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EDITED BY

Andrea Visentin,
University of Padua, Italy

REVIEWED BY

Marzia Palma,
Karolinska Institutet (KI), Sweden
Agnieszka Bojarska-Junak,
Medical University of Lublin, Poland

*CORRESPONDENCE

Maria Teresa S. Bertilaccio
✉ msbertilaccio@mdanderson.org

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Neutrophils unveiled in chronic lymphocytic leukemia

Sheighlah McManus^{1,2}, Priyanka Khare¹
and Maria Teresa S. Bertilaccio^{1*}

¹Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, ²The University of Texas MD Anderson Cancer Center, UTHealth Houston Graduate School of Biomedical Sciences, Houston, TX, United States

This review explores neutrophils' roles in chronic lymphocytic leukemia (CLL), highlighting their functions within the immune system. While neutrophils are known for fighting infections, their altered behavior in CLL significantly impacts disease progression. This review notes the reduced phagocytic abilities of neutrophils and the increased formation of neutrophil extracellular traps (NETs) in patients with CLL. It also examines the effects of CLL treatments, including chemotherapy, immunotherapy and targeted therapies, on neutrophils' count and function, stressing the need for improved strategies to manage therapy-induced immune dysfunction. This review also provides detailed information about the interactions between neutrophils and other immune elements in CLL microenvironment, providing insights for developing therapeutic approaches that can restore immune function and improve patients' quality of life.

KEYWORDS

neutrophils, NETs, CLL, immune dysfunction, infection, disease progression

1 Introduction

Chronic lymphocytic leukemia (CLL) is characterized by not only the accumulation of malignant B cells in the blood and lymphoid tissues but also a complex interplay of various immune cells within the tumor microenvironment [TME; [Figure 1 \(1\)](#)]. The TME is an active and dynamic network that encompasses interactions between lymphoid and myeloid cells. Each of these cells plays a specific role in the progression of CLL, contributing to the immune evasion and dysfunction observed in this disease. The interactions among immune cells are mediated by various cytokines and chemokines that influence their behavior. This interplay shapes the immune response in CLL, aiding clonal expansion and establishing a supportive niche for leukemic cells (2). Among the immune cells of CLL TME, neutrophils play a critical role.

Neutrophils, the most abundant immune cells in the bloodstream, are a vital component of the innate immune system by serving as the body's first line of defense against infections and inflammation (3). However, recent research has unveiled a more intricate and multifaceted role for neutrophils, particularly in the context of cancer (4). Emerging studies suggest that neutrophils can exhibit both pro-tumorigenic and anti-

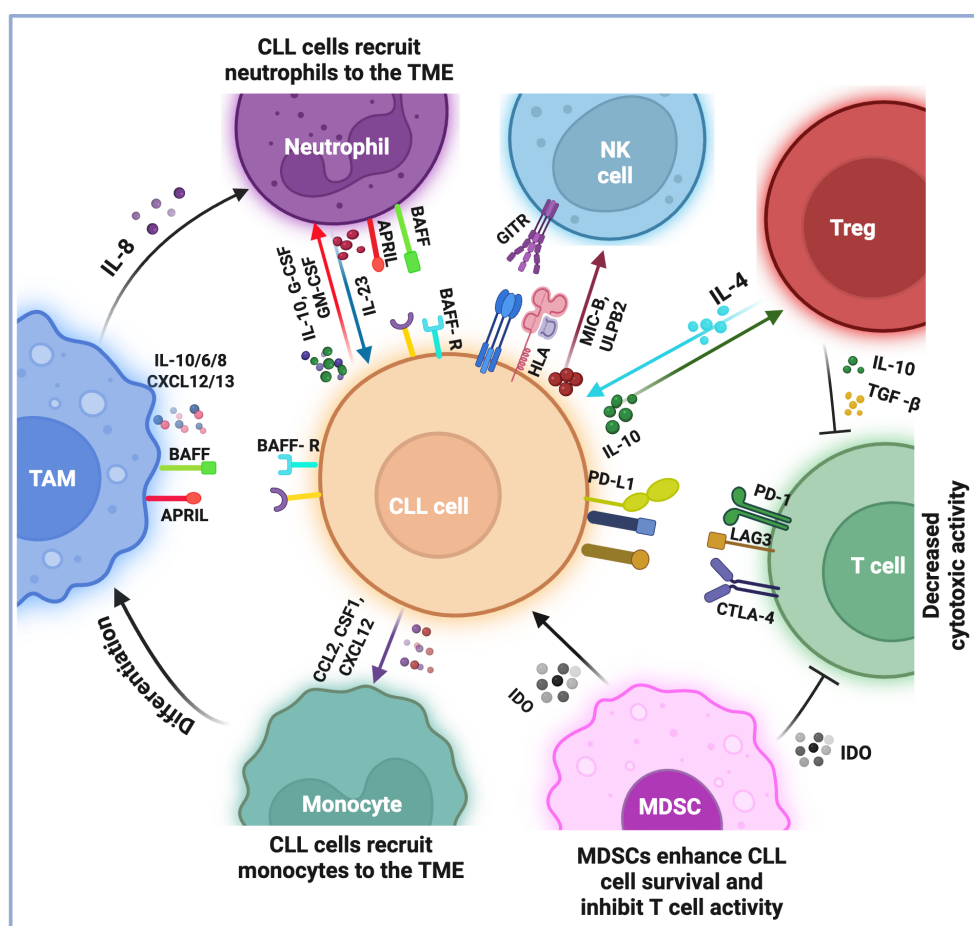


FIGURE 1

Current understandings of the immune microenvironment of chronic lymphocytic leukemia. The immune microenvironment of chronic lymphocytic leukemia (CLL) consists of lymphoid and myeloid cells, which play a role in the survival and growth of leukemia cells. T cells are generally exhausted and show decreased cytotoxic activity, marked by the expression of specific proteins such as PD1, CTLA-4, and LAG3. This exhaustion is further supported by immunosuppressive cells that inhibit T-cell activation by expressing particular molecules (i.e., IL-10 and TGF β by Tregs and IDO by MDSCs). Monocytes are recruited to the microenvironment and transform into tumor-associated macrophages (TAMs), which produce molecules (i.e., BAFF, APRIL, IL-10/6/8, and CXCL12/13) that help CLL cells survive and grow. Neutrophils in the microenvironment also provide support for leukemia cell survival. CLL cells evade NK cell surveillance by expressing HLA class I molecules on their surface and by secreting MIC-B and ULPB2, which compromise NK function. Additionally, the TNFR receptor, GTR, axis with GTRL causes the release of TNF, IL6, and IL8, which act as survival factors for CLL cells. Overall, these interactions between different types of immune cells in the CLL microenvironment reduce the immune system's ability to combat the leukemic cells, thus contributing to the persistence of the disease.

tumorigenic activities, complicating our understanding of their role in various disease states (5). They are also involved in various physiological and pathological conditions, including chronic inflammation, autoimmune diseases, diabetes, and thrombosis (6). Notably, neutrophils significantly influence the TME, affecting cancer progression, treatment response, and prognosis.

In patients with CLL, microbial infections are a leading cause of morbidity and mortality because of immune dysfunction caused and maintained by the disease itself (7–10). Additionally, treatments for CLL can alter immune responses and increase infection risk (9). As an example, the CLL14 (11) and MURANO (12) clinical studies reported adverse events such as neutropenia and various infections, including aspergillus pneumonia, herpes pharyngitis, and candida esophagitis (13). Understanding neutrophil biology is crucial because these cells are essential for patients' defense against pathogens.

Overall, this evidence highlights how neutrophils contribute to disease progression and how this innate immune cell type impacts the intrinsic and therapy-induced immune dysfunction. Therefore, this literature review describes the intricate role of the immune microenvironment in CLL, with a focus on neutrophils and their functions. An enhanced understanding of these innate immune cells can be used to identify novel therapeutically targetable pathways that could restore proper immune function, thereby improving patients' outcomes and quality of life.

2 Immune dysfunction in CLL

Previous studies identified two prevalent themes as the causes of the significant changes affecting the immune system of patients with CLL (14–16). First, intrinsic immune dysfunction arises directly

from the disease itself (14). CLL disrupts the normal functioning of the adaptive immune response by altering the activity of essential lymphoid cells (i.e., B and T cells), which compromises the immune system's ability to fight infections and respond effectively to pathogens. Second, the immune dysfunction in CLL patients can be worsened by various treatments, including chemotherapy (17, 18), immunotherapy (15), and targeted therapy (9, 16), which may inadvertently suppress the immune response or have other unintended consequences, such as increased susceptibility to infections. Consequently, patients with CLL often face a dual challenge of managing their disease while dealing with the repercussions of their treatment on their overall immune health.

2.1 Intrinsic immune dysfunction

Intrinsic immune dysfunction in CLL refers to the inherent alterations and impairments within the immune system that arise during the development and progression of the disease itself. These dysfunctions significantly contribute to the pathogenesis of CLL and its clinical manifestations, leading to a compromised immune response and an increased risk of infection and disease progression (19–23). For instance, leukemic B cells exhibit alterations that cause their accumulation in the blood and lymphoid tissues, which cause defects in their functional capabilities.

B cells naturally recognize specific antigens through the B-cell receptor (BCR), which consists of antigen-binding surface immunoglobulins and accessory molecules that interact with their corresponding antigens, leading to cell activation and antibody production. B-cell antibody production includes V(D)J recombination, somatic hypermutation, and class switching processes. In CLL, defects in antibody production and class switching often occur (24–26). The presence of dysfunctional B cells can reduce the ability to generate protective antibodies, leaving patients vulnerable to infections (27). BCR signaling is constitutively activated in CLL cells, whether this is attributable to self-antigens or environmental factors remains unclear (16–19). Nevertheless, signal transduction through the BCR activates pathways that promote the survival and growth of leukemic B cells, thereby facilitating disease progression.

Furthermore, patients with CLL have been shown to have an imbalance in the ratio of cluster of differentiation 4 (CD4)⁺ to CD8⁺ T cells (28). Although studies have documented a higher number of CD8⁺ T cells in CLL patients compared with age-matched healthy individuals, these cells often express exhaustion markers (i.e., programmed death cell protein 1 [PD-1], cytotoxic T-lymphocyte associated protein 4 [CTLA-4], and lymphocyte-activation gene 3 [LAG-3]) and demonstrate reduced cytotoxic activity, which is perpetuated by improper immune synapse formation (29–31). Thus, CD8⁺ T cells often fail to release the cytotoxic granules necessary for eliminating leukemic cells. CLL patients often have a high frequency of CD4⁺ regulatory T cells (Treg). Treg cells are associated with hindered anti-tumor responses and immunosuppression, as they release immunosuppressive molecules (i.e., interleukin [IL]-10 and transforming growth factor beta [TGF-β]) that reduce CD8⁺ T-cell

activation. Previous studies have demonstrated that removing Tregs leads to effective anti-tumor responses in animal models of CLL (32–34).

Natural killer (NK) cells, which are innate immune cells responsible for anti-tumor and anti-viral responses, are also present in the immune microenvironment of CLL. NK cell functions include degranulation, cytotoxicity, and the release of cytokines (35, 36). In CLL, NK cells' cytolytic activity is impaired owing to defects in their cytotoxic machinery, and leukemic B cells employ immune evasion strategies to escape detection by NK cells (37).

Myeloid cells constitute a significant portion of the immune microenvironment in CLL. Nurse-like cells (NLCs) share lineage and functional similarities to tumor-associated macrophages (TAMs) and have been identified as CLL-specific TAMs (38). NLCs express stromal cell-derived factor 1 (SDF-1), which attaches to leukemic B cells and downregulates its receptor, C-X-C chemokine receptor type 4 (CXCR4), thereby protecting these cells from spontaneous apoptosis. NLCs also release B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL), both of which contribute to the survival of CLL cells (39, 40). Another important myeloid cell component of the CLL TME are monocytes, which include CD14⁺⁺ CD16⁻ classical, CD14⁺ CD16⁺⁺ non-classical, and CD14⁺⁺ CD16⁺ intermediate subsets (41, 42). The recruitment of monocytes to the TME depends on CLL cells' expression of C-C-chemokine receptor type 2 (CCR2) and several monocyte-attracting chemokines such as C-C-motif ligand 2 (CCL2) and CXCL10 (43, 44). When adoptively transferred into humanized MISTRG mice, monocyte subsets from CLL patients differentiate into TAMs (45). Monocytic myeloid-derived suppressor cells (M-MDSCs) are so named because of their common myeloid origin and immunoregulatory properties (46, 47). In CLL patients, an increase in M-MDSCs in the peripheral blood (PB) is associated with a poor prognosis (48, 49). Additionally, in CLL patients, M-MDSCs suppress T cell function and stimulate the production of Tregs. Lastly, CLL cells induce the transition of monocytes into M-MDSCs, suggesting cellular communication between CLL cells, M-MDSCs, and Tregs (50).

The intrinsic immune dysfunction observed in CLL results from a complex interplay of factors that collectively compromise the immune system's ability to respond effectively to the disease. By understanding these intrinsic mechanisms, researchers and clinicians can develop more effective strategies to restore immune function and enhance therapeutic approaches.

2.2 Therapy-induced immune dysfunction

Although chemotherapy, immunotherapy, and targeted therapy are vital for controlling CLL progression and improving outcomes, they often have significant side effects. A primary concern is these therapies' ability to severely compromise the immune system and increase the risk of infection (9, 15, 17). Moreover, such therapy-induced immune dysfunction affects the immune system's ability to perform essential surveillance, hindering its capability to detect and

eliminate residual cancer cells, potentially causing relapse or disease progression.

2.2.1 Chemotherapy

Commonly used chemotherapy drugs for CLL include alkylating agents, such as chlorambucil and cyclophosphamide, and purine analogs, such as fludarabine (18, 51–53). These drugs can lead to significant immune dysfunction. CLL patients receiving chlorambucil often experience bacterial infections, particularly in the respiratory system (54). Chlorambucil treatment can also cause lymphopenia, which significantly impacts the number of T cells, resulting in weakened adaptive immune responses (16). Patients receiving chlorambucil commonly have infections, including respiratory tract infections, bacterial pneumonia, and opportunistic infections (55). Cyclophosphamide combined with other agents, such as fludarabine, has been associated with neutropenia in CLL patients (56).

CLL patients treated with fludarabine alone have a heightened risk of infections, particularly if they previously received treatment for CLL, have advanced disease, or have a low neutrophil count (17). Of note, compared with chlorambucil, fludarabine is associated with a greater degree of neutropenia (52). Furthermore, purine analog-based treatment for CLL is associated with an increased risk of pneumonia, herpes simplex virus infection, and infections from other pathogens, such as *Pneumocystis jirovecii* (a fungal pathogen), *Listeria monocytogenes* (a bacterial pathogen), or cytomegalovirus (CMV; a viral pathogen) (17, 57).

2.2.2 Immunotherapy

The immunotherapy strategies used to treat CLL were reviewed previously (58). The introduction of rituximab, a chimeric monoclonal antibody targeting CD20, combined with chemotherapy, improved patient survival and paved the way for immunotherapy in CLL (18, 53, 56, 58). However, rituximab reduces NK cells' ability to mediate antibody-dependent cellular cytotoxicity against CLL cells (59). Newer generations of anti-CD20 monoclonal antibodies, such as obinutuzumab and ofatumumab, have significantly improved CD20-based immunotherapy (60, 61), but the spectrum of infections patients experience while on these medications is similar to that seen with rituximab. It has been reported that patients with comorbidities receiving obinutuzumab have higher rates of neutropenia than control arms (60). Untreated CLL patients show similar results when treated with ofatumumab as their initial therapy. Immune impairment remains a concern with anti-CD20 monotherapy, as cases of fatal reactivation of hepatitis B virus (HBV) and progressive multifocal leukoencephalopathy (PML) have been reported (62, 63). Additionally, late-onset neutropenia (LON) is a common side effect after the administration of anti-CD20 monoclonal antibodies (mAbs), occurring four or more weeks after the last dose (64–67). Patients with LON may develop subsequent infections, posing a challenge for clinicians and requiring close monitoring during and after therapy.

Another type of immunotherapy, CD19-targeted chimeric antigen receptor (CAR) T-cell therapy, is available for the treatment of CLL. While this therapy effectively eliminates CD19⁺

CLL cells, it also destroys normal B cells, resulting in B-cell aplasia (68). This condition can persist as long as CAR T cells continue to target CD19⁺ cells. The absence of functional B cells impairs the humoral immune response, thereby increasing susceptibility to infections.

2.2.3 Targeted therapy

Targeted therapies, which selectively inhibit crucial signaling pathways responsible for survival and proliferation, have transformed the treatment landscape of CLL. Kinase inhibitors like phosphatidylinositol 3-kinase (PI3K) inhibitors (e.g., idelalisib, duvelisib) (69) and Bruton's tyrosine kinase (BTK) inhibitors (e.g., ibrutinib, pirtobrutinib) (70) specifically target downstream BCR signaling pathways. B-cell lymphoma 2 (BCL-2) inhibitors, such as venetoclax, have also been integrated into CLL therapy (71). Despite their effectiveness, these interventions can exacerbate immune dysfunction (72, 73).

In a retrospective analysis of 378 patients with lymphoid cancers, Varughese et al. found that 53.3% of those receiving ibrutinib as monotherapy developed invasive bacterial infections, and 37.2% developed invasive fungal infections (10). This and other studies' findings suggest that ibrutinib alters neutrophils' ability to respond to infections. Blez et al. found that ibrutinib reduces neutrophils' reactive oxygen species (ROS) production, impairs their phagocytosis ability, and alters their cytokine release in response to *Aspergillus fumigatus*, a type of fungus (74). In a complementary study, Risnik et al. found similar results, as well as a slight impairment in neutrophil extracellular trap (NET) formation and a reduction in the activation of $\gamma\delta$ T cells (75). In addition, several clinical trials have shown that patients receiving BTK inhibitors, like ibrutinib, have an elevated risk of infection with *Aspergillus*, *Cryptococcus*, and *Pneumocystis jirovecii*, as well as decreased NK cell, monocyte, and macrophage function (16). Lastly, in a clinical trial where patients were treated with the PI3K inhibitor idelalisib or placebo in combination with bendamustine plus rituximab, patients receiving idelalisib had a higher rate of infections, including opportunistic infections such as CMV or *P. jirovecii* (76).

3 Neutrophil biology in homeostasis and in cancer

Neutrophils are seen as primary responders to inflammation and infection. They rapidly initiate an immune response through degranulation, phagocytosis, ROS production, and NET formation (77–81). Once they eliminate a threat, they are quickly removed from the site, leading to a high turnover rate. However, emerging evidence suggests that neutrophils play roles beyond frontline defenders (Figure 2). The following section will examine their role in homeostasis and cancer.

3.1 Neutrophil development

Granulopoiesis, the process of neutrophil development, begins in the bone marrow where hematopoietic stem cells (HSC)

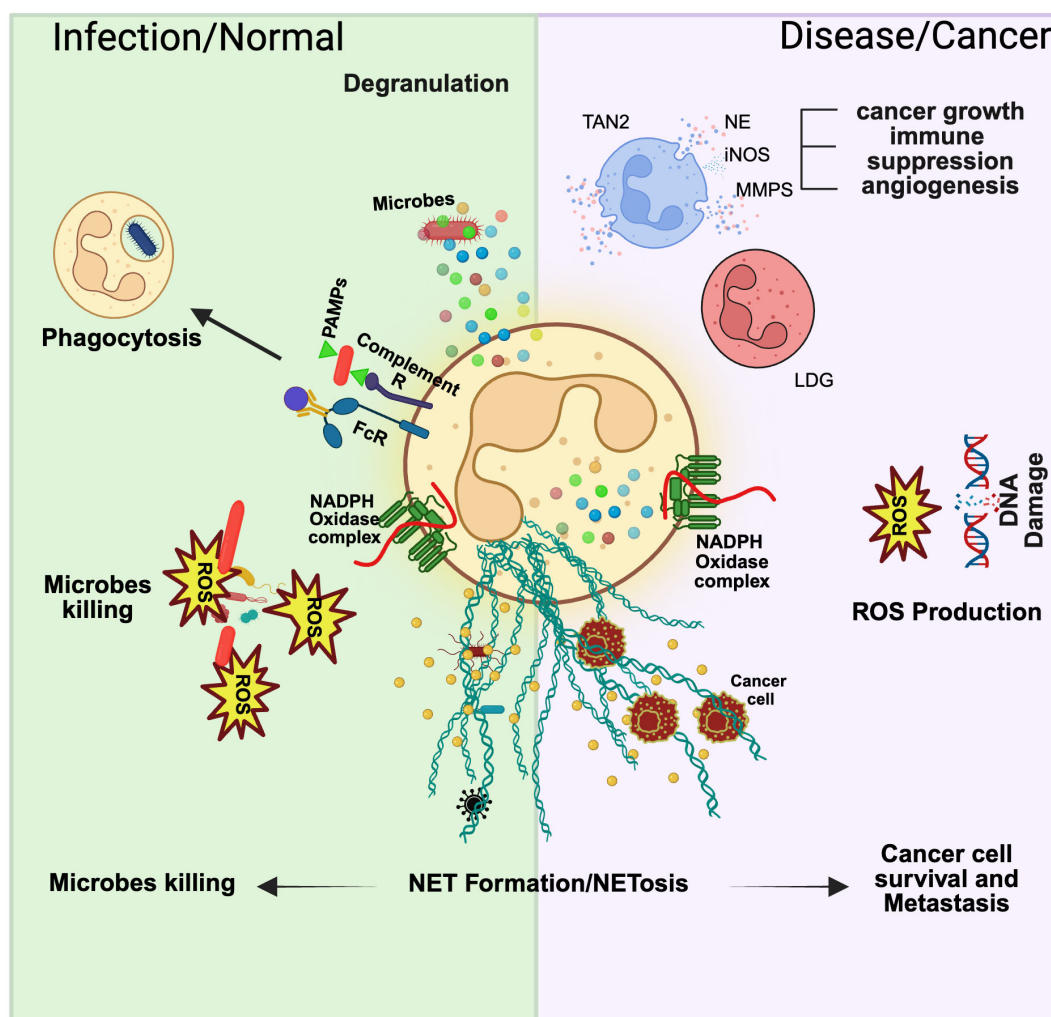


FIGURE 2

Neutrophil function in normal and disease states. Neutrophils display remarkable plasticity in their function, and the state of the host usually governs this. In normal physiological conditions, neutrophils can combat a pathogen via degranulation, phagocytosis, ROS production, and NETosis. In a disease or cancer context, these functions are modified, and various subsets of neutrophils can be identified in the bloodstream. Tumor-associated neutrophil type 2 (TAN2) is a cancer-specific neutrophil subset that can contribute to cancer growth, immune suppression, and angiogenesis. Low-density granulocytes (LDG) isolated from murine cancer models show overall reduced cytotoxic activity. Additionally, ROS production has been linked to DNA damage, which sparks carcinogenesis. Lastly, NET formation has been shown to favor cancer cell survival and metastasis by offering shielded protection during extravasation and migration.

differentiate into common myeloid progenitors [CMP (82)]. CMPs further differentiate into granulocyte-macrophage progenitors (GMP), which ultimately mature into neutrophils through a regulated process governed by various transcription factors. Recent research has identified unipotent cells within the neutrophil lineage; early neutrophil progenitors (eNePs) produce different types of neutrophils, categorized by function (83, 84). During the promyelocyte stage, neutrophils create primary granules such as neutrophil elastase (NE) and myeloperoxidase (MPO); during the myelocyte stage, they produce secondary granules. Once neutrophils develop into band cells, they form tertiary granules, such as arginase 1 (ARG1) and lysozyme. Fully differentiated neutrophils can leave the bone marrow in response to infection or inflammation (85–89). The bone marrow has a substantial reservoir of neutrophils. Granulocyte colony-

stimulating factor (G-CSF) regulates granulopoiesis by inducing the proliferation of granulocytic precursors and influencing the release of mature neutrophils via the CXCR4-CXCL12 axis (88, 90).

Neutrophils are traditionally viewed as short-lived cells (88). However, recent research suggests that their lifespan in a disease condition is longer than previously believed (91). Specific transcription factors, such as GATA binding protein 1 (GATA1) and CCAAT/enhancer binding proteins (C/EBPs), regulate neutrophil production and their release into the bloodstream (92). The factors controlling circadian expression remain unknown. Signals from various cell types (i.e., bone marrow stromal cells, endothelial cells, and osteoblasts) collaborate to regulate the release or withholding of CXCL12 (92). Lastly, neutrophils can survive for extended periods following their activation by cytokines, inflammatory mediators, or microbial products (93). After

completing their antimicrobial functions, neutrophils undergo apoptosis and are cleared by resident macrophages and dendritic cells (94).

3.2 Neutrophil function

Neutrophils rely on pre-existing effector molecules stored in intracellular granules to respond quickly to infection or inflammation (85). Degranulation is highly regulated and occurs hierarchically: secretory vesicles and tertiary granules are released first, followed by secondary and primary granules (95, 96). SNARE and Rab proteins regulate vesicle movement and degranulation (97).

Neutrophils produce ROS by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates superoxide (O_2^-) and other ROS with potent antimicrobial activity (98). For instance, hydrogen peroxide (H_2O_2), in conjunction with MPO, produces hypochlorous acid (HOCl), a highly microbicidal substance (99). Some microbes can thwart ROS production by releasing toxins that prevent the assembly of the NADPH oxidase complex (100). Neutrophils have additional mechanisms to effectively combat pathogens.

Phagocytosis is facilitated by receptors that internalize particles into the phagosome, which is crucial for pathogen elimination (80). Neutrophils identify pathogens through pathogen-associated molecular patterns (PAMPs) or opsonins, which initiate signaling cascades that facilitate particle engulfment (101). The phagosome matures into a microbicidal vacuole known as the phagolysosome, which triggers ROS production by NADPH oxidase (102).

Neutrophils also form NETs to prevent the propagation of infection (81). During NETosis, a dynamic cell death program, neutrophils release chromatin fibers containing nuclear DNA along with antimicrobial proteins, which act as a framework to trap pathogens (103). However, unregulated NET formation can harm the host, contributing to noninfectious inflammatory diseases, such as cystic fibrosis and vasculitis. Excessive neutrophil recruitment or activation is linked to acute and chronic conditions such as myocardial infarction, stroke, systemic lupus erythematosus (SLE), and cancer (4), raising interest in neutrophils as a key player in various diseases.

3.3 Neutrophil phenotypes, plasticity, and function in cancer

Much of our understanding of neutrophils in cancer comes from studies of solid tumors in mouse models, and this information can act as a rationale for understanding the role neutrophils play in hematologic malignancies. Recent research has emphasized the crucial role of neutrophils in cancer, showing that the neutrophil-to-lymphocyte ratio (NLR) acts as a prognostic indicator in various solid tumors (104). For example, a high neutrophil count indicates a favorable prognosis in colon cancer patients, whereas lower neutrophil counts are associated with disease progression in breast cancer patients. This suggests that neutrophils can

significantly influence the progression or regression of cancer (6, 105). NLR is emerging as a potentially useful prognostic indicator in hematologic malignancies, with studies suggesting that higher NLR values indicate increased inflammation and may be linked to poorer outcomes in patients (106). Neutrophils have been found to both combat and promote cancer by interacting with other immune cells within the TME and engaging in a range of functions, such as releasing substances that modulate the host response (107).

Neutrophils show remarkable adaptability in their role, either increasing or reducing inflammation. Their release of ROS is linked to DNA damage in lung cancer (108). In the TME, neutrophils are highly flexible, responding to signals that guide their activation and polarization, thereby influencing mechanisms that promote or hinder cancer development. It remains unclear whether neutrophils' anti- or pro-tumor activities stem from specific activation under different microenvironmental conditions or if certain neutrophil subsets are predisposed to specific functions (3). Further research is needed to clarify the mechanisms behind the diverse functions of neutrophil subsets in both solid and hematologic cancers.

Patients with solid tumors and autoimmune diseases may have distinct subsets of mature neutrophils, including low-density granulocytes (LDGs (3)) and tumor-associated neutrophils (TANs) (5, 107). TANs are categorized as TAN1 and TAN2, which have opposing functions. TAN1 possesses anti-tumor properties; it releases cytokines such as IL-12 and tumor necrosis factor- α (TNF- α), which aid in the recruitment and activation of CD8⁺ T cells. In a murine model of lung carcinoma, TAN1 was associated with early tumor growth, whereas TAN2 emerged during tumor progression, influenced by the TME (109). In another study, the inhibition of TGF- β resulted in more cytotoxic and pro-inflammatory neutrophils, whose phenotype resembled that one of TAN1, whereas the presence of TGF- β leads to a pro-tumor phenotype. In human cancers, early-stage TANs acted as antigen-presenting cells (APCs), facilitating the priming, proliferation, and activation of anti-tumoral T cells (110).

LDGs, or low-density neutrophils (LDN), were first identified in autoimmune diseases as a pro-inflammatory subset linked to SLE progression. LDGs are thought to contribute to SLE pathogenesis by releasing NETs, which cause vascular damage and create autoantigens that induce autoimmunity (111). Increased LDGs are observed in SLE patients with a significant interferon (IFN) signature, a common marker of various autoimmune diseases, especially SLE (112, 113). In SLE, IFN- α production by plasmacytoid dendritic cells primes neutrophils to release NETs via Toll-like receptor 9 (TLR9) signaling (114, 115). NET levels positively correlate with lymphoma progression and childhood acute leukemia development (116, 117). In solid cancer, LDGs from tumor-bearing mice show reduced anti-tumor cytotoxicity, decreased phagocytosis, lower ROS production, increased anti-inflammatory activity, and tumor-promoting properties (118).

Further research clarifying the specific behaviors and roles of neutrophil subsets within the intricate and dynamic human TME would enhance our understanding of how these neutrophil subsets interact with the TME and how they might be therapeutically targeted.

4 Phenotype and functional abnormalities of neutrophils in CLL

Neutrophils play a crucial role in the immune response, during which their recruitment and phenotype undergo significant changes that impact their functionality. Unlike other immune cells, neutrophils are released from the bone marrow as fully functional entities, capable of recognizing and neutralizing pathogens (3). However, in cancer, these cells often enter the bloodstream prematurely (91). This premature release can lead to modifications in their function and phenotype, potentially compromising their effectiveness in immune defense.

Various cytokines and chemokines influence the recruitment of neutrophils to the CLL TME. For example, the pro-inflammatory factors IL-8 (a chemokine) and IL-17A (a cytokine), which are secreted by monocytes and tumor-associated macrophages, play a central role in attracting neutrophils to the TME (119–121). In CLL patients, elevated levels of these cytokines are correlated with poor prognostic outcomes, demonstrating that they not only facilitate neutrophil recruitment but also contribute to disease progression (122, 123).

The phenotype of neutrophils in CLL is characterized by significant alterations that reflect their activated state. Key surface markers associated with neutrophil function, including CD11b, CD64, and CD54, are upregulated on neutrophils isolated from patients with progressive CLL (124). This state of activation is often induced by the microenvironment, where cytokines such as IFN- γ are elevated (125). While this activated phenotype may enhance certain functions, it does not necessarily translate into improved pathogen neutralization, as neutrophils in CLL continue to exhibit functional defects. Podaza et al. investigated how CLL cells affect the neutrophil reprogramming. They identified a specific immunosuppressive subset of neutrophils in the circulation of CLL patients, defined as CD16^{high}CD62L^{dim} neutrophils, whose formation depends on the secretion of IL-10 by leukemic B cells (126).

Recruited and activated neutrophils in CLL appear to have a reduced capacity for apoptosis. They show increased resistance to apoptosis *in vitro*, particularly when cultured with G-CSF (126). This survival advantage may lead to the accumulation of neutrophils in the microenvironment, potentially skewing their functional capabilities and contributing to the overall dysregulation of the immune response. Podaza et al. also assessed how G-CSF and GM-CSF extend the lifespan of neutrophils from healthy donors when exposed to CLL-conditioned media. They found that removing these growth factors neutralizes their antiapoptotic effects, showing that leukemic cell-produced factors are crucial for neutrophil prolonged survival. This evidence is clinically relevant, especially regarding the treatment of patient neutropenia. GM/G-CSF are commonly administered to boost neutrophil counts (127–130); however, a better understanding of the neutrophil-leukemic cell interactions may provide insights into the design of more effective treatment strategies with GM/G-CSF. Building upon this, G-CSF reduces neutrophil chemotaxis in stem cell transplant recipients for up to four weeks (131), underscoring

the importance of exploring the roles of neutrophils in disease and treatment modalities.

Functionally, neutrophils isolated from CLL patients exhibit significant abnormalities that compromise their ability to effectively combat infections. For instance, neutrophils demonstrate impaired phagocytic killing of key non-opsonized bacterial pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa* (7). This impairment underscores the crucial role of neutrophils in the innate immune response, highlighting the challenges in managing bacterial infections in CLL patients. It has been shown that obinutuzumab can significantly modulate the phagocytic function of neutrophils in CLL by enhancing neutrophils' expression of CD16b, which restores recognition and clearance of leukemic cells (132).

In addition to their phagocytic impairments, neutrophils in CLL exhibit reduced random migration and chemotaxis compared to healthy controls in response to stimuli such as *N*-formylmethionine-leucyl-phenylalanine (fMLP) and complement component 5a (C5a) (22). The impaired migration of neutrophils towards infection sites significantly hinders the immune response, making patients more vulnerable to infections.

Studies of neutrophils' capacity to produce ROS in CLL have yielded various results. *In vitro* studies demonstrated that stimulation with fMLP or phorbol myristate acetate (PMA) can significantly enhance ROS generation by neutrophils from CLL patients compared with neutrophils from healthy controls (124). Conversely, *in vivo* studies showed no significant difference in ROS production by neutrophils from the bone marrow of E μ -TCL1 mice compared with that by neutrophils from wild-type control mice (133). This difference emphasizes the need to develop more reliable research models that accurately mimic the complex immune microenvironment of CLL.

Finally, neutrophils from CLL patients release substantially more NETs following stimulation with PMA or a combination of TNF- α and lipopolysaccharide (LPS), compared to healthy donors (134). IL-8, whose levels are elevated in patients with CLL, has been shown to prime neutrophils to release NETs, thus suggesting a potential mechanism through which these cells may inadvertently support tumor progression while attempting to exert antimicrobial effects (121). These findings indicate that the leukemia microenvironment can influence dysfunctions specific to neutrophils, adding further complexity to the immune landscape in CLL.

5 Crosstalk between neutrophils and the immune microenvironment in CLL

Neutrophils play a role in regulating the adaptive immune response by directly interacting with T cells as antigen-presenting cells (APCs) and releasing various mediators that modulate the immune response (85). However, we have a limited understanding of how neutrophils in CLL interact with T cells directly. Podaza et al. documented that neutrophils isolated from patients with CLL

more effectively inhibit T cell activation than those isolated from healthy donors following stimulation with phytohemagglutinin (PHA) (126). The direct mechanism of this inhibition remains unexplored. In addition, Gora et al. demonstrated that Treg cells contribute to the immunosuppressive phenotype of neutrophils *in vivo* through the modulation of essential markers, including CD62L and IL-4R (135). In this study, the depletion of Tregs caused the restoration of the functional neutrophil immunophenotypic profile. Other studies have indicated that neutrophils can express immunosuppressive markers, such as programmed death-ligand 1 (PD-L1), which inhibits T-cell activation, suggesting that neutrophils play an immunosuppressive role in their relationship with T cells (136). Additionally, NETs may induce apoptosis of both CD8⁺ and CD4⁺ T cells, leading to reduced immunity (137). Whether and, if so, at what disease stage this immunosuppressive activity is present in CLL remains to be investigated.

We have a better understanding of the interaction between neutrophils and leukemic B cells, although not all aspects have been completely elucidated. CD16^{high}CD62L^{dim} immunosuppressive neutrophils have been shown to play a significant role in promoting B-cell maturation and survival by binding to BAFF and activating downstream pathways, including the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-dependent pathway, the non-canonical PI3K pathway, and protein B/mammalian target of rapamycin (AKT/mTOR) signaling pathway. At the opposite end of this interaction, leukemic B cells secrete G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF), which increases the lifespan of neutrophils by upregulating the anti-apoptotic protein, Bcl-2-related protein A1 (BFL-1) (126). In a study with E μ -TCL1 mice, neutrophils were predominantly found in the spleen's red pulp and marginal zone (MZ). Splenic peri-MZ neutrophils (also known as B-cell helper neutrophils (138)) expressed BAFF, APRIL, IL-21, and chemokines such as CXCL12 and CXCL13, which induce the immunoglobulin class switching, somatic hypermutation, and activation of MZ B cells (133). Depleting neutrophils in these mice significantly reduced the splenic leukemic burden (133). Further research is necessary to fully comprehend the direct impact of neutrophils on the adaptive immune response.

Although neutrophils' interactions with the myeloid compartment in CLL are largely unexplored, it is likely that they interact indirectly with monocytes, macrophages, and MDSCs. Monocytes in CLL have been shown to produce IL-8, which neutrophils utilize to generate NETs; IL-8 is also involved in neutrophils' chemotaxis and activation, and can enhance their survival and cytokine production (121). Furthermore, MDSCs and TAMs/NLCs have been shown to produce IL-10 in CLL (139, 140). From studies in mouse models of lung disease, which have shown that IL-10 impedes neutrophil recruitment, thereby hindering the clearance of bacterial infections, we can infer that IL-10 likely negatively affects neutrophil behavior in CLL; however, the mechanisms by which it does so are unclear (141, 142). Much work is yet required to understand the full impact neutrophils have on the immune microenvironment of CLL.

6 Targeting NET formation and neutrophil polarization

The role of NETs in cancer development varies depending on the TME. NETs have been found to delay spontaneous apoptosis in leukemic B cells from CLL patients and enhance the expression of activation markers. Various stimuli can initiate the formation of NETs through interactions with surface markers, such as cytokine receptors, TLRs, and damage-associated molecular pattern (DAMP) receptors (143). These receptors activate a series of cellular processes leading to the release of the chromatin-protein mixture into the extracellular space. Each of these steps represents a potential therapeutic target for regulating the tumorigenic role of NETs in solid tumors and eventually in B-cell malignancies. Hence, it is crucial to explore therapeutic strategies that not only effectively target the disease but also restore proper immune function (Table 1).

6.1 PAD4 inhibitors

The protein arginine deiminase (PAD) family consists of a group of enzymes that are responsible for the post-translational modification (PTM) of protein arginine residues through deamination or demethylation, leading to the formation of citrulline. PAD4 is the only member of the family with a nuclear localization sequence. PAD4 competes with other nuclear enzymes to modify multiple arginine residues in histone H3 and H4. The citrullination of histone H3 (CitH3) has been linked to NET formation, highlighting the potential significance of targeting PAD4 in pathological conditions associated with excessive NET formation, such as cancer (144, 145).

BMS-P5 was the first PAD4 inhibitor to be developed. Li et al. demonstrated that administering BMS-P5 to human multiple myeloma (MM) cell lines reduced NETosis. Furthermore, this compound inhibited NET formation in primary bone marrow cells obtained from MM patients. Subsequently, *in vivo* studies revealed a significant reduction in DNA and CitH3 in a BMS-P5-treated mouse model of MM (146).

JB1-589 is another promising selective PAD4 inhibitor, notable for its oral bioavailability. Studies have shown that JB1-589 can stimulate immune responses against lung tumors, resulting in a substantial delay in tumor progression in lung cancer. Like BMS-P5, JB1-589 exhibited a PAD4-mediated preventative effect against NET formation and citrullination in murine neutrophils and arthritis models (147). PAD4 inhibitors could offer a novel approach to treat CLL and restore the proper function of neutrophils by disrupting pro-survival signals that leukemic B cells receive from NETs in the TME.

6.2 Nanoparticle-mediated sivelestat

Nanoparticles have emerged as a promising strategy for the targeted delivery of compounds to cells. Sivelestat was first used to target acute lung injury caused by systemic inflammatory response syndrome (SIRS

TABLE 1 Targeting NET formation and neutrophil polarization.

Molecule Name	Description	References
PAD4 Inhibitors		
BMS-P5	First PAD4 inhibitor; reduces NETosis and decreases DNA and CitH3 levels	144, 145, 146
JB1-589	Oral bioavailability; reduces NETosis and citrullination	144, 145, 147
Potential Targeted Delivery Method		
Nano-particle-mediated Sivelestat	Reduces serum NE, and other pro-inflammatory cytokines; it also decreases NET formation	148, 149
Alternative Uses of FDA-approved Medications		
Prostaglandin E2 (PGE2)	In neutrophils, it can regulate NET release	153, 154, 155
Disulfiram	FDA approved for alcohol use disorder, in neutrophils it blocks GSDMD, which is critical for NET formation	157, 158
Targeting Neutrophil Polarization		
	Expose primary neutrophils to the TAN1 cocktail to repolarize tumor-promoting cells	159

(148)). This compound specifically inhibits the NE stored in neutrophil granules. Cruz et al. demonstrated the efficacy of nanoparticle-mediated sivelestat delivery in inhibiting NET formation (149). They noted a significant decrease in serum levels of NE and other proinflammatory cytokines; furthermore, treatment with nanoparticle-mediated sivelestat protected mice against endotoxic shock. Nanoparticle-mediated agents could serve as innovative therapeutic options able to reduce off-target effects, circumvent resistance mechanisms, and enhance drug effectiveness (150–152). These potential benefits could aid patients with CLL.

6.3 PGE2

Prostaglandin E2 (PGE2) is an endogenous protein with oxytotic properties that is widely seen in various clinical settings, including gastrointestinal cancer (153, 154). Studies with patients who have received autologous and allogeneic hematopoietic stem cell transplants have shown that PGE2 can inhibit NET formation through the induction of protein kinase A and Epac (155). In neutrophils, PGE2 effectively regulates NET release via autophagy induction, highlighting this compound’s ability to regulate NET formation and release. Besides its role in NET formation, PGE2 is linked to several pro-tumor mechanisms, including suppressing antitumor immunity, regulating tumor immune evasion, and promoting tumor progression (156). The specific role of PGE2 in CLL remains unclear; therefore, further research is needed to determine if inhibiting PGE2 could be an effective strategy.

6.4 Disulfiram

Disulfiram, a medication approved by the U.S. Food and Drug Administration for alcohol use disorder, inhibits aldehyde dehydrogenase. Studies have demonstrated its ability to block gasdermin D (GSDMD) in macrophages. In neutrophils, GSDMD is a pore-forming protein crucial for NET formation (157). Adrover et al.

demonstrated that disulfiram effectively inhibited PMA-induced NET formation by neutrophils isolated from the peripheral blood of patients with COVID-19 (158); they also observed similar inhibitory effects in a mouse model of severe transfusion-related acute lung injury. In CLL, it is unexplored whether disulfiram can effectively reduce NET formation. A concern is its impact on macrophages, which may aggravate the immune dysfunction observed in patients. Overall, exploring alternative uses for FDA-approved drugs might present valuable opportunities for patients.

6.5 Neutrophil polarization-targeting therapy

Neutrophils exhibit remarkable plasticity, capable of assuming different phenotypes based on their maturation and surrounding environment (i.e., TAN1 or TAN2). Ohms et al. pioneered the attempt to polarize human neutrophils into distinct subsets. They exposed human neutrophils to either a TAN1 cocktail (containing LPS, IFN-β, and IFN-γ) or a TAN2 cocktail (containing L-lactate, adenosine, TGF-β, IL-10, PGE2, and G-CSF) and demonstrated that each respective cocktail could polarize mature neutrophils into tumor-associated subsets (159). Their findings suggest that interventions designed to polarize tumor-promoting neutrophils into anti-tumor subsets can be used to treat cancer and eventually CLL. Integrating these therapeutic strategies into the current treatment landscape for CLL might have the potential to significantly reduce neutrophil-mediated immune dysfunction in patients. By specifically targeting the formation of NETs and modulating the immune response, these approaches may not only hinder tumor progression but also improve overall patient survival rates.

7 Discussion

This review highlights the complex role of neutrophils in CLL, emphasizing the need to understand their multifaceted functions in

both healthy and diseased states. Neutrophils, as the first responders of the innate immune system, play a crucial role in fighting infections and maintaining immune homeostasis. In CLL, however, their functions are significantly altered, which contributing to an immunocompromised state. This review identifies key abnormalities in neutrophil behavior, including decreased phagocytic abilities and increased formation of NETs, which not only fail to effectively eliminate pathogens but also may inadvertently support tumor progression.

This review also discussed how CLL affects neutrophils' recruitment, phenotype, and function, thereby impacting their responsiveness to infection. Immunosuppressive neutrophil subsets, such as CD16^{high}CD62L^{dim} neutrophils, might be involved in the deep immune dysfunction observed in CLL patients, highlighting the need for targeted therapeutic strategies aimed at immune restoration. The therapeutic landscape for CLL has evolved with the introduction of various treatment modalities, including chemotherapy, immunotherapy, and targeted therapies. While these treatments are essential for managing CLL, they often lead to immunosuppression, presenting a dual challenge for clinicians. Understanding the impact of these therapies on neutrophil function is critical, as it may inform patient management strategies to reduce the increased risk of infections. We emphasize the importance of exploring therapies that can enhance tumor-killing efficacy while addressing the underlying immune fitness. For instance, preclinical studies on the use of lenalidomide in combination with CD23-targeted CAR T-cell therapy showed how to enhance T-cell function and restore normal immune responses. Therapeutic combinations that address both intrinsic and therapy-induced immune dysfunctions should be further explored.

Advanced research is essential to elucidate the interactions between neutrophils and other immune cells in the CLL microenvironment. This knowledge could pave the way for innovative therapeutic approaches aimed at restoring proper immune function. The discovery of neutrophil-specific biomarkers that predict treatment responses and enable tailored therapies could significantly improve patient outcomes; targeting neutrophil-related immune dysfunction presents a promising avenue for advancing CLL treatment. Investigating the mechanisms that regulate neutrophils' behavior and their contributions to the immune landscape may lead to interventions that enhance the effectiveness of existing therapies.

In conclusion, neutrophils play a critical yet often overlooked role in the immune dysfunction associated with CLL. Addressing their dysregulation and understanding their interactions within the immune microenvironment might lead to more effective treatment strategies and improved patient outcomes. As the field of immunotherapy continues to advance, integrating insights from neutrophil biology into therapeutic development will be essential for achieving sustained remission and enhancing the quality of life for CLL patients.

Author contributions

SM: Writing – original draft, Writing – review & editing. PK: Writing – review & editing. MB: Supervision, Writing – review & editing.

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