 2019), have been shown to correlate with poor prognosis in cancers including breast, colorectal, stomach, lung, and pancreatic cancers.

2.2. Tumors Can Induce NETs

NETs in tumors can be the consequence of secretion of factors from cancer cells. Using in vitro coculturing systems with cancer cells and neutrophils, many types of aggressive cancer cells have

been shown to directly induce NET formation, including breast [4T1, BT-549, primary C3(1)-Tag (Park et al. 2016), and D2.A1 (Albrengues et al. 2018)], pancreatic [AsPC-1 (Abdol Razak et al. 2017)], and colon [HT-29, CT26, and LS174T (Teijeira et al. 2020)] cancer cell lines. However, not all cancer cells induce NET formation—for example, 4T07, D2.0R, and MCF-7 cells do not (Albrengues et al. 2018, Park et al. 2016)—and, typically, the lines that do induce NETs form more aggressive tumors than those that do not. Among the tumor-derived factors with effects on neutrophils, G-CSF is highly expressed by many cancers and can trigger neutrophilia, ROS generation, and NET formation (Demers et al. 2012, 2016; Park et al. 2016). IL-8 (also known as CXCL8) is another factor secreted by many cancers, and it not only is a strong chemoattractant for neutrophils but also can induce NETs (Gonzalez-Aparicio & Alfaro 2019). In mice, the homolog of CXCL8, CXCL1, plays a role in NET formation in a sepsis model (Jin et al. 2017). In coculture assays, cancer cells can induce NETsosis through the production of ligands for CXCR1 and CXCR2 (e.g., CXCL1, -2, -5, -6, and -8). Inhibiting CXCR1 or CXCR2 signaling by a blocking antibody or the allosteric inhibitor reparixin prevents formation of cancer cell–induced NETs (Teijeira et al. 2020). Cancer cells also affect neutrophils through the release of HMGB1 under, for example, hypoxic conditions. Neutrophils are attracted to the hypoxia tissue regions in part by HMGB1 and can undergo NETosis through HMGB1-mediated stimulation of TLR2 (Wang et al. 2020) or TLR4 (Tadie et al. 2013), resulting in increased tumor growth and metastasis (Wang et al. 2020, Yazdani et al. 2019). Another mechanism by which cancer cells can induce NETs is via secretion of the protease cathepsin C, which activates PR3 on the membrane of neutrophils to facilitate IL-1β processing, NF-κB activation, and NET formation, together leading to the establishment of a niche that aids metastatic growth (Xiao et al. 2020).

Within the tumor microenvironment, other cell types than the cancer cells can also induce NET formation, such as platelets and cancer-associated fibroblasts (CAFs). Platelets can induce NETs when activated by the release of, for example, HMGB1 (Maugeri et al. 2014) or by the interaction of P-selectin on platelets with anti-P-selectin glycoprotein ligand-1 on neutrophils (Etulain et al. 2015). CAFs can induce NETs through the secretion of amyloid β in mouse models of melanoma, lung, and pancreatic cancer (Munir et al. 2021).

2.3. NET Components' Effect on Cancer

NETs promote cancer progression through a variety of mechanisms. For example, NETs formed after neutrophils were primed by G-CSF-secreting Lewis lung carcinoma cells promoted tumor growth (Demers et al. 2016). Mechanistically, NETs' ability to enhance cancer progression has been linked mainly with the activities of the granular proteins on NETs—including NE, CG, and MMP-9—and with the DNA scaffold. The roles of these components in tumor progression are discussed next.

2.3.1. Neutrophil elastase. NE is important for pathogen-killing whether intracellularly in phagolysosomes, extracellularly after granulation, or when associated with NETs (Weinrauch et al. 2002). NE activity is also required for NET formation (Papayannopoulos et al. 2010) downstream of most NET-inducing stimuli. NE has a variety of protumorigenic effects, including promotion of cancer cell proliferation and migration in breast (Nawa et al. 2012), lung (Gong et al. 2013, Houghton et al. 2010), pancreatic (Gaida et al. 2012, Grosse-Steffen et al. 2012), gastric (Wada et al. 2006), esophageal (Wada et al. 2007), and colorectal (Ho et al. 2014) cancer cells. After binding to the plasma membrane of lung cancer cells, NE can be endocytosed and degrade the intracellular substrate IRS-1, resulting in the activation of the PI3K pathway and the promotion of tumor growth (Gregory et al. 2012, Houghton et al. 2010). Consistently, tumor burden was

markedly decreased and survival increased for the *Kras*^{G12D}-driven mouse model of lung adenocarcinoma deficient for *Elane*$^{-/-}$ (NE$^{-/-}$) (Gong et al. 2013, Houghton et al. 2010). NE can also induce TGF-α secretion from cancer cells, which in turn induces EGFR phosphorylation and ERK1/2 activation in breast (Nawa et al. 2012), gastric (Wada et al. 2006), and esophageal cancer cells (Wada et al. 2007). Of note, sivelestat, an NE inhibitor, can suppress tumor growth in mouse models of colorectal (Ho et al. 2014), gastric (Wada et al. 2006), and breast cancer (Nawa et al. 2012), for example. Thus, based on these studies, NE is a critical protumor component of NETs. However, a recent study showed that NE also can be decidedly antitumor: Human NE can be taken up by cancer cells and proteolytically liberate the death domain of the death receptor CD95 to cause cancer cell death (Cui et al. 2021).

2.3.2. Cathepsin G. CG is another important neutrophil protease in pathogen-killing (Segal 2005). In 1997, it was reported that CG is present in neutrophils and that its activity correlates with tumor grade and clinical stage in non-small-cell lung cancer (NSCLC) (Maksimowicz et al. 1997). Since then, CG has been shown to facilitate tumor angiogenesis (Wilson et al. 2010) and to be involved in the generation of a protumorigenic microenvironment via its ability to activate members of the IL-1 cytokine family (Clancy et al. 2018, Lefrancais et al. 2012, McLoed et al. 2016). The protumorigenic role of CG in metastasis may also relate to its ability to promote aggregation of MCF-7 breast cancer cells in vitro (Morimoto-Kamata & Yui 2017) and to modulate the distant seeding microenvironment by increasing TGF-β signaling at breast tumor–bone interface (Wilson et al. 2008). Tumor-associated neovascularization is required for delivery of nutrients and oxygen to tumors, as well as for outgrowth of disseminated cancer cells (Hanahan & Weinberg 2011). In a mouse model with mammary tumor bone lesions, CG was shown to promote tumor neovascularization via a complex mechanism involving first activation of pro-MMP-9, followed by MMP-9-mediated activation of TGF-β to induce VEGF and CCL2, all of which are factors that can promote angiogenesis (Wilson et al. 2009, 2010). Another mechanism by which CG promotes primary tumor growth is generation of a proinflammatory microenvironment: CG can process pro-IL-1β to induce tumor cell proliferation in an NSCLC model (McLoed et al. 2016), and CG can potentially generate DAMPs from cleaving IL-1 cytokine family members, including IL-1, IL-33, and IL-36 (Clancy et al. 2018).

2.3.3. Matrix metalloproteinase-9. MMP-9 is another protease that can be released by either degranulation or NET formation. MMP-9 degrades extracellular matrix (ECM) proteins and many other substrates (Kessenbrock et al. 2010). In contrast to NE, and possibly CG (Park et al. 2016), MMP-9 is not required for NET formation (Albrengues et al. 2018). MMP-9 is one of the best studied proteases in cancer and can promote tumor incidence, growth, and metastasis (Acuff et al. 2006, Coussens et al. 2000). One major tumor-promoting role of MMP-9 is to trigger an angiogenic switch, as originally shown in the RIP1-Tag2 mouse insulinoma cancer model (Bergers et al. 2000, Nozawa et al. 2006). Importantly, bone marrow transplantations revealed that active MMP-9 mainly comes from myeloid cells (Coussens et al. 2000). In a Lewis lung carcinoma model, neutrophils were specifically found to be the main contributor of MMP-9 expression (Acuff et al. 2006), but whether neutrophils or macrophages are the main contributors of MMP-9 is cancer type dependent (Park et al. 2015). MMP-9 was first observed on NETs in a study showing that MMP-9 on NET-DNA structures activates pro-MMP-2, leading to endothelial damage in lupus (Carmona-Rivera et al. 2015). In cancer, NET-associated MMP-9 can cleave the ECM protein laminin, generating an integrin epitope that can stimulate awakening of dormant breast cancers in the lung (Albrengues et al. 2018). Moreover, a recent study showed that metastatic

4T1 tumor cells can induce CXCR4^{hi} CD62L^{lo} (so-called aged) neutrophils, and that these aged neutrophils promote metastasis by releasing more NETs and MMP-9 (Peng et al. 2021).

2.3.4. The DNA scaffold. Decondensed DNA is the scaffold of the NETs, and the DNA contributes to antimicrobial (Halverson et al. 2015) and protumorigenic functions. Already in 1977, elevated circulating levels of extracellular DNA were demonstrated in cancer patients (Leon et al. 1977), although the cellular source of the DNA is unclear. In 2013, it was shown that pancreatic cancer cells could be coated with extracellular DNA to promote metastasis (Wen et al. 2013), but again, the source of DNA is unclear. DNA from NETs was later shown to promote proliferation and metastasis in pancreatic cancer, not through direct effects on the cancer cells, but rather by inducing autophagy-dependent activation of pancreatic stellate cells, causing increased MMP-2 and -9 production to promote cancer progression (Miller-Ocuin et al. 2019). NET-DNA is distinguished from normal genomic DNA, as it has much higher levels of 8-hydroxy-2′-deoxyguanosine (Yang et al. 2020). CCDC25 was identified as a sensor specifically for NET-DNA, and NET-DNA recognition by CCDC25 in cancer cells causes activation of the ILK-β-parvin pathway, resulting in enhanced cell proliferation, adhesion, and migration (Yang et al. 2020). Multiple intracellular sensors of DNA including cGas/STING (Sun et al. 2013), AIM2 (Fernandes-Alnemri et al. 2009, Hornung et al. 2009), and TLR9 (Chamilos et al. 2012) are expressed by mainly immune cells. Although it has yet to be demonstrated, NET-DNA partially digested by, for example, endogenous DNases can likely be taken up by cells to trigger signaling from these sensors, and this may be important for the regulation of immune responses in cancer.

3. NETs PROMOTE METASTASIS

To metastasize, cancer cells must disseminate from the primary site and invade the basement membrane, intravasate into a blood vessel, survive in the circulation, extravasate at distant tissues, and finally, grow in the new microenvironment (Chaffer & Weinberg 2011). NETs' ability to promote metastasis was first demonstrated in 2013, when targeting NETs with DNase I or an NE inhibitor was shown to abrogate liver metastasis after systemic sepsis in a model of intrasplenic injection of H59 Lewis lung cancer cells (Cools-Lartigue et al. 2013). Since then, many more studies have uncovered prometastatic roles of NETs, as summarized below (**Figure 2**).

3.1. NETs in Cancer Cell Dissemination/Invasion

In order for cancer cells to disseminate from the primary site, they must first gain the ability to migrate and invade through the ECM toward blood vessels. Coculturing NET-forming neutrophils has been shown to increase the migration and invasion of several cancer cell lines, including BT-549, 4T1 (Park et al. 2016), and MC38 (Tohme et al. 2016). Epithelial-to-mesenchymal transition (EMT) is a transdifferentiation process whereby epithelial cells gain mesenchymal properties, including increased ability to migrate and invade. Cancer cells can employ EMT to aid with their dissemination from the primary site (Chaffer & Weinberg 2011). MCF-7 human breast cancer cells can undergo EMT when incubated with isolated NETs: They downregulate the epithelial marker E-cadherin and upregulate mesenchymal markers, including fibronectin (Martins-Cardoso et al. 2020). How NETs induce EMT is unclear, but NE can trigger EMT by cleaving E-cadherin (Gaida et al. 2012, Grosse-Steffen et al. 2012), and NE on NETs might do the same.

In addition to triggering EMT, studies have shown that NETs can stimulate invasion through activation of TLR signaling in cancer cells. In A20 lymphoma cells, TLR9 is required for NET-induced migration (Nie et al. 2019). Although it is unclear how NETs activate TLR9 in A20

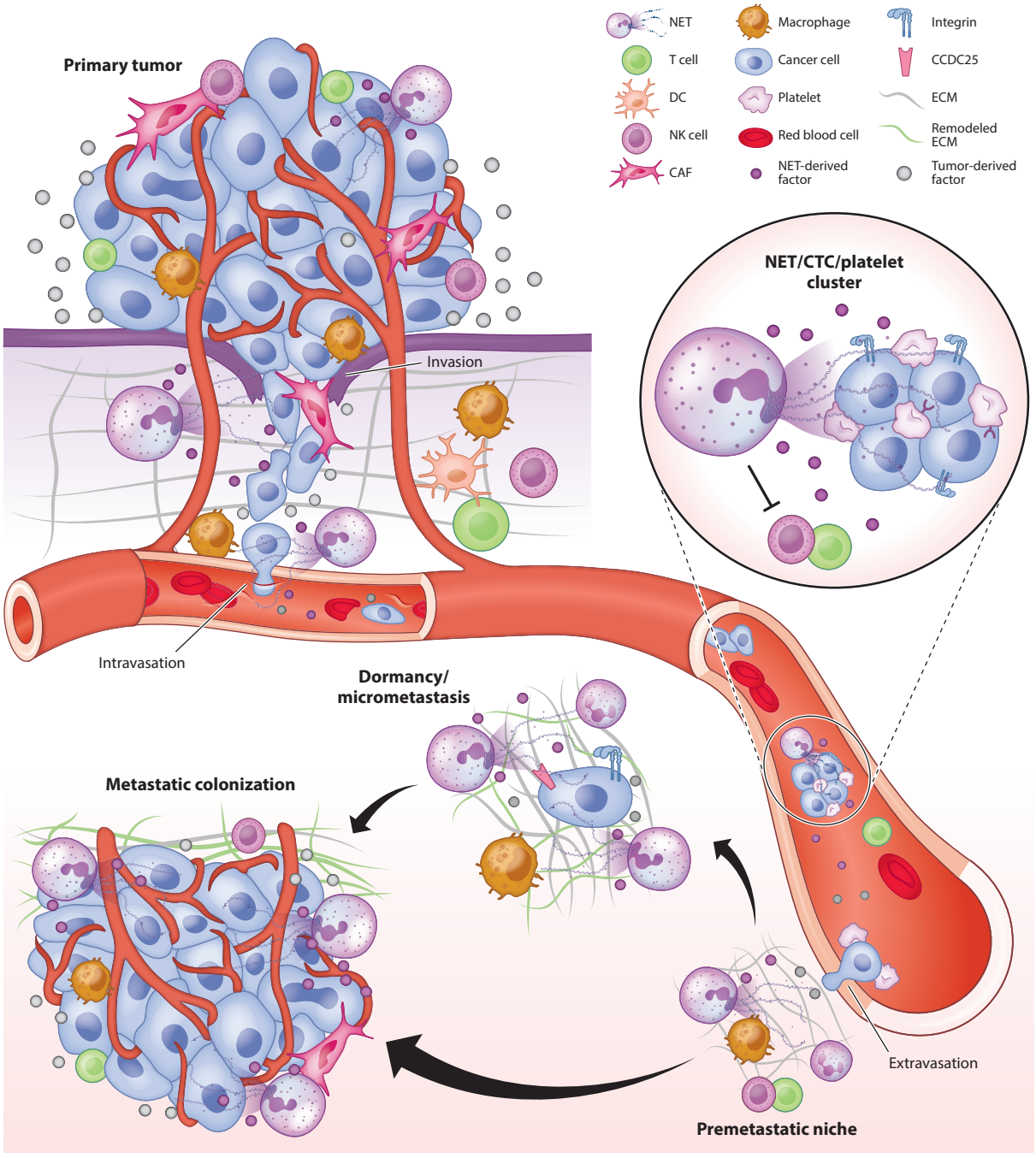

Figure 2

Roles of NETs during tumor progression. At the primary tumor site, NETs induced by tumor-derived factors promote primary tumor growth and invasion. In the bloodstream, CTCs, platelets, and NETs can form clusters, which increase survival and seeding at distant organs. At the seeding site, NETs can generate a prometastatic microenvironment acting on cells and the ECM. Some figure elements were adapted from Servier Medical Art image library (**https://smart.servier.com**) (CC BY 3.0). Abbreviations: CAF, cancer-associated fibroblast; CTC, circulating tumor cell; DC, dendritic cell; ECM, extracellular matrix; NK cell, natural killer cell.

cancer cells, it could be through HMGB1, a known TLR9 ligand, present on NETs (Tohme et al. 2016). For AsPC-1 pancreatic cancer cells, TLR2 and TLR4 have been shown to be required for NET-stimulated migration (Jung et al. 2019). Further research is needed to better understand how components of NETs activate different TLRs to drive migration and other activities in different contexts.

3.2. NETs and Circulating Tumor Cells

Anchorage-independent survival followed by extravasation at distant organs are essential for circulating tumor cells (CTCs) to form metastasis. In circulation, neutrophils can escort and promote cell cycle progression of CTCs to accelerate metastatic seeding (Szczerba et al. 2019). Moreover, neutrophils can aid CTCs' extravasation through secretion of IL-1β and MMP-9 (Spiegel et al. 2016). CTCs can also take advantage of the sticky properties of NETs to protect them from the shear forces in the bloodstream. Systemic inflammation by LPS injection causes increased cancer cell adhesion to liver sinusoidal endothelium through recruited neutrophils (McDonald et al. 2009). Although it was not tested whether NETs were involved, LPS can induce NETs (Brinkmann et al. 2004). Enhanced adhesion between CTC and neutrophils was also observed between H59 cancer cells and LPS-primed neutrophils and this was mediated by neutrophil Mac-1 and cancer cell ICAM-1 signaling (Spicer et al. 2012). In 2013, intravital imaging was used to visualize that NETs entrap CTCs in a lung cancer model of liver metastasis and that DNase I or NE inhibitor treatment abolished the adhesion of tumors to hepatic sinusoids (Cools-Lartigue et al. 2013). The mechanism by which NETs entrap CTCs was subsequently shown to require β1-integrin expression on both cancer cells and NETs (Najmeh et al. 2017). Another mechanism links activated platelets with NETs and CTCs through TLR4-dependent activation of platelets, enabling platelets to adhere to CTCs to form platelet-CTCs aggregates, which then have a higher propensity to be captured by circulating NETs to increase metastasis (Ren et al. 2021). Thus, collectively, it has been clearly demonstrated that NETs can entrap CTCs to promote the development of distant metastases.

3.3. NETs and Metastatic Seeding: Cancer Cell Proliferation and Awakening in the Metastatic Niche

The outgrowth of disseminated cancer cells at the site of seeding is the last step in forming metastasis. It is often hard to untangle this last step from earlier steps of metastasis, but there are studies showing roles of neutrophils and NETs specifically in the metastatic niche. Degradation of the ECM protein thrombospondin-1 (TSP-1) is required for experimental lung inflammation to promote metastatic tumor growth (El Rayes et al. 2015). Degradation is mediated by two neutrophil proteases, NE and CG, which can be released by degranulation and as NET components. Subsequently, it was confirmed that NET-associated proteases can degrade TSP-1 (Albrengues et al. 2018) and that the ability of TSP-1 to suppress tumor spheroid growth can be overcome by adding a NET-conditioned medium (Xiao et al. 2020). NET-proteases can also alter the structure of ECM proteins rather than degrade them. We previously showed that NE and MMP-9 on NETs cleave the ECM protein laminin, exposing an epitope that can activate integrin signaling in cancer cells to trigger awakening, that is, the proliferation of quiescent disseminated cancer cells (Albrengues et al. 2018). In vitro experiments have suggested that degradation of TSP-1 further increased the proliferation of cells in the presence of cleaved laminin (Albrengues et al. 2018).

NETs are not only aiding metastasis of cancer cells that already have seeded in distant organs: studies support that NETs aid in the generation of a pro-seeding microenvironment. Within the liver of breast tumor-bearing mice, NETs are detected at earlier time points than disseminated

cancer cells, and it was proposed that NET-DNA was a possible chemotactic signal, with the abovementioned CCDC25 acting as the NET-DNA sensor on the cancer cells (Yang et al. 2020). Moreover, NETs are detected in the omentum before metastasis in a murine model of ovarian cancer (Lee et al. 2019).

4. CROSS TALK BETWEEN NETs AND OTHER IMMUNE CELLS IN CANCER

The effects of NETs on both the innate and adaptive immune response contribute to their ability to promote cancer and are discussed next.

4.1. Neutrophils/NETs and T Cells

T cells are a crucial component of the adaptive immune system: They kill infected cells, promote the production and release of cytokines to control infection, and generate memory cells so that past infections lead to prevention of future ones. T cells are similarly critical for tumor immunosurveillance. However, T cells must be activated by antigen-presenting cells in order to carry out their functions. Antigen presentation is usually carried out by dendritic cells, but via NET formation neutrophils can also prime, or preactivate, T cells so that they can respond to lower-antigen load (Tillack et al. 2012). In an in vitro coculture system, it was shown that $CD4^+$ T cells that are in direct contact with NETs have lower activation thresholds to CD3 antibody, caused by upregulation of CD25 and CD69, as well as phosphorylation of ZAP70 (Tillack et al. 2012). Moreover, intratumoral injection of NET components (including NET-DNA and proteins) to subcutaneous bladder tumors resulted in the recruitment of more T lymphocytes, the killing of the cancer cells, and an overall smaller tumor size (Liu et al. 2019). Thus, in this context, NETs have antitumor functions, promoting an antitumor immune response. However, neutrophils and NET-forming neutrophils do not always elicit antitumor T cell responses. Tumor-derived G-CSF can lead to the generation of immunosuppressive neutrophil-like cells (Gabrilovich & Nagaraj 2009), which suppress T cells through, for example, arginase activity and the production of ROS (Casbon et al. 2015). Moreover, through production of CCL17, neutrophils can recruit T regulatory cells, which in many contexts dampens antitumor immune responses, including in a mouse mesothelioma model (Mishalian et al. 2014). γδ T cells are critical for epithelial and mucosal pathogen defense and are another T cell subpopulation whose activities are closely intertwined with that of neutrophils. In the presence of cancer-related inflammation, IL-17 from activated γδ T cells increases systemic levels of G-GCF, possibly by increasing the expression from epithelial cells, to drive the expansion of neutrophils (Coffelt et al. 2015, Jin et al. 2019). The expansion of neutrophils has been shown to suppress $CD8^+$ T cells both at the metastatic site in a breast cancer model and at the primary tumor site in a pancreatic ductal adenocarcinoma (PDAC) model (Coffelt et al. 2015, Zhang et al. 2020a). IL-17 derived from γδ T cells can promote NET formation, and these NETs further can suppress $CD8^+$ T cell recruitment to PDAC tumors (Zhang et al. 2020a). In a colorectal cancer liver metastasis model, exclusion of $CD8^+$ T cells in tumors was due to NETs, as degrading NETs with DNase I increased $CD8^+$ T cell infiltration, thereby reducing growth of liver metastatic lesions (Xia et al. 2020). NETs may not only prevent T cells from infiltrating into tumors but also protect cancer cells from being killed by T cells: Taking advantage of live cell imaging and a 3D coculture system of 4T1 cancer cells and NET-forming neutrophils, NETs were shown to encapsulate cancer cells and impede contact with T cells, preventing cancer cell killing by T cells and natural killer (NK) cells (Teijeira et al. 2020). In the 4T1 breast cancer model, the cancer cell killing by NK cells was shown to be inhibited by neutrophils (Spiegel

et al. 2016). These findings identify NETs and neutrophils as components of the tumor immune microenvironment with both inhibitory and stimulatory effects on T and NK cells in cancer.

4.2. Neutrophils/NETs and Monocytes/Macrophages

Monocytes/macrophages differentiate from the same bone marrow precursors as neutrophils, and the cell types have some overlap in their functions. However, during tissue damage–induced inflammation, neutrophils and monocytes/macrophages have complementary roles: At the site of tissue injury, macrophages sense the damage through receptors for DAMPs and then produce several neutrophil-recruiting chemokines, including CXCL family members, TNF-α, and IL-6 (reviewed by Soehnlein & Lindbom 2010). Macrophage-derived factors [including IL-1β, G-CSF, and GM-CSF (granulocyte-macrophage colony-stimulating factor)] can increase the life span of the recruited neutrophils by delaying neutrophil apoptosis at the injury site (Kantari et al. 2008). In turn, recruited neutrophils release granule proteins—including azurocidin, LL-37, and CG—which further activate inflammatory monocytes and trigger their extravasation to the injury site (Soehnlein & Lindbom 2010). A similar macrophage-neutrophil interaction may occur in cancer, as exemplified by the abovementioned study showing that systemic inflammation induced by macrophages increases secretion of IL-17 from γδ T cells to trigger G-CSF-mediated expansion of neutrophils (Coffelt et al. 2015). MicroRNAs, specifically miRNA-142-3p, which exists in NET-enriched supernatants, can also affect macrophages, leading to, for example, increased TNF-α production (Linhares-Lacerda et al. 2020). It is worth noting that IL-1β, IL-8, and IL-18 secreted from macrophages have been shown to induce NET formation (An et al. 2019, Kahlenberg et al. 2013, Mitroulis et al. 2011). Conversely, the NET-associated protein LL-37 (Kahlenberg et al. 2013) and NET-DNA complexes (Hu et al. 2019) can activate inflammasome signaling in macrophages, resulting in the secretion of IL-1β and IL-18. This suggests that a positive feedback loop between NET-forming neutrophils and macrophages may drive sustained inflammation, including in cancer.

A positive feedback loop between neutrophils and macrophages may drive sustained inflammation, but under normal conditions, inflammation resolves. Macrophages can dampen the response, for example, by taking up NETs through endocytosis and degrade them intracellularly in lysosomes with TREX1 (also known as DNase III) (Farrera & Fadeel 2013, Lazzaretto & Fadeel 2019). Proinflammatory macrophages (stimulated with LPS and IFNγ) have enhanced ability to engulf NETs by micropinocytosis (Haider et al. 2020). Macrophages in the tumor microenvironment are generally not of a proinflammatory phenotype, but they can be reprogrammed by, for example, injection with monophosphoryl lipid A (a derivative of LPS) and IFNγ (Sun et al. 2021), causing reduced tumor growth and metastasis. Whether reprogramming also leads to increased turnover of NETs remains to be determined.

5. NETs AND CANCER-RELATED THROMBOSIS

Cancer increases the risk of venous thromboembolisms such as deep vein thrombosis (DVT) by inducing a hypercoagulable state (Seo et al. 2019), and thrombosis is the second leading cause of death in cancer patients (Fernandes et al. 2019). Data support that elevated NET levels drive thrombosis in cancer: First, highly elevated levels of citrullinated histones in blood increases the risk of venous thrombocytic events in patients with cancer (Mauracher et al. 2018), and cancer-associated arterial microthromboses in autopsy samples contain citrullinated histones (Thalin et al. 2016). Second, in the MMTV (mouse mammary tumor virus)-PyMT (polyomavirus middle T antigen) breast cancer and RIP1-Tag2 insulinoma cancer models, kidney tissues become poorly

perfused with tumor progression as NET levels in the circulation also increase, and digesting NETs with DNase I improves the tissue perfusion (Cedervall et al. 2015).

How NETs induce a prothrombotic state has been intensely studied. In general, histones, which are present on NETs, can induce platelet recruitment and aggregation by increasing von Willebrand factor secretion from endothelial cells (Brill et al. 2012). Histones also activate platelets by serving as ligands for TLR2 and TLR4 on the platelets (Semeraro et al. 2011). Proteases present on NETs, such as NE and CG, can promote the formation of thrombi by proteolytically inactivating tissue factor pathway inhibitor, a strong anticoagulant in platelets (Massberg et al. 2010). The sticky DNA structure of NETs itself can act as a scaffold to aggregate red blood cells and activate platelets together to form thrombi (Fuchs et al. 2010). Finally, IL-1β can increase the amounts of G-CSF in circulation, leading to more NETs in circulation, and ultimately more thrombi (Gomes et al. 2019). Inhibiting the IL-1β receptor IL-1R decreased G-CSF levels and prevented the formation of thrombi in a DVT mouse model (Gomes et al. 2019). Consistently, in several mouse models of cancer, G-CSF levels increase with tumor progression and contribute to the prothrombotic stage (Demers et al. 2012).

6. NETs IN OTHER DISEASES

NETs have been implicated in acute and chronic inflammatory diseases other than cancer, including multiple pulmonary diseases (ARDS, asthma, and cystic fibrosis), sepsis, atherosclerosis, thrombosis, and autoimmune disorders like lupus.

NETs are present in human biopsies and tissue from mouse models of ARDS/lung injury, including transfusion-related acute lung injury (Adrover et al. 2020, Thomas et al. 2012), bacteria-induced lung injury (Lefrancais et al. 2018), and influenza infection (Pillai et al. 2016). Notably, targeting NETs with DNase I or PAD4 inhibitors in mouse models of acute lung injury reduces mortality (Adrover et al. 2020, Lefrancais et al. 2018, Thomas et al. 2012). This may also be relevant after infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which has caused the ongoing COVID-19 (coronavirus disease 2019) pandemic. COVID-19 patients with severe disease (up to 10% of cases) develop ARDS with massive tissue damage, likely from a combination of damage from the replicating virus, dysregulated inflammatory responses, excessive cytokine release, and microvascular thrombi formation (Mehta et al. 2020). NETs are found in highly elevated levels in the lung and in blood samples from COVID-19 patients, and NET-forming neutrophils colocalize with activated platelets in microvascular thrombi (Middleton et al. 2020; Veras et al. 2020; Zuo et al. 2020, 2021). Targeting NETs with recombinant DNase I (dornase alfa) is used in treatment of cystic fibrosis (Quan et al. 2001, Yang & Montgomery 2021) and is being tested in humans for COVID-19 patients [e.g., clinical trial identifiers NCT04387786 (Weber et al. 2020), NCT04402970, NCT04359654, NCT04355364 (Desilles et al. 2020) (**https://clinicaltrials.gov/**)]. Thus, the inflammatory reaction and increased thrombosis in COVID-19 may in part be driven by an even more exacerbated response to NET formation than that seen in cancer.

NETs play important roles in atherothrombosis. First, a significant increase in circulating MPO/DNA complexes has been observed in patients with severe coronary artery disease (Borissoff et al. 2013). Second, studies using a mouse model of atherosclerosis (apolipoprotein E–null mice fed a high-fat diet) have demonstrated that NETs are present at cholesterol-rich lesions and can be induced directly by cholesterol crystals (Warnatsch et al. 2015). NETs in this context prime macrophages for cytokine release, including stimulating the release of IL-1β after inflammasome activation, a known driver of atherosclerosis. Importantly, different approaches to target NETs, including depletion of NE (Warnatsch et al. 2015), digestion with DNase I

(Liu et al. 2018), or inhibition of Pad4 with Cl-amidine (Knight et al. 2014), all reduced formation of atherosclerotic lesions and arterial thrombosis in mice. Neutrophil/NET cross talk with macrophages as it occurs in atherothrombosis likely has similarities with similar cross talk in cancer.

7. CLINICAL IMPLICATIONS: TARGETING NETs TO TREAT CANCER

Considering (*a*) the elevated NET levels in cancer patients and (*b*) the ability of NETs to drive metastasis and thrombosis (major causes of cancer related deaths), targeting of NETs is of potential therapeutic interest for the treatment of cancer. In mice, metastasis is reduced by treatment with free DNase I, DNase I–coated nanoparticles (Park et al. 2016), inhibitors of critical enzymes for NET formation, or targeting of NET-modified ECM proteins (Albrengues et al. 2018).

DNase I is an effective NET degrader and has antimetastatic effects in mouse models of cancer, including liver (Sugihara et al. 1993), breast (Park et al. 2016, Yang et al. 2020), and lung (Cools-Lartigue et al. 2013, Patutina et al. 2011) cancer. Importantly, recombinant DNase I (dornase alfa) is an FDA (US Food and Drug Administration)-approved drug already in use and in past or current clinical trials to treat patients with cystic fibrosis (Quan et al. 2001, Yang & Montgomery 2021), lupus nephritis (Davis et al. 1999), and ARDS associated with COVID-19 (Desilles et al. 2020, Weber et al. 2020).

Different from DNase, which disrupts the NET structure after its formation, inhibitors that block the formation or release of NETs include inhibitors of NE, PAD4, and GSDMD. The NE inhibitor sivelestat and various PAD4 inhibitors reduce metastases in mouse models of cancers (Albrengues et al. 2018, Rayes et al. 2019). However, the use of these drugs in patients has been limited: Sivelestat appeared to improve the postoperative status in thoracic esophageal cancer patients undergoing thoracoscopic esophagectomy (Kawahara et al. 2010), while PAD4 inhibitors have not yet been tested clinically. GSDMD is another critical factor for NET formation (Chen et al. 2018, Sollberger et al. 2018), and disulfiram, an FDA-approved drug for alcohol abuse disorder, can also inhibit GSDMD pore formation (Hu et al. 2020). We have recently determined that disulfiram reduces NET formation and improves disease outcome in a mouse model of acute lung injury and in golden hamsters infected with SARS-CoV-2 (Adrover et al. 2022). A clinical trial is currently ongoing to study the effect of disulfiram given to COVID-19 patients (NCT04485130).

The development of approaches to target NETs in cancer are in the early stages, with no NET-targeting drug in clinical testing for cancer. Additionally, there are few preclinical studies to test how targeting NETs affect the responses to other cancer treatments, including chemo-, radio-, or immunotherapy: In a PDAC model, targeting tumor associated neutrophils improved the chemotherapeutic response (Nywening et al. 2018), but whether targeting NETs has an effect is not yet known. However, also in a PDAC mouse model, NETs were found to inhibit the response to immune checkpoint therapy (Zhang et al. 2020a).

8. CONCLUSIONS AND FUTURE DIRECTIONS

As discussed above, there are antitumor effects of NETs, but NETs primarily promote cancer progression. NETs influence the composition of the tumor microenvironment by, for example, interacting with immune cells and modifying the ECM. Targeting NETs in many different mouse models of cancer reduces metastasis, suggesting that developing approaches to target NETs could be beneficial. However, clinical development of NET inhibition is still almost nonexistent. One problem is that cancer is a disease that often lasts for years while the available NET inhibitors require, for example, daily injections. Another problem is that the signaling pathways triggering NET formation are incompletely understood, making it difficult to develop selective inhibitors. It

is also unknown whether NETs are important for specific cancers or metastasis to specific organs, although there are indications that NETs are particularly relevant for metastasis to liver, lung, and omentum—but not to, for example, brain (Yang et al. 2020). The last major problem is the largely unknown effects of NET targeting on patients' ability to clear infections. NETs were only discovered in 2004, and since then we have learned that they are drivers of cancer progression in many different mouse models and present in many types of human cancer. In the next decade, research into the signaling pathways causing NET formation and the effects of NETs on cancer cells and the tumor microenvironment will hopefully allow for the development of approaches to target NETs in cancer.

DISCLOSURE STATEMENT

M.E. is a member of the research advisory board for brensocatib for Insmed, Inc., a member of the scientific advisory board for Vividion Therapeutics, Inc., and a consultant for Protalix, Inc. and holds shares in Agios Pharmaceuticals, Inc. The other authors are not aware of any other affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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