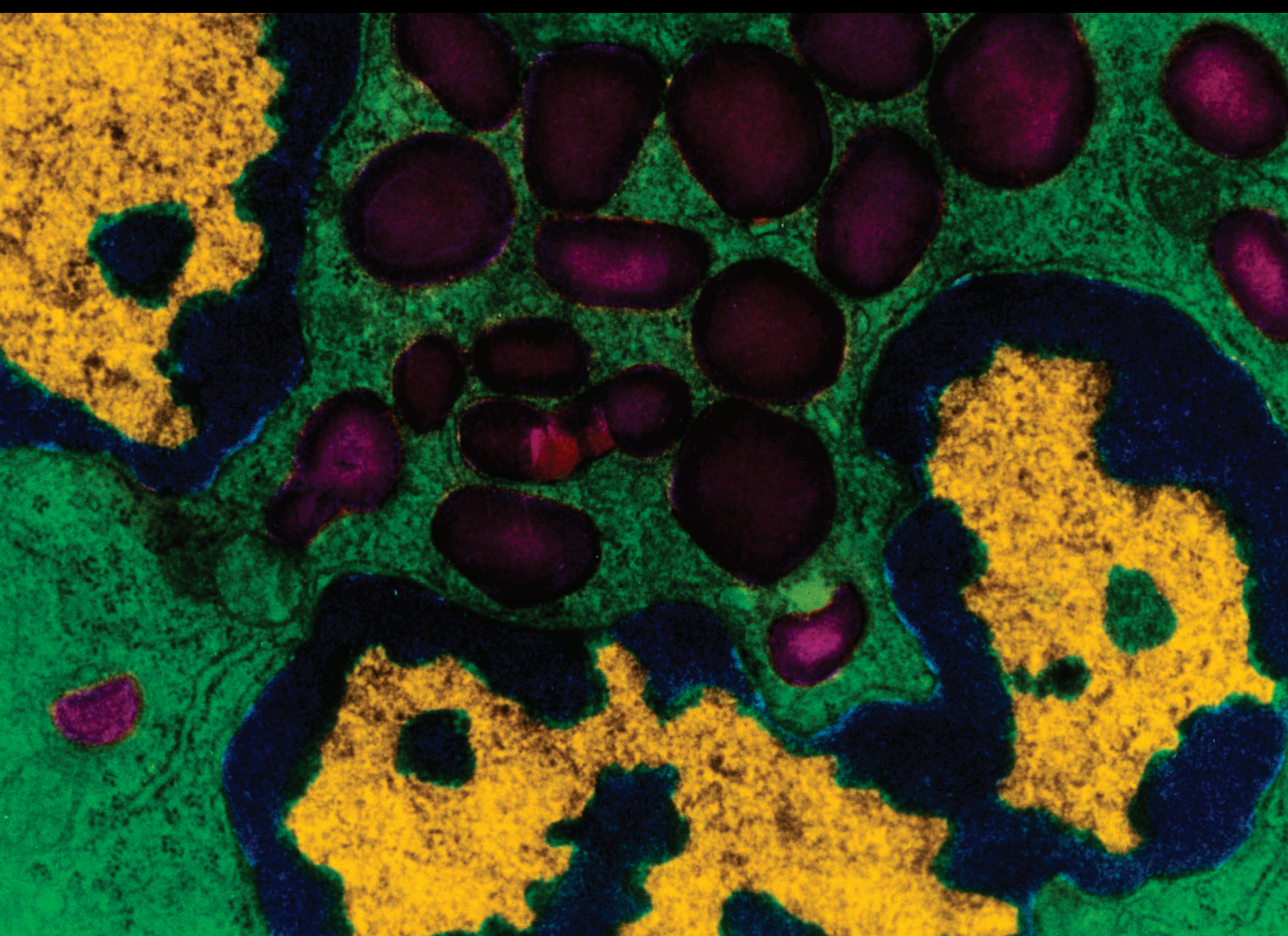
Mediators of Inflammation

Macrophages and Neutrophils: Regulation of the Inflammatory Microenvironment in Autoimmunity and Cancer

Guest Editors: Michal A. Rahat, Seth B. Coffelt, Zvi Granot, Munitta Muthana, and Amedeo Amedei



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Editorial Board

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Editorial

Macrophages and Neutrophils: Regulation of the Inflammatory Microenvironment in Autoimmunity and Cancer

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Macrophages and neutrophils are phagocytes that play major roles in the onset and maintenance of many diseases. These two cell types that belong to the innate immune system are extremely plastic and can move between different modes of activation upon cues received from their immediate microenvironment [1–3]. Once activated, these cells secrete myriad of mediators that shape and regulate the microenvironment, as well as other immune cells, such that this continuous dialogue determines the direction of the immune response and its outcome [4]. This is highlighted in this issue as we focus on the role of macrophages and neutrophils in both cancer and autoimmune diseases. Although these are different diseases, with opposing pathophysiologies and activation of the immune system, some similarities do exist [5]. By comparing these two cell populations in cancer and autoimmune diseases, in the context of their respective microenvironment, we try to examine whether there are similar attributes that could potentially be exploited as new therapeutic strategies. Most of the manuscripts in this issue are dedicated to cancer and the tumor microenvironment (TME), reflecting the abundance of information on macrophages, and the now growing recognition of the role that neutrophils play in the cancerous context. In contrast, the role that both macrophage and neutrophils play in autoimmune diseases is only beginning to emerge and merits more investigation.

We begin with the remote microenvironment. As the TME can remotely affect circulating blood cells, O. Balacescu et al. performed transcriptional analysis in blood samples derived from triple negative (HER2-ve) breast cancer patients (TNBC). These studies revealed distinct molecular signatures according to estrogen and progesterone receptor (ER and PR) status. They found significant enrichment of altered systemic immune-related pathways in the blood of TNBC patients and this correlated with an increase in inflammation and necrosis in primary tumors. The authors also propose that immunotherapy could possibly be synergistic to chemotherapy to improve the clinical outcome of these patients.

In a series of papers, the role of macrophages in cancer is addressed, as well as their potential to become targets or vehicles of therapy. C. Eyileten et al. highlight the role of immune cells and the cellular factors they produce, in promoting or preventing cancer development. They describe how these cells, which include tumor-associated macrophages (TAMs), dendritic cells, neutrophils, T cells, and NK cells, can be targeted or exploited to induce antitumor immunity and discuss the pioneering studies where these cells have been manipulated to exhibit antitumor activity.

The ability of TAMs to produce IL-6 and activate STAT3 has been associated with chemoresistance and poor prognosis in several cancers including colorectal carcinoma. However, it remains unclear whether anticytokine therapy could reverse this, help regain chemosensitivity, and enhance the suppressive effect of chemotherapy on tumor growth. Z.-Y. Wang et al. demonstrate that treatment of carboplatin

increased IL-6 production and STAT3 activation in a dose-dependent manner in the human colorectal LoVo cells, whereas anti-IL-6 neutralizing antibody enhanced their chemosensitivity to carboplatin, abolished STAT3 activation, and increased cell apoptosis. These results suggest a new way to increase the efficacy of chemotherapy.

Nonresolving inflammation is one of the consistent features of the tumor microenvironment and in the intestine; this plays a critical role in the initiation and development of colon cancer. In the study by Y. Wang et al., the inhibitory effect of a novel soy-protein-derived isoflavonoid on inflammation-related colon cancer cell proliferation is described. The anticancer role of a newly synthesized derivative of genistein, GEN-27, was shown to have both antiproliferative and anti-inflammatory properties in colon cancer cells and monocytic cells via modulation of the NF-κB pathway.

N. J. Brady et al. discuss macrophages in the context of normal mammary gland development and mammary tumorigenesis. The review highlights the vital role of macrophages in mammary gland generation and homeostasis, as well as their contribution to tumor formation and progression.

B. Sainz Jr. et al. review the current literature on cancer stem cells and macrophages. This article focuses on the molecular crosstalk between the two cell types within the premalignant niche and established tumors, which influences cancer progression.

We then proceed with the introduction of neutrophils and their function in cancer, as well as their interactions with macrophages. Z. Granot and J. Jablonska review the pro- and antitumor properties neutrophils exhibit, which are regulated by cues in the tumor microenvironment. Much like macrophages, neutrophils are not a homogeneous population of cells and can become either protumoral (N1) or antitumoral (N2). Moreover, neutrophils have a major role in generating the premetastatic niche, as indicated by the large number of neutrophils accumulated in such sites. However, whether neutrophils are activated as N1 or N2 is dictated by the TME. Much remains unknown about the possible activation modes of neutrophils, their biological markers, and their functions in the polarized state in the tumoral context.

M. Orozco-Morales et al. review the interplay between molecular inflammatory mediators and the immune cells recruited to Non-Small Cell Lung Cancer (NSCLC). They highlight the roles played by various factors in regulating the function of TAMs and Tumor-Associated Neutrophils (TANs) in the context of NSCLC. Finally, they discuss the role of tumor cell expressed CD47 in mediating immune evasion.

The inflammatory microenvironment, as studied especially in tumors but also in inflammatory diseases such as rheumatoid arthritis (RA), is very hypoxic, and the lack of available oxygen influences both macrophages and neutrophils, as discussed by A. Egners et al.. Hypoxia induces or activates major transcription factors, such as hypoxia inducible factors (HIFs) 1 and 2 and NF-κB, and those in turn mediate and regulate the hypoxic stress. This is manifested by major changes in every aspect of macrophage and neutrophil functions, including their migration and adhesion to endothelial cells, their ability to kill bacteria, their metabolism and polarization, production of cytokines, and protumorigenic activity, as reviewed in this paper.

The tumor microenvironment (TME) polarizes TAMs and TANs to become protumoral and support tumor growth and progression, invasiveness and metastasis, angiogenesis, and matrix remodeling, while inhibiting the antitumoral immune surveillance. In inflammatory microenvironments and in TME, neutrophils can recruit macrophages, and these, in turn, affect neutrophil functions, thereby exhibiting different degrees of interaction between these two cell types. Kim and Bae explore the biology of TAMs and TANs and their recruitment and polarization and discuss their possible interactions in the TME as well as their role in TME maintenance and their significance in clinical settings. They concluded that the introduction of more sophisticated tumor models and techniques to differentiate different myeloid cell subsets *in vivo* will reveal fundamental information about possible modulation of myeloid cells, including their interaction with platelets in each progression stage of different cancer types.

To finalize the issue, we address the role of macrophages and neutrophils in autoimmune diseases. The involvement of macrophages in autoimmune disease of the brain is highlighted in a review by X. Fan et al. Although current knowledge is quite limited, the ability of macrophages to polarize in different activation modes and carry out different and often opposing tasks renders them important mediators of the pathogenesis of diseases such as neuromyelitis optica (NMO), myasthenia gravis (MG), and Guillain-Barré syndrome (GBS), and burning questions, such as whether macrophages can be targeted or used as future therapeutic agents, must be further explored.

Concluding this special issue is a detailed review by M. A. Rahat and J. Shakya that draws parallels between the cancerous and autoimmune microenvironments. From the point of view of the immune response, cancer and autoimmune diseases, where the immune system is suppressed or hyperactivated, respectively, are opposites. Nonetheless, many elements in the microenvironments are common in both diseases including hypoxia, angiogenesis, the presence of autoantibodies, and cytokine concentrations. Of course, the critical role that myeloid cells, macrophages, and neutrophils in particular play in these diseases and the detailed understanding of the similarities and differences in the two contexts may eventually lead to novel approaches to immunotherapies.

Collectively, these papers highlight the critical importance of macrophages and neutrophils in the microenvironments of both cancerous and autoimmune diseases. These cells can sense the changing microenvironment and interpret the signals and via complex interactions with other tissue cells and infiltrating immune cells, including the interactions between macrophages and neutrophils themselves, they regulate the progression of the immune response in these diseases [6]. These different interactions could become a focus of research in the field in the coming years. Moreover, although the role that macrophages play in the cancer microenvironment has been extensively studied in the last decade, still some areas deserve more attention, for example, the crosstalk between different microenvironmental factors such

as hormones and hypoxia. Neutrophils bear many similarities to macrophages, in their secretome and their killing and proangiogenic abilities and in the way the microenvironment activates them in different and opposing manners. However, this has only recently begun to be unveiled, and more research directed at neutrophil characterization, understanding of their contribution, and deciphering their interactions with different cell populations must be invested. And lastly, the current knowledge and the findings described in this issue point to several new insights into the mechanisms of current and potential therapies and suggest new possible combination therapies that could benefit patients and should be further explored.

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Review Article

Immune Cells in Cancer Therapy and Drug Delivery

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Recent studies indicate the critical role of tumour associated macrophages, tumour associated neutrophils, dendritic cells, T lymphocytes, and natural killer cells in tumourigenesis. These cells can have a significant impact on the tumour microenvironment via their production of cytokines and chemokines. Additionally, products secreted from all these cells have defined specific roles in regulating tumour cell proliferation, angiogenesis, and metastasis. They act in a protumour capacity *in vivo* as evidenced by the recent studies indicating that macrophages, T cells, and neutrophils may be manipulated to exhibit cytotoxic activity against tumours. Therefore therapy targeting these cells may be promising, or they may constitute drug or anticancer particles delivery systems to the tumours. Herein, we discussed all these possibilities that may be used in cancer treatment.

1. Introduction

Neoplasm is a systemic disease where cancer cells act as a leading devil supported by other cells in the surrounding environment. Particularly, inappropriate activation of the stroma and distant metastasis induced by its components can potentiate and accelerate tumour progression towards a high rate of disease mortality [1]. This microenvironment may differ depending on the tumour type and tissue of origin. It is usually composed of the fibroblasts, adipocytes, pericytes, endothelial cells, and immune cells (macrophages, neutrophils, lymphocytes, dendritic cells, natural killers, or myeloid-derived suppressor cells) which contribute to the tumour progression.

2. Macrophages as Drug Targets

Tumour associated macrophages (TAMs), which reside in the tumour mass, play central role in this intratumoural dialog [2]. Cells of the monocyte-macrophage lineage are characterized by considerable diversity and plasticity. In response to various signals, macrophages may undergo classical or alternative activation called M1 or M2, respectively. However, currently it is known that macrophages do not form stable subsets which could be clearly distinguished among each other but respond to a combination of factors present in the tissue which can change their phenotype towards many subforms. Therefore, it is recommended to characterize macrophages by the cytokine used for the activation instead of naming them M1 or M2 [3]. Classically activated macrophages (e.g., LPS activated) have the potential to exhibit antitumour activity whereas alternatively activated (e.g., IL-4 activated) macrophages (called in tumours TAMs) generally have low tumouricidal activity but they promote tissue remodeling and angiogenesis [4]. Therefore they promote tumour development and its spread to distant sites. However, due to high plasticity of macrophages, this process may be reversible and therefore therapeutically exploitable.

The research concerning macrophages in cancer escalated after Lin et al. showed the role of colony stimulating factor 1 (CSF-1) in tumour development, which is normally required for macrophage development. Number and size of primary tumours in CSF-1 knockout mice were similar to the control mice [5]. However CSF-1 deficient mice had lower macrophage number and decreases in tumour progression and metastatic spread. Furthermore, blocking of the CCL2 (chemokine ligand 2), which is secreted by breast cancer cells, in order to recruit metastasis-associated macrophages from the circulation, slows down the growth of tumour metastases [6]. Johnson & Johnson developed CCL2 blocking antibody named CNTO 888 (carlumab) which shows binding affinity to human CCL2 and therefore it decreases macrophage infiltration at the site of challenge. The CNTO 888 is currently in clinical trials for solid tumours; however it does not show antimetastatic activity when used as a single therapy, neither does it block CCL2-CCR2 axis in prostate cancer [7].

Another approach of antimacrophage therapy is to use CXCR4 inhibitors (which are anti-HIV drugs: AMD3100, AMD1498, ALX40-4C, or T22) [8]. The CXCR4 receptor lies downstream in the Hypoxia Inducible Factor (HIF) pathway and therefore increases macrophage infiltration in the tumour and takes part in angiogenesis and cancer progression. Using a mouse model of breast cancer, Welford et al. showed that one of the compounds mentioned above (AMD3100) reduced macrophage recruitment to the tumours and significantly augmented the antitumour efficacy of combretastatin A4P [9]. These results supported previous findings of Welford et al. that TIE-2^+ macrophages limit the efficacy of combretastatin.

Lisa Coussens has developed a completely different drug limiting macrophage infiltration to the tumour. This molecule called PLX3397 (provided by Plexxikon) targets CSF-1R and when used together with standard chemotherapy, in mice with aggressive mammary cancer, reduced pulmonary metastases regulated by macrophages. PLX3397 increased the cytotoxic T lymphocyte infiltration which resulted in reduced primary tumour development, decreased pulmonary metastases, and improved overall survival [10]. Our own experiments showed that targeting of CSF-1/CSF-1R axis may be a good therapeutic approach in cancer cells [11]. We showed that *csf-1r* silencing significantly increased apoptosis, decreased proliferation, and decreased migration of canine mammary cancer cells. It also changed growth characteristics of highly invasive cell lines on 3D matrix significantly decreasing the invasive ability of these cells.

We also showed that manipulating within Wnt signaling may be also a good therapeutic approach. For the first time, tumour associated macrophages mediated a "switch" between canonical and noncanonical Wnt signaling pathway in cancer cells [2]. This "switch" leads to inhibition of canonical Wnt pathway by noncanonical Wnt pathway. Macrophages secrete proteins that inhibit canonical Wnt pathway and decrease cancer cell proliferation and survival. However, the side effect of their function, *ipso facto*, is the activation of noncanonical Wnt pathway. The noncanonical Wnt pathway promotes epithelial-mesenchymal transition, cancer cell motility, invasiveness, and as a consequence their metastasis (Figure 1). Thus, some kind of modulation of macrophage effect on cancer cell by inhibiting of noncanonical Wnt pathway in tumour cells could create a novel and very attractive approach in cancer treatment. This approach would be interesting because it does not impair anticancer activity of macrophages (decrease of tumour growth and survival by inhibition of canonical Wnt pathway) but it may reduce their side effects (metastasis by inhibition of noncanonical Wnt signaling).

An interesting approach to use macrophages in cancer therapy was proposed by Tseng et al. [12] who used macrophages to increase T cell immune response. Exploiting anti-CD47 antibody-mediated phagocytosis of cancer cells by macrophages, the authors showed increased priming of $CD8^+$ cells but decreased priming of $CD4^+$ cells. It resulted in reduction of regulatory T cells level and decreased tumour mass in animals. Therefore, the conclusion is that anti-CD47 antibody treatment not only enabled macrophage phagocytosis of cancer cells but could also initiate an antitumour cytotoxic T cell immune response.

Pahl et al. [13] tried to modify the macrophage phenotype and thus to induce their antitumour response. By treating M1-like macrophages with liposomal muramyl tripeptide (L-MTP-PE) and IFN-γ, authors observed a cytotoxic effect of activated macrophages towards osteosarcoma. This effect was also observed when osteosarcoma cells were treated with supernatants of activated macrophages, suggesting not only direct phagocytosis but also involvement of soluble factors in the cytotoxic effect upon macrophage activation. Whereas stimulation of M2-like macrophages, with LPS and IFN-γ, did not drastically change their low antitumour activity, M2 macrophages, stimulated by IL-10 alone, had no impact on tumour growth. However, Pahl et al. demonstrated that IL-10-polarized macrophages had high cytotoxicity towards osteosarcoma cell lines in the presence of anti-EGFR antibodies, which induced antibody-dependent tumour cell phagocytosis. These data suggest the possible benefits of modifying macrophage function based on its subtype within the tumour.

2.1. Macrophage Transfer. After the development of technology to generate macrophages *in vitro* from blood monocytes, clinical trials in cancer patients have proven the safety of infusing autologous macrophages activated by interferon-gamma or lipopolysaccharide. Adoptive transfer of host cells may be able to correct defective generation of competent immune cells in patients with cancer [14]. Since the safety of M1-activated macrophages therapy has been proven, several studies using macrophages as a delivery system have been published. Griffiths et al. [15] demonstrated the potential of macrophage use as a cell-based delivery system for gene dependent enzyme prodrug therapy. Macrophage-mediated delivery of the CYP2B6 gene under the constitutive CMV promoter resulted in tumour cell killing in the presence of the prodrug, cyclophosphamide.

In 2011 Muthana et al. [16] showed a novel system that used infiltration of classically activated macrophages, transduced with hypoxia-regulated oncolytic adenovirus in which proliferation was restricted to prostate tumour cells. Using this system, authors markedly inhibited tumour

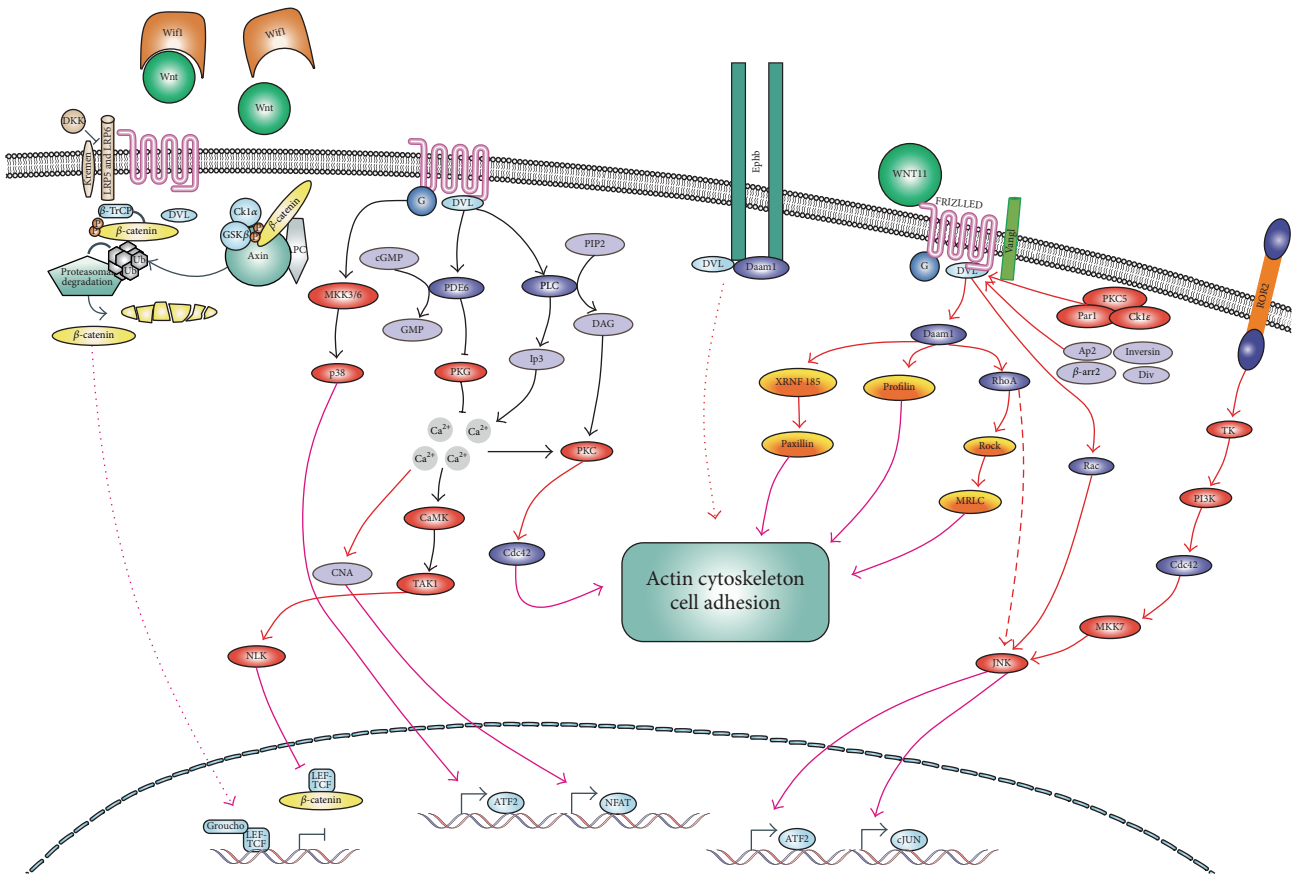

FIGURE 1: Wnt signaling and macrophages. Wnt signaling pathway is one of the most important pathways regulating cells proliferation, differentiation, polarity, and migration. At least two distinct pathways transduce Wnt signals: canonical Wnt/β-catenin pathway and the β-catenin independent noncanonical Wnt pathway (Wnt/Ca^{2+} signaling and Wnt/planar cell polarity (PCP) signaling). Wnt/β-catenin signaling pathway is upregulated in many cancers. Lack of β-catenin degradation and its nuclear accumulation is an evidence of activated Wnt/β-catenin pathway. β-catenin acts in the nucleus as a transcription factor increasing cancer proliferation and survival. Activation of Wnt/PCP signaling during development results in cytoskeleton remodeling (by Rho, Ras, and JNK) promoting cell movement. Calcium dependent Wnt increases the motility of various cell types by regulating the formation of lamellipodia. It also increases expression of vimentin and therefore induces an epithelial-mesenchymal transition, the crucial step in metastasis. Macrophages infiltrated to tumour mass inhibit cell proliferation as effect of inhibition of Wnt/β-catenin pathway. However canonical and noncanonical Wnt pathways work on the principle of antagonism, so macrophages inhibiting β-catenin pathway activate noncanonical Wnt pathway and lead to cytoskeleton remodeling in cancer cells and facilitate their motility.

growth and reduced metastases. The same group successfully tested this system after chemotherapy or irradiation during increased tumour infiltration by macrophages [17]. Using this method, authors were able to significantly increase life-span of tumour-bearing mouse as compared to control.

Seo et al. [18] established genetically engineered stable macrophages of RAW264.7 cell line and used them to deliver the prodrug-activating enzyme to the lung melanomas. Animals were treated with inactive prodrug which underwent activation in macrophages. The therapy reduced tumour weights and numbers of melanoma foci.

Firstly, these data indicate that macrophages can constitute targets of anticancer therapy; however the disadvantage of this method is reduction of their positive and physiological activity in live organism. Therefore modulation of their activity seems to represent more appropriate approach. Secondly, macrophages can be exploited as carriers in a gene therapy or as carriers of enzymes activating prodrugs. Macrophages infiltrate diseased tissue and may respond to the hypoxic microenvironment by expression of a therapeutic gene or enzyme. However, the use of viruses in this kind of approach might create unpredictable risk, not only to the treated individuals but also to the population as a whole. The clinical application of oncolytic viruses should be regulated by specific guidelines at international levels. Furthermore, because of the biological limitations of animal models, safety of their preclinical testing should be widely discussed. Cancer is a disease demanding aggressive approaches. However, the balance of risk and benefit must always be of prime consideration, not only for the patients but also for the whole population [19].

3. Neutrophils

Neutrophils are traditionally considered as the first line of host defense against invading pathogens [20]. They kill

invading pathogens by releasing activating cytokines along with reactive oxygen species (ROS). Despite the role in host defense, they have impact on tumour development being part of its microenvironment [21] and they also have powerful antitumoural effect under certain circumstances [22]. However, the role of neutrophils in the tumour microenvironment is not yet fully understood. Recent studies demonstrated that tumour associated neutrophils (TANs) can promote tumour development, increase metastasis, and enhance angiogenesis [23]. On the other hand some studies showed that neutrophils can be cytotoxic for the tumour cells *in vitro* and *in vivo* [24, 25]. Similarly to macrophages the first are called TAN-2; the latter are called TAN-1.

3.1. Neutrophils in Tumour Progression. Neutrophils were shown to have angiogenic effect through the release of multiple factors. TANs have influence on tumour cells via oncostatin M which is a cytokine belonging to interleukin-6 (IL-6) family [26]. In the experiment of TANs coculture with breast cancer cells, this cytokine induced angiogenesis and invasiveness of the latter [27]. Additionally, ROS released by neutrophils may play an important role in tumour progression. Güngör et al. demonstrated that major neutrophilic oxidant hypochlorous acid (HOCl) induced three different types of DNA damage and mutagenicity *in vitro* in human alveolar epithelial lung cells [28]. It was also reported that the proteinase of neutrophil elastase (NE) produced by TANs promotes tumour cell proliferation in both human and mouse lung adenocarcinomas [29]. Another potential direct effect of neutrophils on tumour progression is secretion of matrix metalloproteinase-9 (MMP-9) enzymes [30]. Bekes et al. demonstrated that highly metastatic human fibrosarcoma and prostate cancer cells recruit neutrophils to primary tumours, which increased angiogenesis and intravasation of cancer cells due to secretion of MMP-9. In their study, inhibition of neutrophil influx by interleukin-8 (IL-8) neutralization decreased tumour angiogenesis and intravasation [31]. In addition, it was shown that secretion of MMP-9 by neutrophils prevents apoptosis of tumour cells and induces carcinogenesis [32]. More recently a publication by Bald et al. showed the involvement of neutrophils in induction of migration and metastasis of melanoma cells [33]. In that study, UV-damaged epidermal keratinocytes released nuclear proteins (high mobility group box 1, HMGB1) that caused recruitment and activation of neutrophils. Activated neutrophils produced TNFα that increased motility of melanoma cells.

3.2. Neutrophils in Therapy. The first reports of antitumoural effect of neutrophils were published in 1970; Bubeník et al. and Godleski et al. showed neutrophil activity against human bladder tumours and rat mammary gland carcinosarcoma, respectively [34, 35]. In 1972 Pickaver et al. [36] described the first direct evidence of the cytotoxic effects of neutrophils on tumour cells. They demonstrated that rat neutrophils collected from the peritoneum and incubated with syngenic tumour cells were able to kill them. Neutrophils produce proteases, ROS, and defensins [37] that can directly damage targeted cells [37, 38]. Dallegri et al. showed apoptosis and necrosis of tumour cell due to increased secretion of HOCl by neutrophils [39]. Moreover, the cytotoxic effects of neutrophils on tumour cells can be increased via target-specific antibodies [40, 41] interacting with the Fcγ receptors on the surface of neutrophils via their Fc tail [42] inducing antibody-dependent cellular cytotoxicity. Repp et al. showed that neutrophils obtained from patients treated with recombinant human G-CSF expressed FcγRI receptor which is a high affinity receptor for IgG [43]. Two years later the same group found that neutrophils from patients treated with recombinant human G-CSF are more effective in inducing antibody-dependent cellular cytotoxicity against glioblastoma, squamous cell, and ovarian and breast carcinoma in contrast to the neutrophils from healthy, untreated donors [44]. Another study showed that the concurrent administration of G-CSF and rituximab (a chimeric antibody against the CD20 antigen on normal and malignant B cells) increased the survival rates of mice with non-Hodgkin's lymphoma. The experiments conducted *in vitro* and *in vivo* demonstrated the role of neutrophils stimulated by G-CSF in enhancing the biological antitumour activity of rituximab [45]. Another approach to achieve the antitumoural effect of neutrophils is to change the immunity of tumours. For example, Cavallo et al. transduced mouse mammary adenocarcinoma cell line (TSA) with the IL-2 genes inducing local inflammatory reactions. TSA-IL-2 cells caused neutrophil infiltration to the tumour mass [46]. Similarly, using IL-10-expressing mouse mammary adenocarcinoma model, it was demonstrated that neutrophils play the key role in the early rejection of the tumour [47]. To determine the biological importance of IL-8 which is a strong chemoattractant for neutrophils [48], Schaider et al. examined melanoma cells from primary and metastatic lesions. These cells, when transduced to produce low levels of IL-8, showed impaired growth *in vivo* due to massive neutrophil infiltration [49]. Similarly, ovarian cancer cells, transduced with the IL-8 human and the murine MIP-1a genes, showed impaired tumourigenicity when injected into nude mice. That was accompanied by the massive neutrophil infiltration in the tumour injection site [50].

Another effective approach to increase number of tumour infiltrating neutrophils is to use live bacteria or certain bacterial products. For example, *Mycobacterium bovis* [51], *Corynebacterium parvum* [52], *Clostridium novyi* [53], *Salmonella typhimurium* [54], and *Salmonella choleraesuis* [55] induced neutrophil infiltrations to the tumour microenvironment. Lee et al. administered *S. choleraesuis* to the mouse with orthotopic hepatocellular carcinoma in order to stimulate a potent inflammatory response. It caused reduced intratumoural microvessel density, increased infiltration of neutrophils, induced cancer cell death, and significantly prolonged survival [55]. Since the 1970s *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) vaccine has been commonly used as an adjuvant treatment for bladder cancer after surgery. Just after BCG administration the massive neutrophil infiltration occurs in the bladder [56]. Suttmann et al. supported that neutrophils are compulsory for efficient BCG immunotherapy of bladder cancer and local immune responses [57]. Since then, several other studies also confirmed that BCG-stimulated neutrophils are highly effective in immunotherapy for bladder cancer [58, 59]. Furthermore,

Jinesh et al. [60] showed that RT4v6 bladder cancer cells are resistant to BCG-activated TANs. They demonstrated the critical role of increased TNF-α in the anticancer effects of BCG-stimulated neutrophils. Using Smac mimetic compound for neutrophil stimulation they effectively killed bladder cancer cells. Antitumour effect of TANs was also demonstrated by Fridlender et al. [22]. They treated mice with SM16, which is a transforming growth factor-β (TGF-β) inhibitor, and used monoclonal anti-Ly6G antibody for the systemic depletion of neutrophils. This strategy showed that, after TGF-β blockage, TANs can undergo N1 phenotype which produce higher levels of TNF-α, MIP-1α, NO, and H_2O_2 and have antitumourigenic and proinflammatory characteristics. Andzinski et al. [61] showed that IFN type I stimulation induced neutrophils polarization towards antitumour phenotype both in mice and in human.

Jaganjac et al. [62] reported that neutrophil infiltration at the site of W256 carcinoma cells in Sprague-Dawley rats was associated with spontaneous tumour regression. Similarly, injection of Sephadex (a granulocyte attractant) reduced the incidence of the W256 carcinoma cells regression due to neutrophils infiltrating the Sephadex injection site instead of the tumour. As stated before, neutrophil recruitment can have a negative impact on the tumour; however use of proinflammatory stimuli (e.g., bacterial products or neutrophil attractants) leads to neutrophil infiltration accompanied by the classical inflation and antitumour responses.

3.3. Neutrophils as Delivery Systems. Neutrophil-based drug delivery was also studied in conjunction with microbial resistance against antibiotics. Wendel et al. showed that neutrophils loaded with chlorhexidine (antibacterial drug) can effectively kill *E. coli* and *F. necrophorum* in the mouse liver [63]. Because neutrophils are continuously recruited to the tumour, further studies may focus on using them as drug delivery systems.

Tumour progression is modified by a wide variety of host myeloid cell types, including neutrophils. Recent studies showed serval mechanisms of protumourigenic effects of neutrophils on tumour cell proliferation, increased metastasis, and enhanced angiogenesis, although some studies demonstrated that proinflammatory polarization of neutrophils via multiple signals results in antitumour effects.

4. DC in Cancer Immunotherapy

Dendritic cells (DCs) are at the centre of immune system ability to react against cancer cells. DC-based immunotherapy is a type of a vaccination where tumour antigens are loaded into DCs, followed by administration of these modified DCs to the patient, aiming to stimulate specific T cell immunity against cancer cells. Many improvements in this field have been done but effectiveness of such therapies still awaits the major breakthrough. Successful strategies will require combination of this sophisticated cell-based therapy with other, more blunt approaches.

The principle of DC therapy is to exploit basic ability of DCs to stimulate T cell-based anticancer response. Crucial importance of this process is the ability of DCs to cross-present antigens. This process relies on presentation of exogenous antigens (normally presented on MHC class II) via MHC class I, enabling direct $CD8^+$ T cell stimulation. For the successful DC-based immunotherapy three main conditions must be met. Firstly, the activated and antigen-loaded DCs must have immunostimulatory potential. Secondly, effector T cells must be able to unleash cytotoxic activity and lastly tumour cells must be susceptible for the immune attack of effector T cells. Currently there is only one DC-based immunotherapy that has been approved for the treatment of cancer. Sipuleucel-T is a biological used in the treatment of hormone-refractory prostate cancer [64]. The methods consist of *in vitro* stimulation of blood-derived autologous antigen presenting cells (APCs) with GM-CSF and their antigen loading and reinfusion to the patient. However, therapeutic benefit of this protocol seems to be limited, since overall survival of patient with phase III clinical trial was increased by 4.1 months as compared with placebo [65].

Selection of the specific cancer antigen has become a criticalstep in DC vaccine design. An "ideal" cancer antigen should be specific for tumour cells and associated with malignant phenotype. Overexpression of cancer antigen should be restricted to all tumour cells in patients treated for a given tumour type, although the primary feature of cancer antigen, in the context of their therapeutical potential, is ability to induce T cell immunity against tumour with confirmed immunological and clinical relevance [66, 67]. There is a growing list of tumour specific antigens; however only 46 of 75 representative cancer antigens, proposed by the National Cancer Institute, induced T cell response in clinical trials, and 20 of them showed evidence of benefits for patients [66]. On the top of the list of tumour antigens suitable for DC-based cancer therapy is WT1 protein (overexpressed in AML) [67] or highly immunogenic MUC-1 (overexpressed and/or hypoglycosylated in numerous cancer types) [68]. Cancer antigens can be delivered to DCs by pulsing with peptides, proteins, and lysates of apoptotic cancer cells. This exogenous supply of proteins and short peptides used to be the favorite and most common method of antigen loading, which allows peptides to be presented in the context of both MHC class I and MHC class II molecules. Some data favor the use of whole antigen over synthetic short peptides, because there is no need to match the MHC haplotype of the patients [69, 70].

Most of the therapeutic protocols use monocyte-derived DCs (moDCs), which require their differentiation into immature DCs and subsequent maturation to DCs [71]. Recent publications have shown that shortening of the time of differentiation/maturation increases the costimulatory potential of DCs [72] with lowered expression of immunosuppressive PD-L1 molecule [73, 74]. The choice of differentiation protocols is absolutely crucial for maximizing immunostimulatory potential of moDCs. Most of the protocols use IL-4 and GM-CSF [75]. However, this classical method can be improved as shown by replacement of IL-4 with IL-15 or IFNα [76, 77]. These unconventional differentiation protocols were shown to improve cytotoxicity against AML cells [77, 78]. Not only differentiation but also activation of moDCs by various cytokine cocktails must be optimal. For that TNFα, IL-1β, IL-6, and PGE2 were shown

to deliver positive effects. However, as with differentiation protocol, this classic protocol can also be improved, for example, by stimulation with cytokines or toll-like receptors (TLRs) agonists [79]. Maturation is also affected by induction of chemokine receptors that facilitate movement into regional lymph nodes (e.g., CCR7) and by the synthesis of cytokines that stimulate T cell differentiation and proliferation (e.g., IL-6, IL-12, or IL-10). TLRs have an essential role in the recognition of and in bridging innate and adaptive immunity. DCs play an important role in activation of immune response against viral infections and can recognize such PAMS as ssRNA by TLR7 and TLR8. Synthetic agonist of TLR7/8 (imiquimod) exerts antiviral and anitumour properties and is marketed for the treatment of external genital warts caused by human papillomavirus [80]. In response to TLR ligands, costimulatory molecules, for example, CD86, CD40, and CD83, are rapidly upregulated and lead to a maturation of DCs, increasing formation of MHC-peptide complexes. Use of TLR agonist was shown not only to stimulate T cell-based cytotoxicity but also to dampen Treg immunosuppression and activation of NK cells [72, 81]. Several TLR ligands [poly(I:C), OK-432, and R848] have been included in clinical trials of DC-based immunotherapies [79].

Another tool to improve immunostimulatory potential of DCs is to modulate expression of stimulatory and inhibitory molecules. Example of this strategy is transfection of OX40L or IL-12 to DCs [82, 83]. Alternatively, shRNA was used to target immunosuppressive molecule as PD-L1 or IL-10 [84, 85]. Because the main problem encountered with anti-CTLA-4 treatment is the resistance of advanced tumours, due to the strong tumour-induced T cell tolerance, effectiveness of DC-based therapy could be improved by combination with immune checkpoint inhibitors that target PD-1/PDL-1 or B7/CTLA-4 pathways [86–88]. Several preclinical tumour models are showing that CTLA-4 blockade in combination with DC vaccination primes immune response and potentiates a specific antitumour response. A single dose of DC vaccine/anti-CTLA-4 inhibits tumour growth in 60% of the challenged mice with EL4 lymphoma cells; moreover the vaccine or CTL-4 blockage administrated alone has no potent antitumour effect [89]. Combination of anti-PD-L1 antibody tumour peptide-pulsed DCs (B16 melanoma) resulted in a higher number of melanoma peptide-specific cytotoxic T cells, unfortunately without significant reduction in tumour growth [90]. Blockade of PD-1 reduced Treg cell numbers attenuating their immunosuppressive activity and also encouraged the ability of DCs to stimulate leukemia antigen-specific T cells [85, 91]. Combinatorial therapy of anti-PD-1mAb pidilizumab and DCs vaccination is currently under phase II clinical trial and when used in patients with AML, remission occurs [92]. Furthermore, clinical trial of PD-L1/2-silenced DC vaccination in combination with donor lymphocyte infusions for the treatment of posttransplant leukemia relapse has also been registered [93]. These data suggest that combination of immune checkpoint inhibitors that target CTLA-4 and PD-1 with DC vaccination enhances the efficacy of T cell immunity.

Another checkpoint pathway that was shown to regulate antitumour immune response axis is CD200-CD200R. Lack of CD200R signaling inhibits outgrowth of an endogenous tumour irrespective of CD200 expression by the tumour cells [94]. Tumour-expressed CD200 suppresses antitumour responses, implying the potential of anti-CD200 antibodies for CD200-expressing cancers [95]. Blockade of CD200-CD200R interaction by antibodies leads to decreased tumour growth in immune competent mice [96]. The CD200 surface molecule is a key mediator of immune escape in AML and CD200 contributes to AML-induced immunosuppression through a multifaceted mode of action, which includes alteration of cytokine profile from TH1 to TH2, induction of Treg cells, and suppression of NK cell function [97–99]. An *in vitro* study of AML showed that abrogation of CD200-CD200R interaction enhances the T cell-stimulatory capacity of DCs [100], whereas inhibition of CD200-CD200R interaction was already investigated in clinical trial phase I/II in patients with relapsing or refractory B cell chronic lymphocytic leukemia or multiple myeloma. The antibody was well tolerated, however, without major therapeutic effects. Interesting way to boost CD200-CD200R blockade is its combination with stimulation of TLR7 pathway, as it was shown that lack of CD200 increases TLR7-dependent immune response [101]. However an adverse effect of CD200-CD200R blockade is also possible as CD200 expression increases with progression of squamous cell carcinoma, suggesting that CD200-expressing tumour cells engage and modulate tumour associated myeloid-derived suppressor cells [102, 103].

Recent years have shown major advances in the field of DC-based immunotherapy; however we still wait for the real change delivered to the patients. Most likely successful use of DC-therapies will depend on the combination with other therapies, not necessarily focused on immunostimulation themselves.

5. T Cell-Based Therapy

Cytotoxic T lymphocytes (CTLs) are the immune effector cells that mostly contribute to cancer rejection. They can recognize specific antigens presented by the APCs with class I major histocompatibility complex (MHC class I) molecules and then can mediate elimination of the cell that they have specifically recognized [104]. Therefore, since 1964 scientists tried to treat established tumours in mice, by the transfer of CTLs. Over time, this led to development of strategy termed adoptive cell therapy (ACT). Transferred T cells exhibit specific antitumour activity in cancer patients. There can be two sources of such T cells: (1) natural host T cells identified in the tumour, the autologous tumour infiltrating lymphocytes (TILs), and (2) T cells from patients' blood that have been genetically engineered *ex vivo* with specific antitumour T cell receptors (TCRs) or chimeric antigen receptors (CARs) [105].

5.1. TILs Used in ACT. This strategy is currently the most effective treatment for patients with metastatic melanoma, which is considered as the most immunogenic tumour [106, 107]. The gene aberrations that cause high mutational heterogeneity of melanoma malignancies might be associated with higher probability of the presence of antigen-specific T cells within the tumour [108]. Indeed, melanoma seems to be the

only tumour that reproducibly allows obtaining of TILs capable of specific antitumour recognition [107]. After homogenization of a tumour, TILs are cultured for 1-2 weeks *ex vivo* with high dose of interleukin 2 (IL-2). Then, T cells are tested for their antitumour reactivity in coculture assays. These cultures that respond to the tumour antigens (i.e., by production of IFN-γ) are activated and expanded to large numbers (1×10^{11} cells). After 5-6 weeks T cells are infused back into the patient followed by administration of IL-2, which is the most potent lymphocyte growth factor. Bolus infusion of IL-2 enhances efficacy of ACT, enabling survival and proliferation of adoptively transferred lymphocytes *in vivo* [109]. Further significant improvement of ACT effectiveness using TILs is achieved when the specific preconditioning of cancer patient is applied. This includes lymphodepletion achieved either by chemotherapy (high dose cyclophosphamide and fludarabine) or by total body irradiation (TBI) of nonmyeloablative (2 Gy) or myeloablative (12 Gy) dose [110, 111].

5.2. ACT Using Genetically Engineered T Lymphocytes. ACT using genetically modified T cells allows for treatment of other types of cancers, that is, cervical cancer and lymphoma and leukemia and prostate and bile duct cancer and neuroblastoma [109]. It became possible by the introduction of lymphocyte genes encoding conventional T cells receptor (TCR) or chimeric antigen receptor (CAR) [112]. The transduction is based on retroviral or lentiviral vectors or CRISPR technology [113]. Transduced T cell expresses the receptor for specific antigen that can be recognized in MHC independent manner. First target for chimeric antigen receptor was CD19, the molecule present on B cells. CAR therapy was therefore used primarily for the patients with high-risk B cell malignancies; however, by using different targets, use of CARs is being extended to solid tumours. Since the first generation of CAR was used, a lot of improvement in terms of its construction has been made. Second and third generation of this receptor contain not only main domain zeta but also one or more costimulatory domains (CD28, ICOS, and 41BB) that provide complete activation signal for T cells. Thus, transferred T cells are able to proliferate and survive *in vivo* without becoming anergic [114].

All these reports suggest that T cells may play key role in tumour elimination. The treatment of cancer with genetically modified T cells was already demonstrated with both preclinical and clinical studies. However, further researches and new clinical trials are needed to fully understand the antitumour effect of T cells.

6. NK Cell-Based Cancer Immunotherapy

Natural killer (NK) cells play a key role in cancer immune-surveillance as they exhibit natural cytotoxicity against many tumour cells even in the absence of preimmunization or stimulation and are virtually able to eradicate malignant cells [115]. As such, NK cell-based immunotherapy holds a great promise for cancer treatment. Thus far, however, the therapeutic potential of NK cell-based immunotherapy has yet to be realized. The major impairment is due to the cancer cell response itself that entails mechanisms to escape NK cell action or induce defective NK cells. Early investigations, using autologous lymphokine-activated NK cells, achieved limited clinical success in cancer patients [116]. Current approaches have thus evolved towards the use of expanded allogeneic NK cells, which are not inhibited by self- histocompatibility antigens like autologous NK cells or stable allogeneic NK cell lines that are more suitable for large-scale production. Alternatively, genetically engineered NK cell lines that are able to express high levels of cytokines, Fc receptors, and/or chimeric tumour antigen receptors have been recently proposed [117]. Progress in understanding NK cell biology and function is, however, needed to foster the development of novel approaches able to address therapeutic NK cells protocols.

6.1. Biological Role of NK Cells in Tumour Immune-Surveillance and Therapeutic Perspectives. NK cells comprise 5–15% of circulating lymphocytes and provide a first line of defense against cancer. They display potentially powerful weapons that may provide immediate, short-lived responses by delivering toxic enzymes or releasing cytokines that directly lyse tumour cells or mediate T or B cells immune responses [118]. As previously reviewed deeply by Cheng et al. NK cells are activated by initial recognition of altered receptor patterns on the surface of the target cell, NK cell recognition of tumour cells by inhibitory and activating receptors is a complex phenomenon, and at least three recognition models have been proposed, namely, "missing-self," "nonself," and "stress-induced self." In fact, upon cellular transformation, surface MHC-I expression on tumour cells is often down-regulated or eventually not present ("missing-self"), in order to evade recognition by antitumour T cells. Human tumour cells with poor self-MHC-I expression or bearing "altered-self" stress-inducible proteins are thus the preferred NK cell targets for potential therapy. NK cell inhibitory receptors are able to detect the absence of MHC-I expression and mediate cytotoxicity against defective cancer cells [117]. Activation and expansion of NK cells via cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21, and type I IFNs have been studied *ex vivo* [119, 120]. Lang at al. showed the increased activity of NK cells via IL-2 stimulation *in vitro* [119]. After that, Leong et al. demonstrated that preactivation of NK cells with IL-12, IL-15, and IL-18 has shown significantly enhanced antitumour effects [121]. Similarly Kobayashi et al. studied very high doses of IL-15 to observe any meaningful antitumour effects of activated and expanded NK cells *in vitro* and these cells were effective *in vivo* in a lung metastasis mouse model [122].

Therapies designed to induce either a passive or active antitumour response by harnessing the power of NK cells are a most appealing strategy to control tumour development. Despite the multiple properties of NK cells, malignant cells can develop mechanisms to evade immune-surveillance and establish an immune-privileged environment. Some tumour cells may in fact produce immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β), thus impairing the adaptive antitumour immune response, or eventually shift the immune response towards a Th2 response with less antitumour capacity [123]. In order to overcome these limitations, novel generations of genetically modified NK cell

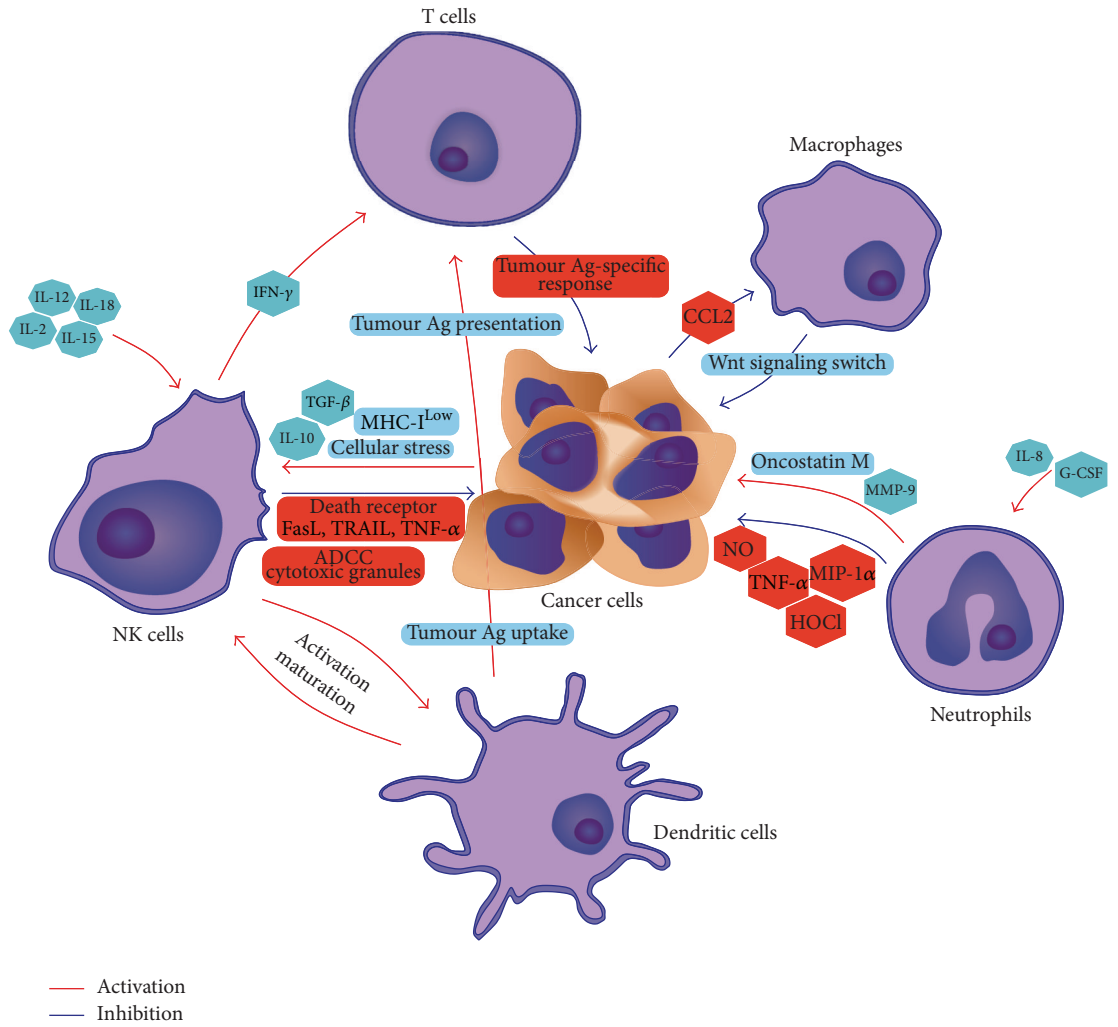

Figure 2: Immune cells in tumour microenvironment. The figure shows the potential roles of immune cells in tumour immunosurveillance. NK cells activated by cancer cells (cellular stress and low expression of MHC-I and IL-10 and TGF-β) directly recognize and attack cancer cells through at least four mechanisms: cytoplasmic granule release, death receptor-induced apoptosis, effector molecule production, or ADCC. Interaction of NK cells with DCs leads to improving their antigen uptake and presentation, facilitating the generation of antigen-specific T cells responses. Tumour associated neutrophils secrete oncostatin M inducing angiogenesis and invasiveness of tumour cells. Potential direct effect of neutrophils on tumour progression is secretion of matrix metalloproteinase-9 (MMP-9) enzymes. Inhibition of neutrophil influx by interleukin-8 (IL-8) neutralization can decrease tumour angiogenesis and intravasation. Infiltration of macrophages to tumour microenvironment inhibits canonical Wnt signaling leading to decreased proliferation and survival of cancer cells but as "side effect" noncanonical Wnt signaling is activated inducing metastasis. Ab, antibody; ADCC, antibody-dependent cellular cytotoxicity; DC, dendritic cell; IFN, interferon; and NK, natural killer.

lines are being exploited in order to obtain high numbers of functional NK cells that have the potential to survive *in vivo* and are capable of expressing cytokine or over-expressing activating receptors. Retargeting NK cells via chimeric receptors by genetic manipulation approaches has also been proposed to modulate and enhance NK-tumour cell interaction [124, 125]. Clinical trials are currently carried out in haematological malignancies including leukemia and myelodysplastic/proliferative diseases and recently applied also to solid tumours [126].

The design of new strategies, including adjuvant therapies or genetic engineering of NK cells, is currently pursued in order to maximize the cytotoxic potential of NK cells to treat human malignancies.

7. Future Perspectives for Using Immune Cells in Cancer Therapy

The role of immune cells in cancer development cannot be underestimated. Thus, prospective therapies targeting these cells may increase effectiveness of cancer treatment. The three most important directions in the development of therapies concentrating around immune cells should be considered. Firstly, there are attempts to decrease the number of immunosuppressive cells, promoting the tumour growth and metastasis, by blocking activity of chemokines recruiting immune cells. Secondly, there are therapies switching off metastasis promoting activities of immune cells, which will modulate their activity by blocking or activating desired

functions. Searching for ligands that will enable these changes is of the highest importance. Also genetically engineered, derived from the host, activated immune cells cultured *ex vivo* with knocked-out or knocked-in genes may be utilized as an element of complex therapy. Finally, some of immune cells may be considered as delivery systems transporting antitumour factors directly to the destination place.

Improvement of knowledge about processes taking place in tumour microenvironment will allow creation of specific, personalized therapy. Better understanding of suppressive tumour environment may allow combining therapy, for example, DC or TIL vaccinations with agents tackling immunosuppressive mechanisms (GM-CSF, IFN, IL-2, IL-15, and TNF). It is important to improve knowledge of tumour microenvironment biology, enabling a wider use of recombinant immune cells, cytokines, tumour associated antigens, viruses, and so forth. One of the most important aspects is gathering of information about the escape strategies used by tumours.

Next step will be identification of mechanism(s) used by the tumour of individual patients in order to select the most appropriate approach for each patient to counteract tumour escape. Spreading of tumour immunotherapy will allow using it in earlier stages of the treatment or even in minimal residual disease. It may provide improvement of therapy due to less compromised immune system by chemo- or radiotherapy pretreatment.

8. Conclusion

Tumour progression is modified by a wide variety of host cell types, where the key role is played by tumour associated macrophages, T lymphocytes, natural killer cells, tumour associated neutrophils, and dendritic cells. Despite earlier studies showing that these cells might exhibit cytotoxicity towards tumour cells, recent discoveries have indicated that they promote tumour progression by increase of cancer cell proliferation, metastasis, and enhanced angiogenesis. Therefore, they may constitute targets for further anticancer therapy. Figure 2 summarizes interactions between the immune cells in the tumour microenvironment. However, better understanding of the phenotypic and functional properties of these cells is still required.

Competing Interests

The authors declare that they have no conflict of interests.

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Review Article

Double Roles of Macrophages in Human Neuroimmune Diseases and Their Animal Models

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Macrophages are important immune cells of the innate immune system that are involved in organ-specific homeostasis and contribute to both pathology and resolution of diseases including infections, cancer, obesity, atherosclerosis, and autoimmune disorders. Multiple lines of evidence point to macrophages as a remarkably heterogeneous cell type. Different phenotypes of macrophages exert either proinflammatory or anti-inflammatory roles depending on the cytokines and other mediators that they are exposed to in the local microenvironment. Proinflammatory macrophages secrete detrimental molecules to induce disease development, while anti-inflammatory macrophages produce beneficial mediators to promote disease recovery. The conversion of the phenotypes of macrophages can regulate the initiation, development, and recovery of autoimmune diseases. Human neuroimmune diseases majorly include multiple sclerosis (MS), neuromyelitis optica (NMO), myasthenia gravis (MG), and Guillain-Barré syndrome (GBS) and macrophages contribute to the pathogenesis of these neuroimmune diseases. In this review, we summarize the double roles of macrophage in neuroimmune diseases and their animal models to further explore the mechanisms of macrophages involved in the pathogenesis of these disorders, which may provide a potential therapeutic approach for these disorders in the future.

1. Introduction

Macrophages distributed in tissues throughout the body play a key role in immune response, tissue homeostasis, metabolism, and repair [1]. Mature macrophages in different tissues present with different phenotypes, such as microglia in the brain, alveolar macrophages in the lungs, Kupffer cells in the liver, and osteoclasts in bone tissue [2]. In addition, macrophages can switch their phenotypic and functional properties depending on the signals in their microenvironment in homeostasis and disease [3]. The polarization of macrophages is determined by the cytokines and other mediators they encounter. Different subsets of macrophages exert either proinflammatory or anti-inflammatory roles. Recently, the studies have demonstrated that macrophages take part in the pathological process of neuroimmune diseases. This review outlines the double roles of macrophages in human neuroimmune diseases, such as multiple sclerosis (MS), neuromyelitis optica (NMO), myasthenia gravis (MG), and Guillain-Barré syndrome (GBS) as well as their animal models.

2. An Overview of Macrophages

2.1. The Origin of Macrophages. Historically, macrophages were considered to derive primarily from hematopoietic stem cells (HSCs) via bone marrow progenitors and circulating blood monocytes intermediates [4]. However, more and more evidences have revealed that there are dual origins of tissue macrophages, either from embryonic progenitors or from blood monocytes (Figure 1). The major macrophage populations are established prior to birth [5]. These cells develop from either primitive yolk sac macrophages or embryonic fetal liver monocytes and self-replenish themselves [1, 6]. Hoeffel and colleagues have shown that yolk sac macrophages derive from early erythromyeloid progenitors (EMPs), while late c-Myb$^+$ EMPs seed the fetal liver and give rise to fetal

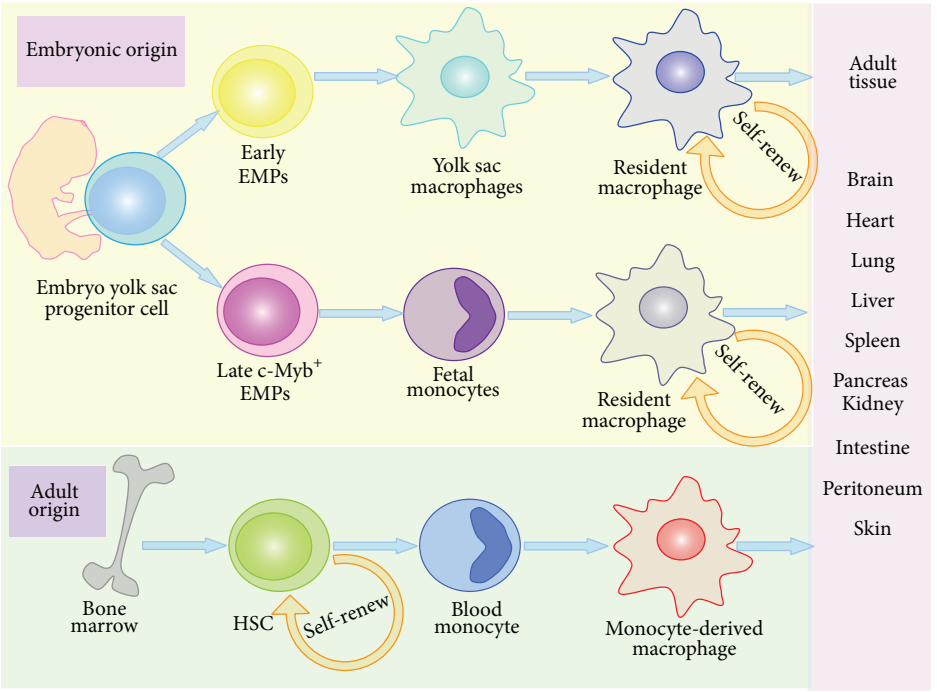

FIGURE 1: Origin and self-renewal of macrophage. Tissue macrophages have dual origins. One part develops from embryonic progenitors in the yolk sac and fetal liver and self-renew. The other part derives from hematopoietic stem cells (HSCs) in bone marrow and blood monocyte intermediates. HSCs also can self-replenish themselves. Monocyte-derived macrophages can give rise to some subsets of resident macrophages under certain conditions. Resident macrophages and monocyte-derived macrophages ultimately constitute macrophages in all tissues, such as microglia in the brain, Langerhans cells in the skin, and Kupffer cells in the liver. EMPs, erythromyeloid progenitors; HSCs, hematopoietic stem cells.

monocytes. Both early EMPs and late c-Myb$^+$ EMPs are generated in the yolk sac [6]. Yolk sac macrophages are the main precursors of microglia, while fetal monocytes differentiate into most other macrophages (alveolar macrophages in the lung and Kupffer cells in the liver, for example) [6–8]. In dermis and gut tissues, macrophages are renewed by adult HSC-derived monocytes [9, 10]. Besides, in spleen, kidney, and pancreas, macrophages with dual origins coexist [11]. However, most studies on origin of macrophages are focused on rodents and cells, so the exact origin of human macrophages is urgent to be clarified.

2.2. The Polarization and Roles of Macrophages. Macrophages not only present antigens as other antigen presenting cells (APCs) such as dendrite cells, but also eliminate microbes and tumor cells together with natural killer cells, T cells and B cells. What is more, macrophages contribute to tissue repair and remodeling, as well as restoration of pathogen-disturbed homeostasis [12]. The activated state, or polarization, of the macrophages depends on numerous factors from the microenvironment they reside in during normal homeostasis and in the pathological conditions [3]. Pathogen- and self-local environment-derived stimuli induce the macrophage phenotypic polarization [13]. Proinflammatory subtype/anti-inflammatory subtype polarization is the most well-described and commonly reported paradigm of macrophage polarization [14] (Figure 2). Proinflammatory subtype, also known as classically activated macrophages, is generally instigated by the presence of microbial products, such as lipopolysaccharide (LPS), proinflammatory cytokines, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), as well as damage associated molecule patterns high mobility group box 1. Anti-inflammatory subtype, regarded as alternative activated macrophages, is activated by T helper 2 (Th2) cell-associated cytokines (IL-4 and IL-13), anti-inflammatory molecules (IL-10 and glucocorticoids), and immune complexes (IC) [15, 16]. Proinflammatory macrophages, characterized by their expression of high levels of TNF-α, IL-1, IL-6, IL-12, IL-23, nitric oxide (NO), and reactive oxygen intermediates (ROI), by their upregulation of major histocompatibility complex-II (MHC-II), costimulatory molecules, and T helper 1- (Th1-) recruiting chemokines, have a strong microbicidal and tumoricidal activity [17–19]. By contrast, anti-inflammatory macrophages, which upregulate surface molecules including mannose receptor CD206 and scavenger receptor CD163 and produce high levels of IL-10, transforming growth factor-β (TGF-β), and chemokines, are supposed to contribute to parasite infestation, tissue remodeling, and tumor progression [14, 17, 19, 20]. Anti-inflammatory macrophages can be further subcategorized into M(IL-4), M(IC), M(IL-10), and so on [15, 19]. M(IL-4), activated by IL-4, produces CCL24 and CCL22 in mice and CCL17 and CCL18 in human, resulting in the recruitment of eosinophils, basophils, and Th2 cells [19]. M(IC), stimulated by immune complexes (IC), produces CCL1 in mice, recruiting regulatory T cells (Tregs) [19]. M(IL-10) is activated by IL-10, which

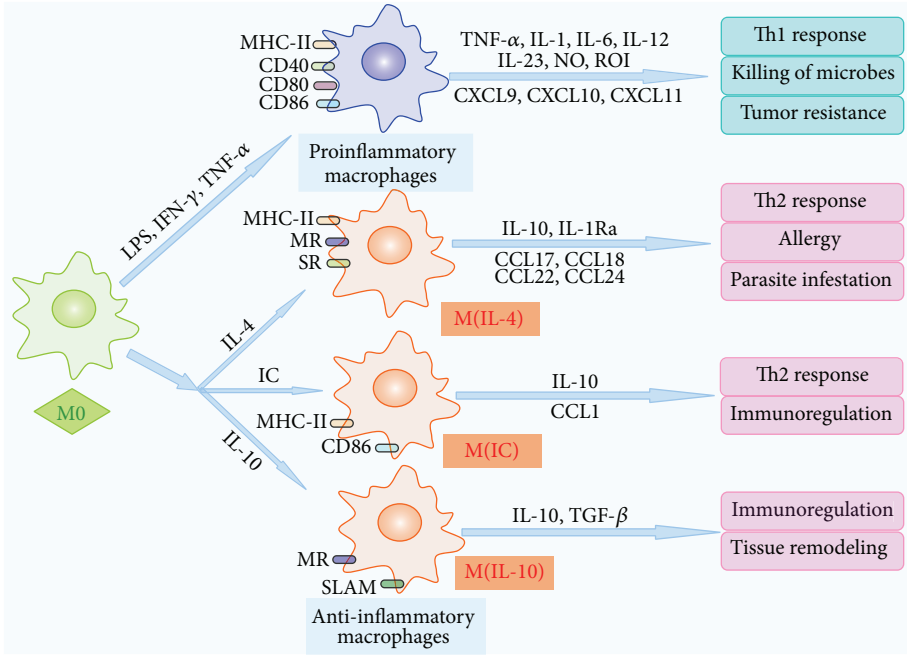

Figure 2: Macrophage polarization into proinflammatory and anti-inflammatory macrophages. Macrophages polarize and acquire different functional properties in response to numerous factors from the microenvironment. Macrophages activated by IFN-γ, LPS, or TNF-α can develop proinflammatory macrophages, with strong microbicidal and tumoricidal properties. In contrast, anti-inflammatory macrophages contribute to Th2 response, immunoregulation, and tissue remodeling. Anti-inflammatory macrophages have different subsets. M(IL-4) macrophages (induced by exposure to IL-4) secret TNF-α, IL-1, and IL-6 and induce Th2 cell response and allergy. M(IC) macrophages (induced by IC) secret IL-10 and exert immunoregulatory function. M(IL-10) macrophages (induced by IL-10) secret IL-10 and TGF-β, suppress immune responses, and promote tissue remodeling. CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; IC, immune complexes; IFN-γ, interferon γ; LPS, lipopolysaccharide; MHC-II, major histocompatibility complex-II; MR, mannose receptor; NO, nitric oxide; ROI, reactive oxygen intermediates; SLAM, signaling lymphocytic activation molecule; SR, scavenger receptor; TGF-β, transforming growth factor-β; TLR, toll-like receptor; TNF-α, tumor necrosis receptor-α.

is immunosuppressive and engaged in extracellular matrix remodeling [14]. Diverse microenvironmental factors shape macrophage different activation states, which induce the dynamic switch of macrophage phenotype and function, showing different extremes of a continuum ranging from proinflammatory subtype to anti-inflammatory subtype [21, 22]. Transcription factors including STAT1, STAT6, C/EBPb, IRF-4, IRF-5, and PPAR-γ can regulate transcription programs which control the polarization of proinflammatory/anti-inflammatory macrophage [23, 24]. Proinflammatory subtype/anti-inflammatory subtype polarization status is regulated by the complex and interacting endogenous cellular signaling pathways in the microenvironment, such as C-Jun N-terminal kinase (JNK) signaling pathway, phosphatidylinositol-3-kinase (PI3K)/Akt signaling pathway, Notch signaling pathway, and JAK/STAT signaling pathway [2].

Macrophages are dispersed in many tissues and have distinct functions influenced by their location in the body [25]. Kupffer cells in liver contribute to the uptake of lipoprotein for maintenance of homeostasis and the endocytosis of pathogens and waste materials for host defense [26]. Alveolar macrophages in lung are involved in the uptake of inhaled particle and host defense against many borne microorganisms [27]. In homeostasis, Kupffer cells achieve immune surveillance and liver tolerance through IL-10 secretion [28]. Perturbation of homeostasis results in the activation of Kupffer cells by β-glucans from bacteria and fungi or lipopolysaccharide (LPS), the endotoxins of Gram-negative intestinal bacteria [29]. Activated Kupffer cells present either proinflammation or anti-inflammation phenotype [17]. Upon activation, microglia acquire an amoeboid shape and exert proinflammation or anti-inflammation roles dependent on different cytokines and other mediators they are exposed to [30].

In disease state, identifying different subsets of macrophages, activated states of macrophages, and macrophage polarization is crucial for understanding the pathogenesis and treatment of human disease.

3. Macrophages in Human Neuroimmune Diseases and Their Animal Models

Macrophages represent a ubiquitous yet complex population of immune cells that play major roles in both disease and homeostasis throughout the body. They contribute to both pathology and resolution in all acute and chronic inflammatory diseases including infections, cancer, obesity, atherosclerosis, and autoimmune disorders [31]. Neuroimmune diseases are a series of complex autoimmune diseases which involve the nervous system, including MS, NMO, GBS, and

MG. The exact pathogenesis of these diseases is essentially ambiguous. But emerging data has suggested that macrophages may be associated with the development of these diseases (Table 1).

3.1. Macrophages in Multiple Sclerosis and Experimental Autoimmune Encephalomyelitis. MS, one of the most frequent central nervous system (CNS) diseases in young adults, is a progressive autoimmune disease caused by damage to the myelin and axons of brain and spinal cord [56]. MS patients show various neurological symptoms which originate in different areas of the CNS, such as motor deficits, sensory disturbances, visual disturbances, and neuropsychological symptoms [57]. So far, the etiology of MS is still not well understood; genetic, metabolic, environmental, and immunological factors have all been implicated [58]. The pathological hallmarks of MS consist of lymphocytes and macrophage infiltration, axonal demyelination, neuronal impairment, and remyelination [59, 60]. Different functional subpopulations of macrophages, with various roles including phagocytosis, antigen presentation, and lymphocyte stimulation, are abundantly present in inflammatory MS lesions [61]. Macrophages not only induce lesion formation and axonal damage, but also contribute to remyelination. On one hand, macrophages exert proinflammatory, neurotoxic, and myelin-attacking properties through secretion of inflammatory mediators, reactivation of pathogenic T cells, and suppression of Tregs expansion [32]. On the other hand, macrophages present repair mechanisms through the production of neurotrophic factors and clearance of myelin debris [33, 34]. Experimental autoimmune encephalomyelitis (EAE) is an animal model used to explore the mechanisms of MS and translate them into therapeutic interventions [62]. EAE can be induced either by active immunization with myelin components coupled with adjuvant or by passive transfer of myelin-reactive T cells [63]. EAE shares many pathological features with MS, such as chronic demyelination, neuronal damage, and neuroinflammation [64, 65]. It has been demonstrated that macrophages have a pathogenic role in initiating EAE, and eliminating macrophages significantly inhibits disease [35]. Another study showed that macrophages predominated in demyelinated areas and the macrophage number was correlated with tissue damage in EAE [36]. However, macrophages are also beneficial to remyelination. Undoubtedly, macrophages in MS or EAE consist of different phenotypical and functional subpopulations (Table 2).

3.1.1. Microglia and Monocyte-Derived Macrophages. Historically it was difficult to distinguish activated microglia from activated macrophages in CNS lesion sites because they both present similar antigenic markers [87]. Thanks to chimeric mice, whose bone marrow (BM) cells are replaced by donor BM cells containing mismatched-MHC of fluorescently labeled myeloid cells, microglia can be distinguished from monocyte-derived macrophages [88, 89]. Microglia and monocyte-derived macrophages are functionally distinct populations of macrophages with unique origins. Microglia are located in the parenchyma and rely on local self-renewal, while monocyte-derived macrophages are renewed by blood derived monocytes and situated in both the parenchyma and the CNS barriers of the choroid plexus, perivascular space, and the meninges [30]. In addition, a TGFβ-1 dependent microglial signature of microglia can provide the ability to distinguish microglia from infiltrating myeloid cells in the CNS [90]. Also, an evolutionarily conserved protein TMEM119 serves as a reliable microglial marker that differentiates microglia from monocyte-derived macrophages in human brain [91]. Interestingly, there is virtually no background trafficking of monocyte-derived macrophages in the CNS parenchyma of healthy organism [36]. Perturbation of CNS homeostasis can result in the recruitment of monocyte-derived macrophages which are associated with axonal loss, astrogliosis, and neurodegeneration in the CNS [30]. Once homeostasis is restored, these monocyte-derived macrophages seem to vanish [30]. A recent study revealed important physiological roles of microglia in learning and memory by promoting learning-associated synaptic structural remodeling using CX_3CR1^{CreER} mice which express tamoxifen-inducible Cre recombinase [92]. Now it has been generally accepted that EAE is characterized by activation of resident microglia and extensive infiltration of monocyte-derived macrophages. Monocyte-derived macrophages are important in the effector phase of EAE and actively initiated demyelination. But the activation of microglia precedes the massive immune cells infiltration and the demyelination cascade and finally dominates the remyelination and repair of disease [93]. Microglia not only boost inflammatory and degenerative events in the CNS, which are correlated with axon and oligodendrocyte pathology, but also exert neuroprotective role in EAE [30]. Ponomarev et al. found that activated microglia promote the development and maintenance of inflammatory lesions in the CNS before the infiltration of circulating monocytes/macrophages into the CNS, implying the contributions of microglia in the early stages of EAE [78]. However, another study showed that microglia eliminated debris and suppressed cellular metabolism at EAE onset, presenting a beneficial role [36]. After myelin internalization, microglia gain a less-inflammatory phenotype and support tissue repair [94–96]. In addition, microglia express high levels of TGF-β and low levels of activation markers CD45, CCR1, and CCR5, which induces a protective process [37]. Monocyte-derived macrophages are phagocytic and inflammatory cells which initiate demyelination at EAE onset [36]. Monocyte-derived macrophages can present antigens and activate myelin-reactive T cells in CNS of EAE and then express high levels of adhesion molecules (ICAM-1 and VCAM-1) and chemokines (CCL2 and CCL3), attracting leukocyte infiltration into CNS [79–81]. Moreover, monocyte-derived macrophages induce the activation of resident microglia to accelerate inflammation, indicating that they are important population in EAE pathology [82]. These results show that macrophages play a key role in disease processes. The intervention of macrophage/microglia activation prior to disease induction had modest effects in disease progression; nevertheless the intervention at disease onset significantly improved disease severity [97]. Furthermore, inhibiting the activation of microglia induced a delayed

Table 1: Roles of macrophages in neuroimmune diseases and their animal models.

Neuroimmune diseases	Beneficial roles of macrophages	Harmful roles of macrophages	Refs
MS	Production of neurotrophic factors Clearance of myelin debris	Secretion of inflammatory mediators Reactivation of pathogenic T cells Suppression of Tregs expansion	[32–34]
EAE	Eliminated debris and suppress cellular metabolism Production of anti-inflammatory TGF-β	Presenting a pathogenic role in initiating EAE Eliminating macrophages inhibits EAE A positive correlation between macrophage number and tissue damage in EAE	[35–37]
NMO		Participate in CDC and ADCC Mediating proinflammatory immune mechanism through the expression of intense immunoreactivities for IFI30 and CD163	[38–40]
Rats or spinal cord cultures models for NMO		Macrophages exacerbated the severity of NMO lesions in spinal cord cultures Depletion of macrophages reduced the severity of NMO pathology in rats Phagocytosis Secretion of proinflammatory cytokines	[33, 41, 42]
MG		Poliovirus-infected macrophages in thymus of several MG patients, which may be involved in the intrathymic alterations leading to MG	[43]
EAMG	Induction of apoptosis in activated T cell blasts in vitro by large suppressive macrophages which were generated from restimulating spleen cells from EAMG	Acting as APCs during the acute phase Promoting the production of antibodies to AChR in the chronic phase	[44–46]
GBS	Secretion of anti-inflammatory cytokine IL-10	Professional antigen presentation Secretion of cytokines and other inflammatory mediators Phagocytosing myelin in AIDP and axons in AMAN	[47–51]
EAN	Inducing T cell apoptosis by secreting proapoptotic mediators Secretion of IL-10 and TGF-β which both can reduce the severity of EAN	Acting as APCs Secreting proinflammatory cytokines IL-12, TNF-α, MMP-9, and iNOS that propagate inflammation and induce myelin and axonal damage Attacking myelin or axon in a complement-dependent manner	[52–55]

AChR, acetylcholine receptor; ADCC, antibody-dependent cellular cytotoxicity; AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; APCs, antigen presenting cells; CDC, complement-dependent cytotoxicity; EAE, experimental autoimmune encephalomyelitis; EAMG, experimental autoimmune myasthenia gravis; EAN, experimental autoimmune neuritis; GBS, Guillain-Barré syndrome; IFI30, interferon gamma-inducible protein 30; IL-10, interleukin-10; IL-12, interleukin-12; iNOS, inducible nitric oxide synthase; MMP-9, matrix metalloproteinase-9; MS, multiple sclerosis; NMO, neuromyelitis optica; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis receptor-α; Tregs, regulatory T cells.

Table 2: Roles of different functional subpopulations of macrophages in MS and EAE.

	Tissue resident macrophages (microglia)	Monocyte-derived macrophages	Proinflammatory macrophages	Anti-inflammatory macrophages	Refs
MS	As competent APCs Secretion of proinflammatory and neurotoxic molecules Maintenance of CNS homeostasis Immunosuppression	Eating myelin remnants Secreting proinflammatory cytokines Displaying an intermediate activation to suppress neuroinflammation and promote CNS repair	Excessive secretion of proinflammatory cytokines, ROI and NO	Phagocytosing debris Promoting tissue repair Increased anti-inflammatory macrophages in MS after treatment with glatiramer acetate	[66–77]
EAE	Promoting the development and inflammatory lesions in CNS in the early stage of EAE Eliminating debris and suppressing cellular metabolism at EAE onset Secretion of TGF-β to induce a protective process	Presenting antigen Activating myelin-reactive T cells Expressing adhesion molecules and chemokines to attract leukocyte infiltration into CNS Activating some microglia to accelerate inflammation	Contributing to the establishment of early inflammation in EAE Associating with EAE severity	Promoting the differentiation of Th2 cells and Tregs to suppress EAE severity Inhibiting the development of Th17 cells	[30, 36, 37, 78–86]

APCs, antigen presenting cells; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; NO, nitric oxide; ROI, reactive oxygen intermediates; TGF-β, transforming growth factor-β; Th2, T helper 2; Th17, T helper 17; Tregs, regulatory T cells.

onset of EAE [98]. Another study showed that conditional depletion of microglia-endogenous TGF-β-activated kinase 1 (TAK1) suppressed disease, strongly diminished CNS inflammation, and decreased tissue damage by cell-autonomous inhibition of the NF-κB, JNK, and ERK1/2 pathways in EAE [99, 100]. Through CD11b-HSVTK mice which express herpes simplex thymidine kinase in macrophages and microglia, Heppner et al. found that microglial paralysis suppressed the development and maintenance of inflammatory CNS lesions in EAE [101]. A recent study has demonstrated that CXCR7 suppression modulated microglial chemotaxis to ameliorate the clinical severity of EAE [102]. In addition, hydroxychloroquine treatment suppressed the activation of human microglia and attenuated EAE [103]. 18β-glycyrrhetinic acid can attenuate EAE through suppressing microglia activation-mediated CNS inflammation and promoting neuroprotective roles of microglia [104]. Fingolimod treatment of EAE resulted in diminished microglial activation in vivo PET imaging [105]. From these studies, it has been speculated that microglia/macrophages, which display double roles in the disease course of EAE, are quite important for exploring the pathogenesis and progression of MS.

In MS, microglia turn into competent APCs for T cells after eating myelin and axonal remnants, which promote their expression of MHC-II and costimulatory molecules and their secretion of inflammatory and neurotoxic molecules, resulting in neuroinflammation and demyelination [66, 67]. Moreover, microglia play a crucial role in the maintenance of CNS homeostasis [68]. Microglia in normal appearing white matter of MS patients display features of immunosuppression and expressed molecules to prevent activation and tissue damage [69]. Monocyte-derived macrophages are found in active demyelinating lesions of MS patients [106, 107]; one part contains myelin remnants [70] and the other secretes inflammatory cytokines and expressed costimulatory molecules, both inducing MS lesion development [71, 72]. Besides, some monocyte-derived macrophages display an intermediate activation which suppress neuroinflammation and promote CNS repair, presenting a neuroprotective role in MS [73, 74]. Glucocorticoids, IFN-β, glatiramer acetate, and fingolimod, commonly used drugs for MS, can effectively inhibit macrophage or microglia activation and alleviate disease severity in early stage of MS [108–111]. Therefore, targeting macrophages or microglia is an attractive therapeutic option for the treatment of MS.

3.1.2. Proinflammatory and Anti-Inflammatory Microglia/Macrophages. The current concept of macrophage polarization describes two subtypes with distinct but opposing functions [112], the proinflammatory subtype with secretion of TNF-α, IL-1β, IL-12, and IL-23 and the anti-inflammatory subtype with secretion of IL-10, TGF-β, and sIL-1Rα [113–115]. It has been demonstrated that proinflammatory microglia/macrophages induce tissue damage due to excessive secretion of proinflammatory cytokines, ROI and NO [75, 76]. In contrast, anti-inflammatory microglia/macrophages can phagocytose debris and promote tissue repair and termination of neuroinflammation, leading to a neuroprotective response [77].

In EAE, microglia/macrophages also can be classified into proinflammatory and anti-inflammatory microglia/macrophages. Proinflammatory and anti-inflammatory microglia/macrophages predominate differentially during disease course. For instance, proinflammatory microglia/macrophages contribute to the establishment of early inflammation in EAE, whilst anti-inflammatory microglia/macrophages induce the resolution of inflammation [83]. What is more, proinflammatory microglia/macrophages are associated with increased EAE severity, whereas anti-inflammatory microglia/macrophages are correlated with ameliorated clinical disease [84]. Anti-inflammatory microglia/macrophages promote the differentiation of Th2 cells and Tregs, which can suppress EAE severity [85]. Anti-inflammatory microglia/macrophages also participate in the development of relapses in EAE [116]. Administration of ex vivo activated anti-inflammatory macrophages may not only suppress ongoing severe disease but also promote immunomodulatory expression pattern in CNS lesions, indicating their anti-inflammatory role in the recovery of EAE [116]. Adoptive transfer of anti-inflammatory macrophages could inhibit the development of T helper 17 (Th17) cells and induce the differentiation of Th2 cells and Tregs which both reverse EAE, confirming their direct therapeutic relevance [85, 86].

Recent studies also have shown that there are $CD163^+$ and $Arg\text{-}1^+$ anti-inflammatory microglia/macrophages in MS brain [94, 117]. In addition, primary cultures of human monocyte-derived macrophages were exposed to IFN-γ and LPS for the activation of M1 and to IL-4 for the activation of anti-inflammatory macrophages. Anti-inflammatory macrophages migrated over longer distance and with higher velocity towards CCL5, CXCL10, CXCL12, and C1q, all of which were key factors for monocytes recruitment into MS lesions, whereas proinflammatory macrophages did not respond and remained sessile [118]. Upon stimulation with CCL2, anti-inflammatory macrophages were able to make filopodia, while proinflammatory macrophages adapted a spherical morphology, suggesting that the cytoskeleton of proinflammatory and anti-inflammatory macrophages was rearranged [118]. So, the activation status of macrophage induced the cytoskeleton rearrangement and affected macrophage migration, which may involve the pathological process of MS [118]. Intriguingly, another study showed that, in active demyelinating MS lesions, although macrophages and activated microglia predominantly displayed proinflammatory characteristics, the majority of these cells coexpressed the markers of proinflammatory and anti-inflammatory macrophages, suggesting an intermediate activation status [59]. The balance between proinflammatory and anti-inflammatory microglia/macrophages is proposed to predict the development of disease and relapse [66]. Furthermore, anti-inflammatory microglia/macrophages are increased in MS after treatment with glatiramer acetate. Induction of anti-inflammatory microglia/macrophages may suppress neuroinflammation and promote CNS repair. Hence, the treatment of MS may focus on shifting proinflammatory microglia/macrophages into anti-inflammatory microglia/macrophages.

In conclusion, the double roles of microphages and identifying the beneficial subset in disease course should be clarified. Of course, future studies should shed light on the double roles of microglia and CNS-infiltrating macrophages, proinflammatory and anti-inflammatory microglia/macrophages in different stages of disease process, and the cell intrinsic and extrinsic pathways that regulate the roles and phenotype change. Most of all, shifting the phenotype of macrophages into the beneficial one is an attracting therapeutic hint.

3.2. Macrophages in Neuromyelitis Optica and Its Animal Model. NMO is a neuroimmune disorder characterized by recurrent episodes of optic neuritis and transverse myelitis, resulting in significant blindness and/or paralysis [119]. Antibodies against aquaporin-4 (AQP4) are found in the serum of most NMO patients [120]. AQP4 is a water channel protein expressed on astrocytic end-feet in CNS, as well as skeletal muscle cells and epithelial cells in kidney, lung, and gastrointestinal tract [121]. Anti-AQP4 autoantibody (NMO-IgG) plays a key role in the pathogenesis of NMO [122]. NMO-IgG binds to AQP4 on astrocytes, then induces complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), and finally leads to blood-brain barrier disruption, demyelination, and neuronal injury [38]. The pathological features of NMO include vasculocentric deposition of immunoglobulin and activated complement, loss of AQP4 and glial fibrillary acidic protein, marked granulocyte and macrophage infiltration, and demyelination with axon loss [39]. Macrophages also participate in CDC and ADCC of NMO. So far, no single rodent model has proven to be a perfect representation of NMO in humans [123]. Commonly used experiments are obtained through passive transfer of NMO-IgG in certain contexts in rats or spinal cord cultures [124]. A study showed that macrophages exacerbated the severity of NMO lesions in spinal cord cultures exposed to NMO-IgG and complement [41]. In a model of NMO in rats produced by intracerebral injection of NMO-IgG, depletion of monocytes and macrophages (both proinflammatory and anti-inflammatory subtypes) could reduce the severity of NMO pathology [42]. Macrophages exacerbate astrocyte damage of NMO lesions through phagocytosis and secretion of proinflammatory cytokines or oxidative metabolites [33]. In the brain lesions of patients with NMO, $CD68^+$ macrophages and microglia expressed intense immunoreactivities for interferon gamma-inducible protein 30 (IFI30) and CD163, suggesting that severe fulminant activation of macrophage-mediated proinflammatory immune mechanism exerted a crucial role in the generation of NMO lesions [40].

Only a few of studies have shown that macrophages involve NMO and its animal models, let alone the roles of different subsets of macrophages, such as microglia/macrophages, and the different polarization of macrophages in NMO. Future studies should focus on the roles of macrophage subsets and clarify whether macrophages can become the therapeutic target of NMO.

3.3. Macrophages in Myasthenia Gravis and Experimental Autoimmune Myasthenia Gravis. MG, an antibody-mediated neuroimmune disease of the neuromuscular junction, is characterized by fluctuating muscle weakness and abnormal fatigability [125]. Pathogenic autoantibodies consist of antibodies against acetylcholine receptor (AChR), muscle-specific tyrosine kinase (MuSK), lipoprotein receptor-related protein 4 (LRP4), and so on [126]. The autoantibodies are produced in T cell dependent and B cell mediated pathogenic processes, which further activate the complement system and induce inflammation of the postsynaptic muscle membrane. The abnormalities of the thymus are related to the pathogenesis of MG, including thymoma and thymic hyperplasia [127]. Experimental autoimmune myasthenia gravis (EAMG), induced by immunization with Torpedo AChR, is a conventional animal model of MG, commonly used to investigate the mechanism underlying the pathophysiology of MG for the development of novel therapeutic strategies [128]. A previous study indicated that the pathologic features of EAMG in the acute phase included macrophage infiltration and inflammation of muscle endplates and muscle fiber necrosis [44]. Macrophages act as APCs during the acute phase of EAMG, while they promote the production of antibodies to self-AChR in the chronic phase [45]. However, large suppressive macrophages generated from restimulating spleen cells from EAMG could induce apoptosis in activated T cell blasts in vitro, indicating a potential immunotherapy of EAMG [46]. In human, there are poliovirus-infected macrophages in thymus of several MG patients, which may be involved in the intrathymic alterations leading to MG [43]. Future studies may be conducted with respect to analysis of the macrophage subsets and polarization in the pathogenesis and treatment of MG.

3.4. Macrophages in Guillain-Barré Syndrome and Experimental Autoimmune Neuritis. GBS is an acute inflammatory demyelinating neuropathy, resulting from a complicated immune response to incompletely characterized antigens in the peripheral nervous system [129]. Acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN) are typical subsets of GBS [47]. Both cellular and humoral immunity contribute to disease development, resulting in neuroinflammation, demyelination, and axonal damage in the peripheral nervous system (PNS) [47, 130]. AIDP is majorly related to $CD4^+$ T cell induced macrophage associated demyelination, while AMAN mostly involves autoantibodies against ganglioside [48]. Experimental autoimmune neuritis (EAN) which is a T cell mediated inflammatory demyelinating disease induced by immunization with proteins and peptides of PNS myelin together with Freund's complete adjuvant is regarded as a useful animal model of GBS [131, 132].

Macrophages exercise their functions through professional antigen presentation and secretion of cytokines and other inflammatory mediators [47, 49, 50]. Macrophages express high levels of MHC-II in EAN [131]. What is more, macrophages secrete proinflammatory cytokines IL-12 and TNF-α, matrix metalloproteinase-9 (MMP-9), and inducible nitric oxide synthase (iNOS), which propagate inflammation and induce myelin and axonal damage in EAN [52, 53]. Interestingly, macrophages in PNS not only contribute to

the inflammatory pathology and tissue destruction, but also promote recovery in EAN [52]. In EAN, macrophages induce T cell apoptosis by secreting proapoptotic mediators if they contact with their targets [54]. Macrophages also secrete IL-10 and TGF-β in EAN, which both inhibit the disease and reduce disease severity [48, 55]. What is more, macrophages are involved in the pathogenesis of GBS. Macrophages phagocytose myelin in AIDP and axons in AMAN [51]. Macrophage-mediated segmental demyelination and axonal loss are the pathological features of GBS [133]. Macrophages express high levels MHC-I and MHC-II in GBS [134]. In addition, macrophages are directed towards myelin or axonal targets by antibodies and attack targets in a complement-dependent manner [53]. Interestingly, macrophages in PNS promote recovery in GBS [48].

There are resident endoneurial and monocyte-derived macrophages in GBS and EAN. Different from microglia, most of these resident macrophages in the PNS are renewed by monocyte-derived macrophages [135]. In PNS, resident endoneurial macrophages express MHC-I, MHC-II, and complement receptors [136]. Monocyte-derived macrophages are important for full-brown inflammatory disease in EAN because elimination of these cells reduced disease severity [137]. A study indicated that TNF-α exacerbated EAN by inducing proinflammatory macrophages. However, TNF-α deficiency attenuated EAN by inducing a switch of macrophage phenotype from proinflammatory subtype to anti-inflammatory subtype [52]. Similarly, compound A which is a plant origin ligand of glucocorticoid receptors also could relieve the severity of EAN by inducing anti-inflammatory macrophages [138].

So, it is better to understand the roles of resident and blood derived macrophages, as well as M1 and M2 cells in the development of GBS and EAN.

4. Conclusion

Macrophages, both proinflammatory and anti-inflammatory, participate in the complex immunopathological framework in the pathologies of neuroimmune diseases. The change of microenvironment in disease process dictates macrophage polarization, such as functional and hypotypic differentiation. Future studies are needed for the exploration of the exact double roles of macrophage subsets and the shift between them, indicating a macrophage-centered therapeutic strategy for neuroimmune disorders.

Competing Interests

The authors report no conflict of interests.

Authors' Contributions

Xueli Fan and Hongliang Zhang contributed equally to the work.

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Review Article

The Response of Macrophages and Neutrophils to Hypoxia in the Context of Cancer and Other Inflammatory Diseases

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Lack of oxygen (hypoxia) is a hallmark of a multitude of acute and chronic diseases and can be either beneficial or detrimental for organ restitution and recovery. In the context of inflammation, hypoxia is particularly important and can significantly influence the course of inflammatory diseases. Macrophages and neutrophils, the chief cellular components of innate immunity, display distinct properties when exposed to hypoxic conditions. Virtually every aspect of macrophage and neutrophil function is affected by hypoxia, amongst others, morphology, migration, chemotaxis, adherence to endothelial cells, bacterial killing, differentiation/polarization, and protumorigenic activity. Prominent arenas of macrophage and neutrophil function, for example, acute/chronic inflammation and the microenvironment of solid tumors, are characterized by low oxygen levels, demonstrating the paramount importance of the hypoxic response for proper function of these cells. Members of the hypoxia-inducible transcription factor (HIF) family emerged as pivotal molecular regulators of macrophages and neutrophils. In this review, we will summarize the molecular responses of macrophages and neutrophils to hypoxia in the context of cancer and other chronic inflammatory diseases and discuss the potential avenues for therapeutic intervention that arise from this knowledge.

1. Introduction

Oxygen is of central importance for life and oxygen availability impacts on various physiological and pathophysiological processes across a wide range of species. To guarantee a sufficient supply of cells and tissues with O_2, intricate oxygen delivery systems emerged during the evolution of biological complexity [1]. As the majority of organs and functional tissues display multicelled structures, local oxygen concentration is markedly different already in the healthy state. Indeed, local variances in O_2 concentration are of central importance for embryonic development and normal organ function, for example, in cartilage, liver, and kidney [2, 3]. Lack of oxygen (hypoxia) is a hallmark of a multitude of acute and chronic diseases and, depending on degree and duration, can be either beneficial or detrimental for organ restitution and recovery [4]. The physiological differences in local oxygen concentration and the dynamic nature of oxygen in cells and tissues result in a wide range of oxygen partial pressure in mammalian organisms: values from 150 (lung apices), 100 (alveoli and arterial blood), to <20 mmHg (bone marrow) were reported [5]. In the context of inflammation, oxygen metabolism and, eventually, hypoxia are particularly important and significantly influence the course of inflammatory diseases. In this review, we will summarize how two prominent representatives of myeloid cells, macrophages and neutrophils, respond to hypoxia in the context of inflammation. We will focus on cancer and certain chronic inflammatory diseases. The intriguing importance of hypoxia/HIFs for myeloid cell function during infectious diseases has been covered by excellent reviews before [6, 7] and will not be discussed in detail here.

2. Inflammation and Hypoxia

The term "Inflammation" refers to a complex and highly ordered sequence of events by which the organism reacts to potentially harmful situations with the aim to defend and reconstitute tissue integrity. Inflammatory reactions can be triggered by microorganisms, chemicals, radiation, and

mechanical force, to name a few. As chief effectors of the innate immune system, macrophages and neutrophils are of paramount importance in the inflammatory process and can be found in high numbers and strongly activated states in inflamed tissues. To fully comprehend the pivotal role of macrophages and neutrophils it is important to note that they not only engulf and kill microorganisms, but also orchestrate the activation of other cell types important for tissue/organ reconstitution, for example, lymphocytes, fibroblasts, and endothelial cells [8, 9]. Inflammation is intricately linked to oxygen metabolism [10]. Calor (heat), tumor (swelling), and rubor (redness), three of the four classical signs of inflammation, are based on enhanced blood flow and vascular permeability and are hence directly associated with altered oxygen distribution in inflamed areas. It is important to note that, while enhanced blood flow suggests boosted oxygen delivery, inflamed areas are usually severely hypoxic, most prominently in the acute stage [11–13]. Traditionally, this has been attributed to reduced oxygen diffusion due to higher interstitial pressure (swelling) and enhanced oxygen consumption of cells in their struggle to survive the harsh conditions of inflamed areas. Intriguing results from Campbell and colleagues have substantially expanded our perception of the mechanisms and functional relevance of hypoxia during inflammation in recent years [14]. While the neutrophil respiratory burst had been hypothesized to contribute to inflammation-associated hypoxia before, Campbell et al. presented convincing experimental evidence for a functional role of activated neutrophils in (a) oxygen depletion during colitis and (b) the induction of a transcriptional hypoxic response in intestinal epithelial cells [14]. Furthermore, mice with a defective respiratory burst (Nox2 −/− mice, a model system for chronic granulomatous disease) displayed severe impairment of inflammatory resolution in the gut, supporting the notion that hypoxia and hypoxia-induced transcriptional responses are functionally relevant for various aspects of the pathogenesis of inflammation [14]. The intricate link between hypoxia and inflammation is furthermore demonstrated by the observation that hypoxic conditions *per se* are able to induce inflammatory reactions [10]. Exposure of mice to 5% O_2 for 60 minutes resulted in significantly enhanced protein expression of IL-6, TNF-α, and IL-1 in both serum and isolated macrophages [15]. Similar observations have been made in humans as healthy volunteers showed increased serum levels of proinflammatory factors after three overnight stays at high altitude [16]. The *in vitro* response of macrophages to hypoxia is complex and very much determined by macrophage phenotype and source as well as the culture conditions. In general, hypoxia exerts profound effects on various important aspects of macrophage biology, for example, expression of cell surface markers, viability, phagocytosis, metabolic activity, and cytokine release (comprehensively reviewed in [17]). The notion that ischemia-associated inflammatory reaction of lung and kidney grafts increases the risk of transplant failure and graft rejection demonstrates the clinical relevance of hypoxia-induced inflammation [18, 19]. All of the above translates into the possibility of a vicious circle where hypoxia and inflammation cooccur and mutually boost each other [20]. It is reasonable to assume that the molecular mechanisms that fine-tune the hypoxia-inflammation circle represent attractive targets for the treatment of chronic, nonresolving inflammation. The latter notion is further supported by alleviation of experimental colitis via delivery of oxygen [21, 22].

3. Hypoxia and Cells of the Innate Immune System

The history of research on the metabolism of immune cells resembles the history of cancer metabolism research as both topics were highly investigated in the beginning of the 20th century, followed by decades of faded interest and a surge in exciting and innovative results in the last 15 years (partly explained by unprecedented technical improvements and the widespread availability of omics methods). It was first reported a little over a century ago by Levene and Meyer that leukocytes display high glycolytic activity [23, 24]. This observation was confirmed by independent researchers in subsequent studies [25, 26] and led to the conclusion in 1938 that in leukocytes "fermentative metabolism was high in comparison to the oxidative metabolism and that splitting of sugar into lactic acid took place under aerobic conditions" [27]. As glycolysis represents the principal means to generate energy when oxygen is scarce, these findings argued for a pronounced dependence of leukocytes on the molecular mechanisms behind the response to hypoxia. While various transcription factors are induced upon oxygen depletion [28], hypoxia-inducible factors 1 and 2 (HIF-1, HIF-2, collectively termed HIFs) represent the principle molecular mediators of the hypoxic response [29, 30]. Genetic inactivation of HIF-1α in myeloid cells (via lysozyme M-Cre [31]) resulted in the notion that HIF-1α is indeed essential for inflammation in different acute and chronic murine model systems [32]. A similar genetic approach revealed that HIF-2α in macrophages is fundamental for proinflammatory cytokine expression upon LPS treatment as well as the *in vivo* response to cutaneous and peritoneal irritants [33]. These two fundamental studies established the functional importance of the HIFs for myeloid cell function and kicked off a huge number of follow-up studies that significantly broadened our understanding of the interplay between hypoxia and inflammation. The following chapters attempt to summarize parts of this work with special emphasis on the response of neutrophils and macrophages to hypoxia in the context of cancer and other inflammatory diseases.

4. Neutrophils

Neutrophilic granulocytes, or shortly neutrophils, are part of the mammalian innate immune system and recruited to wounds and infections during the early disease phase. With 50–70% they constitute the most abundant circulating white blood cell population. Chemical signals such as the chemokine IL-8, complement factor C5a, N-formylated peptides, platelet-activating factor, and leukotriene B4 attract neutrophils to inflammatory sites [34]. After sensing of bacteria or mediators of inflammation, neutrophils phagocytose

microbes followed by assembly of an electron transport chain (NADPH oxidase) which shuttles electrons across the membrane to molecular oxygen for the generation of hypochlorous acid (HClO) and reactive oxygen species leading to lysis of microbes [35]. This process is termed "respiratory burst" and requires a significantly elevated consumption of molecular oxygen [36]. The respiratory burst represents an essential antimicrobial pathway of neutrophils. Furthermore, neutrophils can kill invading pathogens via release of granule contents, activating cytokines like TNF-α, IL-1, interferons, defensins, or reactive nitrogen species, and in some instances they generate extracellular traps [34]. As outlined in detail above, the presence of activated neutrophils at sites of inflammation results in oxygen depletion, a phenomenon aptly referred to as "inflammatory hypoxia," underscoring the taut link between inflammation and hypoxia [37].

5. Neutrophils, Hypoxia, and Inflammation

Inflamed lesions often become severely hypoxic due to increased cellular oxygen demand and reduced availability caused by trauma, compression, or thrombosis [10–13]. Hypoxia and HIFs, in turn, influence various aspects of neutrophil biology. The rather short-lived naïve cells are activated and possess increased survival times within inflammatory environments [34]. Hypoxia-associated inhibition of neutrophil apoptosis was demonstrated to be NF-κB-dependent, indicating NF-κB as a regulator of the hypoxic response in neutrophils [38]. Furthermore, the neutrophil activating and survival factor MIP-1β (macrophage inflammatory protein-1β) was shown to be induced under hypoxic conditions, operating as an alternative mediator of neutrophil survival [38]. Neutrophil binding to the epithelium is facilitated by HIF-1-promoted β_2 integrin expression [39]. Moreover, neutrophils mainly rely on high rates of glycolysis for the generation of ATP in which HIF-1α is critically involved by regulating the expression of key glycolytic enzymes [32]. The absence of HIF-1 causes depletion of intracellular ATP pools resulting in profound impairment of the inflammatory response due to decreased neutrophil aggregation, motility, bacterial killing, and invasion, once more suggesting HIF-1α to be crucial for neutrophil functionality [32, 40]. In addition, HIF-1 increases neutrophil expression of antimicrobial molecules, which is, for example, suggested by experiments showing that myeloid-specific HIF-1α deficiency increases susceptibility to local as well as systemic bacterial infections [32, 40]. Interestingly, neither neutrophil development nor differentiation is affected by specific deletion of HIF-1α in the myeloid progenitor lineage [32].

Far less is known about the role of HIF-2α during neutrophilic inflammation, although when isolated from patients they were shown to express increased amounts of HIF-2α [41]. Thompson et al. reported that HIF-2α-deficient murine inflammatory neutrophils displayed no impairment of chemotaxis, phagocytosis, or respiratory burst but elevated sensitivity to apoptosis leading to reduced neutrophilic inflammation [41]. In line with this notion, neutrophils carrying HIF-2α gain-of-function mutations had lower apoptosis rates. This study suggests a predominant role of HIF-2α for the resolution of inflammation. Certainly, further investigations of the functions of HIF-2 are needed to broaden our understanding of its influence on neutrophil performance during inflammation.

Hypoxia not only influences neutrophil activity; but neutrophils also shape the tissue microenvironment through depletion of local molecular oxygen. As they migrate across the epithelium they change the mRNA expression profile of epithelial cells, which consequently stabilize HIF and upregulate genes responding to hypoxia [14]. Infiltrating neutrophils further modulate the host response to inflammation, resulting in effective inflammatory resolution and tissue protection for which oxygen depletion proved to be critical [14]. Taken together, therapeutic targeting of neutrophils at inflammatory sites has to be carefully executed and precisely timed to prevent nonresolving inflammation or other potentially harmful outcomes.

6. Hypoxia and Tumor-Associated Neutrophils (TANs)

Neutrophils comprise a significant proportion of the inflammatory infiltrate in cancerous lesions and high levels of blood neutrophils were observed in patients suffering from advanced stage tumors [42]. In many cancer types, such as bronchoalveolar carcinoma [43], metastatic melanoma [42], and renal carcinoma [44], neutrophil accumulation was associated with poor prognosis, related to increased aggressiveness [45] or, as in human gliomas, to tumor grade [46]. In contrast, high neutrophil counts in gastric tumors correlate with favourable prognosis [47].

The potent influence of TANs on cancer development, progression, and outcome is becoming more and more appreciated [48–50]. In consideration of the prominent evidence for hypoxia affecting neutrophil behaviour and activity in tumors, it is surprising that until now only a small number of studies focused on this topic. Adherence of neutrophils to the endothelium, their activation, and elevated vessel extravasation leading to tumor infiltration were attributed to hypoxia-induced signalling in endothelial cells [51]. Furthermore, a report by Atai et al. suggests that HIF-1-dependent induction of osteopontin is crucial for the recruitment of neutrophils to neoplastic lesions [52]. IL-8, the main neutrophil attracting chemokine, is also induced in the course of the hypoxic response [53, 54]. Analysis of HIF-1-regulated, hypoxia-associated genes revealed augmented gene expression in TANs compared to splenic myeloid derived suppressor cells (MDSCs) for iNOS, IL-10, and IL-6 [55]. The neutrophil-specific serine protease elastase supports cancer cell proliferation [56] and its release is triggered under hypoxia [57]. Interestingly, in the hypoxic microenvironment of tumors, TANs are suggested to influence the classical (M1) versus the alternative (M2) polarization of macrophages [58, 59].

In analogy to the classification of macrophages, tumor-associated neutrophils were subdivided into two different polarization states: N1 and N2 [60]. Protumorigenic N2 TAN formation by TGF-β, which is another HIF-1 target and considered the master mediator of this process, was demonstrated to be induced under hypoxia [60]. In turn, TANs

take on a proinflammatory and antitumorigenic N1 phenotype under conditions of TGF-β blockade and, by secreting reactive oxygen species, exhibit the potential to induce tumor cell lysis and growth arrest.

7. Neutrophils in Other Inflammatory Diseases

Macrophages were for a long time attributed to be the central players during inflammatory disease like rheumatoid arthritis (RA), whereas the influence of neutrophils in this context was largely elusive. However, compared to macrophages neutrophils are often found at much higher numbers at inflammatory sites and they are similarly capable to present antigens to and activate T-cells [61]. Neutrophils represent the most abundant immune cell type in the synovial fluid from joints of RA patients and were found at active sites of bone and cartilage destruction in this setting [62, 63]. Elevated secretion of ROS by neutrophils was suggested to be part of the disease driving processes during RA progression [64].

In the course of inflammatory liver disease, neutrophils were directly implicated in hepatocellular death mediated by the respiratory burst. They were shown to be recruited through TNF-α and other factors released by tissue-resident Kupffer cells [65]. Even in cases where other stimuli led to destruction of the liver parenchyma, the involvement of neutrophils often aggravated disease outcome [66]. Ischemia-reperfusion liver injury taking place, for example, during transplantation is another type of inflammatory process in which primed neutrophils take part to a significant extent [67, 68]. Furthermore, as a consequence of extensive alcohol consumption, neutrophil influx into the liver, hepatocyte degeneration, and necrosis finally result in neutrophilic steatohepatitis [69, 70]. Mechanistically, osteopontin was suggested to be critically involved as it is induced in rat hepatocytes after feeding the animals with an ethanol-containing liquid diet and its cleaved form correlated with neutrophil infiltration [71].

In patients suffering from chronic obstructive pulmonary disease (COPD), neutrophils are the most abundant inflammatory cells in the bronchial wall and lumen and neutrophil accumulation was reported to correlate with the decline of lung functionality [71–74]. In response to pollutants or infective agents, pulmonary epithelial cells or resident alveolar macrophages secrete chemoattractants inducing the recruitment of neutrophils and other immune cells [75]. However, not only elevated tissue invasion but also impaired neutrophil clearance was implicated in the pathogenesis of COPD as alveolar macrophages exhibit a loss in phagocytic activity and cigarette smoke has directly been linked to reduced phagocytosis of apoptotic neutrophils [76, 77]. Clinical trials investigating the efficiency of drugs that promote neutrophil apoptosis and clearance in patients with chronic obstructive pulmonary disease are ongoing.

8. Macrophages

Macrophages are phagocytic cells and crucial effectors of innate immunity in the primary response to pathogens besides their key role in acute and chronic inflammatory responses. Many pathological processes with macrophage involvement (e.g., inflammation, wound healing, atherosclerosis, and tumors) are characterized by hypoxia [29]. Hypoxic zones arise especially in inflamed tissues, driving cellular metabolism to adapt to this hostile microenvironment. It is therefore not surprising to note that HIFs are found stabilized in macrophages at various stages of activation and polarization [78, 79] and that inhibition of HIF impacts on a plethora of archetypical macrophage functions such as aggregation, migration, and invasion [6, 32, 40].

9. Response to Hypoxia: Macrophages and HIFs

Hypoxia influences various aspects of macrophage function, including energy metabolism and different immune responses. Myeloid cell-specific inactivation of HIF-1α via Cre/loxP-mediated conditional gene inactivation resulted in notably reduced inflammatory responses in skin and joint inflammation [32]. In this experimental setting, significantly reduced intracellular ATP levels were detected in HIF-1α-deficient macrophages, enforcing the pivotal importance of HIF-1α-controlled glycolysis for energy generation in myeloid cells [24, 25]. Besides sterile inflammation, hypoxia also commonly occurs in areas of infection [6]. As macrophages are of paramount importance in the first line defence against invasive microorganisms, it has long been hypothesized that these cells must be especially equipped to cope with and function in hypoxic areas. It was convincingly shown, again via conditional gene ablation in mice, that HIF-1α is of paramount importance for bacterial killing activity of macrophages (and neutrophils) [32, 40]. Of note, the antimicrobial effect of HIF-1α was not limited to hypoxic culture conditions, but clearly evident under ambient air, further supporting the above outlined hypoxia-independent importance of HIF-1α in macrophages. In line with this notion, bacterial infection of macrophages under normoxic culture conditions results in robust stabilization of HIF-1α protein [40]. This effect is (partly) mediated by lipopolysaccharide (LPS, a component of the outer membrane of gram-negative bacteria) as LPS represents a potent inducer of both mRNA expression and HIF-1α protein accumulation in murine macrophages and human monocytes [80, 81]. This suggested a functional importance of toll-like receptor 4 (TLR-4, the archetypical LPS receptor) for HIF-1α activation and resulted in the publication of a plethora of interesting publications addressing this point. We know today that HIF-1α and TLRs interact bidirectionally on many biologically relevant levels [82]. On the one hand, HIF-1α regulates the surface expression of various TLRs (e.g., TLR-2, -4, -6, and -9) [83–85]. On the other hand, intracellular signal transduction of several TLRs is (partially) mediated by HIF-1α (e.g., TLR-2, -3, -4, -7/8, and -9) [85–88]. Of special interest in downstream TLR signalling is the NF-κB family of transcription factors. NF-κB represents a pivotal control element of the immune system and is potently induced by LPS [89]. Of note, LPS-induced HIF-1α activation is dependent on NF-κB in human monocytes and murine macrophages [80, 90].

Taken together, the paramount importance of HIF-1α for TLR activation qualifies as a molecular explanation for the outlined function of HIF-1α in microorganism defence.

Compared with the vast amount of literature available regarding the importance of HIF-1α, the role of HIF-2α for the hypoxic response of macrophages is only beginning to emerge. Hypoxic culture conditions lead to robust accumulation of HIF-2α protein in various myeloid cell types, for example, human monocyte-derived macrophages (MDM) and primary murine bone marrow-derived macrophages (BMDM) [33, 91]. Functional inactivation of HIF-2α, by either RNA interference in MDM or Cre/loxP-mediated deletion in BMDM, resulted in significantly reduced transcriptional responses to hypoxia (and to proinflammatory stimulation with LPS plus interferon-γ) [33, 78]. Comparable to other cell types, the function of HIF-1α and -2α for the hypoxic response of macrophages is not redundant at all times, but distinct regarding the regulation of selected factors [30]. For example, loss of HIF-2α in macrophages does not impact on the expression of two classical HIF-1α target genes, the inducible NO synthase (iNOS) and VEGF-A [33, 92]. On the other hand, HIF-2α activates soluble VEGF receptor-1, a potent inhibitor of VEGF, while HIF-1α is without effect [92].

10. Macrophage Polarization and Arginine Metabolism

Macrophages are highly plastic cells and can rapidly change their polarization in response to microenvironmental cues [93]. In recent years, the concept of a Th1-driven, proinflammatory macrophage (termed M1) and a Th2-driven, proangiogenic/immune-evasive M2 macrophage has evolved. The above outlined connection of hypoxia and inflammation led to the question how/if HIFs are involved in macrophage plasticity. Takeda and colleagues were the first to address this point and intriguingly found that HIF-1α and HIF-2α contributed to macrophage polarization in opposing ways [94]. While Th1 cytokine-induced M1 skewing of murine BMDM is paralleled by HIF-1α protein stabilization, M2 induction with interleukin-4 resulted in HIF-2α protein accumulation [94]. While hypoxia potentiated this response, the cytokine-induced HIF stabilization was clearly detectable under normoxic conditions, further supporting the notion of hypoxia-independent HIF accumulation. Analysis of HIF-1α- or HIF-2α-deficient murine macrophages further strengthened the opposing roles of the 1α and 2α isoform for macrophage polarization [94]. It is important to note that macrophage polarization was not the focus of the experimental setup applied by the Johnson group as their primary goal was to unravel the role of HIFs for NO homeostasis in macrophages. In principle, two factors determine extracellular NO abundance (via competition for the precursor L-arginine): the family of NO synthases (most prominently iNOS) produces NO while arginase-1 metabolizes L-arginine into ornithine and polyamines, effectively reducing extracellular NO levels [95]. Interestingly, these factors are differently expressed in polarized macrophages: iNOS in M1 and arginase-1 in (murine) M2 macrophages [93, 96]. Via the identification of arginase-1 as a HIF-2α target gene, Takeda et al. provided a molecular mechanism for the opposing effect of the HIFs on macrophage polarization. It was first reported in the late 1980s that activated murine macrophages are able to kill tumor cells via iNOS-derived NO [97, 98]. The rapid progression of the majority of malignant tumors led to the assumption that macrophage-mediated killing is somehow compromised during malignant progression [95]. Indeed, it could be shown that NO production in many tumors is reduced due to diminished iNOS activity in macrophages [99]. Convincing data from independent research groups argue for a time- or stage-dependent effect: at early stages, (M1) macrophage-derived NO results in tumor cell killing. Dying tumor cells release various factors (e.g., TGF-β, interleukin-10, or sphingosine-1-phosphate) that lead to M2 polarization of macrophages, which express high levels of arginase-1, ultimately resulting in reduced intratumoral NO abundance, thus contributing to tumor progression [95]. The work by Takeda et al. complements as well as expands this concept by pointing to HIF-2α as an important molecular mechanism in the switch from M1 to M2 during tumor progression. Using spheroids from human breast cancer cells, Werno and colleagues presented additional experimental evidence for a role of HIF-1α for M1 polarization [100]. *In vivo* confirmation of these results is missing thus far, but as more and more reliable antibodies against M1/M2-specific markers are available and FACS-based characterization of the tumor immune infiltrate prevails this should only be a matter of time.

11. Tumor-Associated Macrophages (TAMs)

Hypoxia is a hallmark of solid tumor formation and a potent driver of the malignant phenotype. In certain entities, cervical, breast, prostate, and head and neck cancer as well as melanoma, hypoxia represents an independent prognostic factor [101]. Macrophages are attracted by and accumulate in hypoxic regions and intratumoral hypoxia is a pivotal regulator of TAM function [102, 103]. Among these, angiogenesis induction is probably the best studied phenomenon. In human breast cancer, the proangiogenic factor VEGF-A is expressed almost exclusively in macrophages in hypoxic areas, a process largely dependent on HIF-1α, as suggested by experiments performed with murine macrophages [32, 104]. In addition to VEGF-A, the expression of additional proangiogenic molecules in TAMs like basic fibroblast growth factor (bFGF), CXCL8/IL-8, adrenomedullin, and matrix metalloproteinase 9 (MMP-9) is induced by hypoxia in a HIF-1α-dependent manner [105]. Furthermore, HIF-1 activity was shown to enhance the expression of the chemokine CXCL12 and of its receptor CXCR4, both crucially involved in angiogenesis and cancer metastasis [106]. Angiogenesis inhibitors were among the first molecular targeted drugs approved for cancer therapy and their market launch was paralleled by enormous expectations. Unfortunately, initial enthusiasm soon dissipated as the antiproliferative efficacy did not meet the anticipations [107, 108]. The demonstration of enhanced hypoxia and HIF stabilization in rodent tumor models upon application of angiogenesis inhibitors lead to the assumption that HIFs are causally involved in the resistance to

antiangiogenic therapy [109]. It was subsequently shown by various groups that inhibition of HIF-1 was able to enhance the efficacy of angiogenesis inhibitors [110–112]. Clinical studies are under way to confirm these observations in patients with advanced cancers [113]. Besides angiogenesis, TAMs are able to fuel cancer progression via their suppressive effect on adaptive immunity [114]. Macrophages play a crucial role in this setting as they can inhibit T cell-mediated tumor cell killing in a hypoxia/HIF-1α-dependent manner.

Compared to the available information on HIF-1α, the importance of HIF-2α for TAM biology has been explored to a far lesser extent. An immunohistochemical study with human breast cancer samples displayed HIF-2α protein in TAMs and reported a correlation between high TAM HIF-2α and tumor vascularity and tumor grade [115]. The same group showed HIF-2α-positive TAMs in human head and neck squamous carcinoma, albeit without association with clinical parameters [116]. Celeste Simon and coworkers used conditional gene targeting to address the functional importance of HIF-2α in TAMs. Myeloid-specific loss of HIF-2α resulted in reduced numbers of TAMs in two murine model systems (DEN-induced liver tumors and inflammation-associated intestinal tumors (via AOM+DSS)) [33]. The authors identified reduced migratory and invasive ability of macrophages as the underlying mechanisms. Interestingly, intracellular ATP levels were not affected, in contrast to macrophages displaying a functional loss of HIF-1α [32]. Murine intestine is currently the best studied organ with respect to the role of HIF-2α in inflammatory and proliferative conditions and the existing data were comprehensively summarized in a recent review [117].

12. Macrophages in Other Inflammatory Diseases

Rheumatoid arthritis (RA) is a chronic inflammatory joint disease that causes bone and cartilage destruction. Hypoxia occurs in the course of RA in synovial tissues, potentially affecting inflammation, angiogenesis, synovial responses, and resolution [118, 119]. Increased HIF-1α protein levels were detected in macrophages of RA patients [120] and myeloid-specific deletion of HIF-1α reduces joint swelling and inflammatory activity in a murine arthritis model [32].

Despite impressive improvements in therapy and prevention, cardiovascular diseases are still the leading cause of death in developed countries. Atherosclerosis is a pivotal process in the pathogenesis of cardiovascular diseases and inflammation is centrally involved in atherosclerosis development. Interestingly, a causal role for hypoxia in the pathogenesis of atherosclerotic plaque formation was hypothesized more than 60 years ago [121]. Indeed, while the healthy arterial media is already characterized by reduced oxygen partial pressure (20–50 mmHg), even lower values have been measured in atherosclerotic plaques [122, 123]. Macrophages with ingested lipids (foam cells) are a histopathological hallmark of atherosclerotic plaques. Numerous studies have analyzed the role of HIF-1α in macrophages and foam cells for processes important in atherosclerosis development (reviewed in [124]). While the majority of these studies suggest a functional importance of HIF-1α, *in vivo* studies with macrophage-specific HIF1α null mice have failed to confirm this notion. Unfortunately, the results of these studies have thus far only been presented on scientific conferences and not as peer-reviewed publications. Hence, a causal role of HIF-1α in macrophages for the pathogenesis of atherosclerosis remains elusive at this time.

13. Potential Therapeutic Implications

The protumorigenic role of hypoxia, the functional connection of HIFs with "cancer genes," and the observation that the majority of HIF-regulated biological pathways are positively associated with the malignant phenotype made the HIFs an attractive target for drug development [125]. Currently, 75 studies are listed in https://www.clinicaltrials.gov/ that aim to analyze the efficacy of HIF-1 inhibitors for a wide spectrum of diseases, for example, cancer, wound healing, and cardiovascular diseases. As outlined above, anticancer molecular targeted drugs were thus far not able to meet the gigantic expectations associated with their approval. Against this background it would not be surprising to observe resistance against and, subsequently, diminished antiproliferative efficacy of HIF-targeting substances in the clinical setting. Indeed, we and others have shown that cancer cells are able to compensate for the loss of HIFs very effectively [126]. We therefore strongly believe that it is of crucial importance to analyze the mechanisms that underlie resistance/compensation towards/of HIF inhibition in order to identify combination partners with the potential to result in long-lasting, effective, and well tolerable HIF-based cancer therapy.

14. Perspective

Hypoxia is a hallmark of the hostile microenvironment of inflammation and macrophages and neutrophils, the chief effectors of innate immunity, have evolved to cope with and function in these conditions. Albeit several oxygen-sensitive transcription factors have been described, tissue-specific knock-out mouse models have enabled in-depth deconstruction of the hypoxic response, demonstrating that HIFs are absolutely essential for proper myeloid cell function under hypoxic conditions. Compared with the large amount of literature on the role of HIF-1α, the functional importance of HIF-2α remains elusive for several inflammatory conditions. Another key question is how effective HIF-modifying agents will prove to be in the therapy of acute and chronic inflammation and what kind of side effects will emerge. Will resistance against HIF inhibitors result in diminished antiproliferative or anti-inflammatory efficacy over time and will we be able to deconstruct the underlying mechanisms to design smart combination therapies? These questions, among others, will have to be addressed in order to achieve a successful translation of the exciting science that we had the pleasure to witness after the initial publication of HIF-1 in 1992 and of HIF-2 five years later.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

GEN-27, a Newly Synthetic Isoflavonoid, Inhibits the Proliferation of Colon Cancer Cells in Inflammation Microenvironment by Suppressing NF-κB Pathway

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Nonresolving inflammation is one of the consistent features of the tumor microenvironment in the intestine and plays a critical role in the initiation and development of colon cancer. Here we reported the inhibitory effects of GEN-27, a new derivative of genistein, on the inflammation-related colon cancer cell proliferation and delineated the mechanism of its action. The results indicated that GEN-27 inhibited the proliferation of human colon tumor HCT116 cells stimulated by culture supernatants of LPS-induced human monocytes THP-1 cells and significantly decreased LPS-induced secretion of proinflammatory cytokines interleukin-6 and interleukin-1β in THP-1 cells. The HCT116 cell proliferation elicited by THP-1-conditioned medium could be blocked by the interleukin-1 receptor antagonist (IL-1RA). Further mechanistic study revealed that GEN-27 remarkably inhibited the nuclear translocation of NF-κB and phosphorylation of IκB and IKKα/β in both HCT116 and THP-1 cells. In addition, GEN-27 markedly suppressed the HCT116 cell proliferation stimulated by IL-1β treatment, which was dependent on the inhibition of NF-κB/p65 nuclear localization, as verified by p65 overexpression and BAY 11-7082, an NF-κB inhibitor. Taken together, our findings established that GEN-27 modulated NF-κB signaling pathway involved in inflammation-induced cancer cells proliferation and therefore could be a potential chemopreventive agent against inflammation-associated colon cancer.

1. Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the leading cause of cancer-related mortality among both men and women worldwide [1, 2]. Patients with inflammatory bowel disease (IBD), such as ulcerative colitis (UC) or Crohn's disease, have an increased risk of CRC [3]. The cumulative probability of CRC in UC patients ranges from 2% after 10 years of disease up to 18% after 30 years of disease [4].

It has been suggested that the leukocyte infiltrates exist in neoplastic tissue and there is a close association between chronic inflammation and cancer [5]. Chronic inflammation may be involved in all three stages of tumor development, which contributes to the tumor initiation by inducing DNA damage and chromosomal rearrangement or amplification. It also facilitates tumor promotion by inducing the formation of small clusters of malignant cells. Additionally, inflammation promotes tumor progression by inducing angiogenesis, invasion, and metastasis [6]. Overall, increasing evidence from experiments and epidemiological, preclinical, and clinical studies indicates that chronic inflammation is closely related to tumorigenesis, with CRC being one of the paradigms of the link between inflammation and cancer [7].

Inflammatory cytokines in tumor microenvironment regulate the communication between tumor and stromal cells, and tumor interactions with the extracellular matrix, thereby promoting tumor development [5]. Greten et al. found the evidence of cytokine-regulated tumor promotion in AOM/DSS mouse model of CAC [8]. Primary transcription factors

such as nuclear factor κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3), which are driven by inflammatory cytokines including tumor-necrosis factor α (TNFα), interleukin-6 (IL-6), and interleukin-1β (IL-1β), are key orchestrators controlling inflammation-related cancer [9].

NF-κB plays a crucial role in the immediate-early pathogen responses and regulates many cellular processes including immune signaling, inflammation, cell proliferation, apoptosis, and cancer development. It is sequestered in the cytoplasm which forms an inactive complex with its inhibitor IκB under basal conditions. Upon stimulation with corresponding ligands, such as LPS, IL-1β, or TNFα, the IκB kinase (IKK) complex is activated, which leads to the phosphorylation and proteasomal degradation of IκB, followed by translocation of NF-κB into the nucleus to initiate specific target gene transcription [10]. Dysregulation of NF-κB activation has been strongly related to several autoimmune diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and type I diabetes. In addition, chronic exposure to inflammatory signals in the tumor microenvironment leads to NF-κB activation in malignant cells, further driving tumor cells survival and proliferation. Thus NF-κB pathway has attracted much attention due to its important role in inflammatory diseases and cancers.

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one), a isoflavonoid isolated from dietary soybean, has shown a wide variety of biological activities, such as antioxidant, anti-inflammatory, and anticancer properties, particularly in cancer prevention [11]. In CRC, previous studies have shown that genistein is capable of inducing G2/M phase cell cycle arrest and programmed cell death, inhibiting cell proliferation, and reducing metastasis [12, 13]. Its mechanisms include inhibition of topoisomerase I and topoisomerase II and DNA polymerase II and downregulation of genes encoding cyclins: B1 and D1 [14]. It also suppresses NF-κB pathway, activates ATM/p53-p21 cross-regulatory network, and attenuates WNT signaling by upregulating sFRP2 protein [15, 16].

GEN-27 (5-hydroxy-7-[2-hydroxy-3-(piperidin-1-yl)propoxy]-3-{4-[2-hydroxy-3-(piperidin-1-yl)propoxy]phenyl}-4H-chromen-4-one), a newly synthesized derivative of genistein, was synthesized from genistein through two steps as indicated in Figure 1. Initially, the phenolic hydroxy groups at the C7 and C4′ of genistein were alkylated with (chloromethyl) ethylene oxide in dry ethanol in the presence of K_2CO_3. Then piperidines were coupled with the epoxy substrate to afford GEN-27. It was identified by IR, 1H-NMR, MS, and elemental analysis. The purity was 99.31% determined with HPLC (mp: 140–143°C).

Here in this study, we aimed to study the inhibitory effects of GEN-27 on the proliferation of human colorectal carcinoma HCT116 cells in the inflammatory microenvironment and the underlying mechanisms of the interaction between inflammatory cells and tumor cells.

2. Materials and Methods

2.1. Reagents and Antibodies. GEN-27 was obtained from College of Science, China Pharmaceutical University (Nanjing, China). GEN-27 (purity > 99.5%) was applied in DMSO to 0.1 M and stored at −20°C. The concentrations used here were 1, 5, 10, and 20 μM in vitro and freshly diluted with DMEM to final concentration. LPS, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), and BAY 11-7082 were purchased from Sigma-Aldrich (St. Louis, Mo, USA). LPS was dissolved in distilled phosphate-buffered saline (PBS) at 10 mg/mL and stored in small aliquots at −20°C. Primary antibodies against NF-κB p65 (C22B4), p-IκBα (Ser32), IκBα, p-IKKα/β (Ser176/180), and IKKα were obtained from Cell Signaling Technology (Danvers, MA); antibodies against cyclin D1 (L283), bcl-2 (P65), PCNA, β-actin, and goat anti-rabbit IgG (H&L) HRP were obtained from Bioworld Technology (St. Louis, MN). Recombinant human interleukin-1 receptor antagonist (IL-1RA) and recombinant human IL-1β were purchased from Genscript Corp. (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) kits for determining IL-6 and IL-1β were from Boster Biotech Co. Ltd. (Wuhan, China). Fetal bovine serum and RPMI-1640 were from Gibco (Grand Island, NY, USA).

2.2. Cell Lines and Culture Condition. Human colorectal cancer cell line HCT116 and human acute monocytic leukemia cell line THP-1 were purchased from the Cell Bank of Institute of Cell Biology (Shanghai, China). These two cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C in a humidified incubator containing 5% CO_2.

2.3. Colorimetric MTT Assay. The cytotoxicity was measured by the modified MTT assay. Briefly, the logarithmic cells were plated into 96-well plates at a density of 4000~5000 cells/well in a final volume of 100 μL medium for 12 h at 37°C and then treated with various concentrations of GEN-27 at indicated durations. After 24 h or 48 h incubation, the absorbance (A) was measured at 570 nm by the Universal Microplate Reader (ELx800, BioTek Instruments Inc., Winooski, VT). Percentage of cytotoxicity was determined as follows: percentage of cytotoxicity = $[1 - (A_{570}$ of test sample)/$(A_{570}$ of control sample)$] \times 100\%$. The IC_{50} was taken as the concentration that caused 50% inhibition of cell proliferation and was calculated by SAS statistical software. All assays were performed in triplicate.

2.4. Cell Cycle and Apoptosis Assay. HCT116 cells were plated into 6-well tissue culture plates at approximately 2×10^5/well and treated with various concentrations of GEN-27. After incubation, they were harvested and resuspended with PBS. Apoptosis-mediated cell death of tumor cells was examined using double staining with recombinant FITC-conjugated Annexin-V and propidium iodide (PI), according to the manufacturer's protocol of the Annexin-V-FITC Apoptosis Detection Kit (KeyGen, Nanjing, China). For cell cycle assay, cells were trypsinized, washed with PBS, and fixed in 1.5 mL 95% ethanol at 4°C overnight followed by incubation with RNase and staining by PI. Data acquisition was performed with FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA).

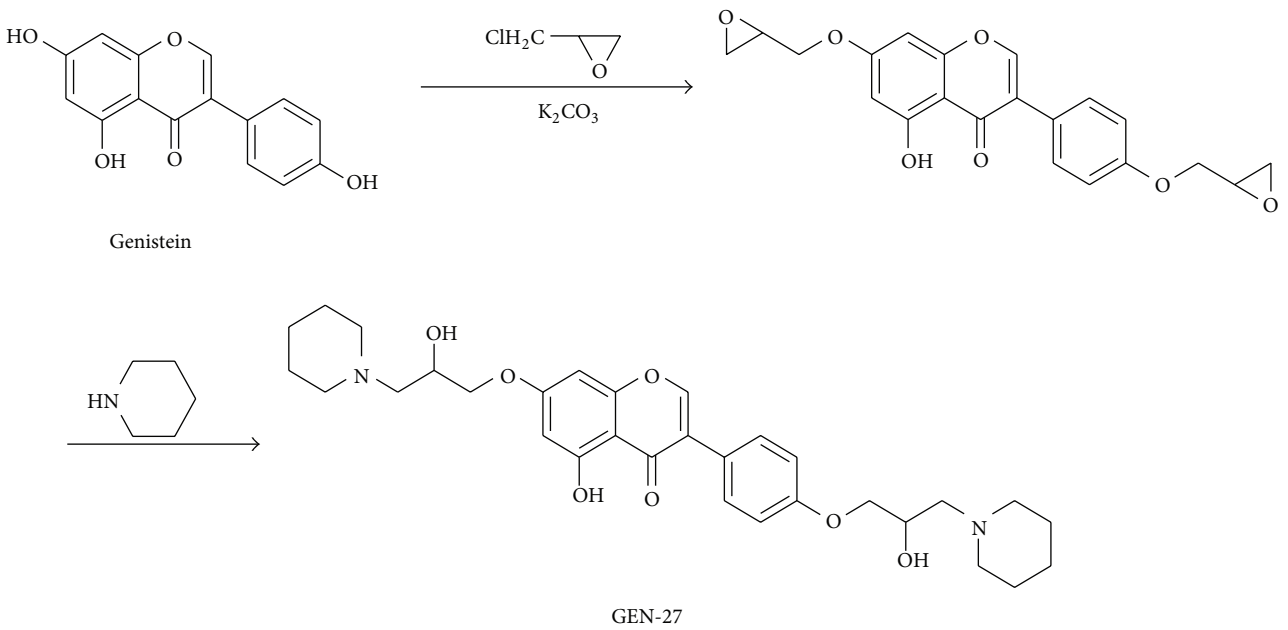


Figure 1: The synthetic route of GEN-27.

2.5. Total RNA Isolation and Real-Time PCR. Human monocyte THP-1 cells were incubated with different concentrations of GEN-27 in the presence or absence of LPS (10 μg/mL). After incubation for 24 h, total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Then the concentration and purity of total RNA were measured by the ratio of A_{260}/A_{280} using an Epoch Microplate Spectrophotometer (BioTek, USA). Real-time PCR was performed as follows: RNA samples were reverse transcribed to cDNA and subjected to quantitative PCR, which was performed with the LightCycler® 96 Real-Time PCR System (Roche, Basel, Swiss) using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The program for amplification was 1 cycle of 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 95°C for 10 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control in the analytical gels. Primer sequences used in this study were listed as follows:

IL-6: 5′-TGTAGTGAGGAACAAGCCAGAG-3′ (forward), 5′-TACATTTGCCGAAGAGCC-3′ (reverse);

IL-1β: 5′-AGGCTGCTCTGGGATTC-3′ (forward), 5′-GCCACAACAACTGACGC-3′ (reverse);

GAPDH: 5′-AAGGTCGGAGTCAACGGATTT-3′ (forward), 5′-AGATGATGACCCTTTTGGCTC-3′ (reverse).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). For analysis of cytokine production, THP-1 cells were cultured at 1×10^5 cells/mL for 24 h. Cells were centrifuged at 2,000 rpm at 4°C for 10 min and the supernatants were carefully collected and applied onto the precoated human IL-6 or IL-1β microplate. ELISAs were conducted according to the manufacturer's recommendations. All reactions were performed in triplicates and the experiments were repeated three times for statistical analysis. Levels of cytokines were expressed in ng/mL.

2.7. Preparation of Cytosolic and Nuclear Extracts and Whole Cell Lysates. HCT116 or THP-1 cells were cultured to 70% confluence and then treated with LPS (10 μg/mL) alone or in combination with GEN-27 for indicated times. Following treatments, cells were harvested by centrifugation and then washed with ice-cold PBS three times. Whole cell lysates were obtained according to the method as described in the following: prepared cells were lysed on ice for 1 h in lysis buffer (100 mM of Tris-Cl; pH 6.8, 4% (m/v) SDS; 20% (v/v) glycerol; 200 mM of β-mercaptoethanol; 1 mM of PMSF; 0.1 mM of NaF and 1 μM DTT). The lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4°C, and the supernatant was collected. The isolation of cytosolic and nuclear extracts was performed according to the method of Nuclear-Cytosol Extraction Kit (KeyGEN Biotech, China) with more modification. Specific steps are as follows: after washing, prepared cells were lysed with membrane lysis buffer (10 mM Hepes-PH 8.0, 10 mM KCl, 1.5 mM $MgCl_2$, and 1 μM DTT), incubated for 15 min on ice, and then added to 1% NonidetP-40 (NP-40) for 10 sec; the supernatant was collected as cytoplasmic fractions after centrifugation at 13,000 rpm for 20 min at 4°C. The precipitate was added with nuclear lysis buffer (2 mM Hepes-PH 8.0, 1.5 mM $MgCl_2$, 42 μM NaCl, 1 μM DTT, 25 μL glycerol, and 0.2 μM EDTA) for 1 h on ice and vortexed every 10 min. The concentration of protein was detected using BCA assay with a Varioskan multimode microplates spectrophotometer (Thermo, Waltham, MA).

2.8. Western Blot Analysis. For immunoblot analysis, equal amounts of protein samples (40~60 μg) were separated

electrophoretically using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The gels were then transferred to 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA) using a semidry transfer system (Bio-Rad, Hercules, CA). The membranes were blocked for non-specific binding with 3% bovine serum albumin (BSA) in PBS for 90 min at 37°C. The blots were incubated with specific primary antibodies overnight at 4°C. After being washed with PBST three times, the blots were incubated with horse radish peroxidase- (HRP-) conjugated immunoglobulin G (IgG) for 1 h at 37°C, and chemiluminescence was detected with Pierce ECL Western blotting substrate (Thermo Scientific) and visualized by ChemiDoc MP Imaging system (Bio-Rad, Hercules, CA, USA).

2.9. Immunofluorescence. For detection of NF-κB p65 translocation to the nuclear, HCT116 cells were planted at 1~2 × 10^5 cells/mL on glass cover slips in 6-well plates and treated with 10 μM GEN-27 for the indicated time period with or without LPS (10 μg/mL). After treatments, cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA) at room temperature for 20 min. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 at 4°C for 10 min, blocking (PBS containing 3% BSA for 1 h) was followed by washing thrice with PBS for 5 min, and then the cells were incubated with the primary antibody NF-κB p65 at a dilution of 1 : 200 in PBS containing 0.5% BSA at 37°C overnight. The next day, cells were washed three times with PBS followed by incubating with Green-Fluorescence Alexa Fluor 488 dye labeled donkey anti-rabbit IgG antibody for 1 h at 37°C. After the immunoreactions, the cover slips were mounted onto microscope slides using Ultra Cruz™ Mounting Medium (Santa Cruz Biotechnology Inc., CA). Immunofluorescence photomicrographs were captured using fluorescent microscope (Olympus IX51, Olympus Corporation, Tokyo, Japan).

2.10. Culture of Human Colon Cancer HCT116 Cells with Conditioned Media from LPS-Treated Human Monocytes THP-1 Cells. HCT116 cells were seeded into 96-well plates at a density of 4000~5000 cells/well in 100 μL medium, grown to 60~70% confluence one day before treatment. THP-1 cells were cultured in 6-well plates and then stimulated with LPS (10 μg/mL) combination of various concentrations of GEN-27 (1, 5, and 10 μM). After the cells were collected by centrifugation, the cultured supernatant was aseptically stored at 4°C for use. Prepared HCT116 cells were (1) left untreated or treated with (2) 10 μg/mL of LPS only, (3) various concentrations of GEN-27 (1, 5, and 10 μM), and (4) cultured supernatant from THP-1 cells which were processed as described above. After HCT116 cells were cultured for 24 h, the cell culture supernatant was removed and the proliferation was determined by MTT assay as described above.

2.11. Plasmids Transfection. NF-κB/p65 plasmid and control plasmid (Addgene, Cambridge, MA, USA) transfections were performed according to the manufacturer's instructions of ExFectTM Transfection Reagent (Vazyme Biotech). The extent of gene overexpression was determined by Western blot.

2.12. Statistical Analyses. Data were expressed as means ± SDs from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical significance was done using an analysis of variance that was followed by Student's t-test and Newman-Keuls test. $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered to be statistically significant.

3. Results

3.1. GEN-27 Inhibits the Cell Viability of THP-1 Cells. Initially, we determined the cytotoxicity of GEN-27 in THP-1 cells using MTT assay. As shown in Figure 2(a), GEN-27 inhibited the growth of THP-1 cells with IC_{50} values of 24.49 ± 0.21 μM (24 h) and 11.28 ± 0.26 μM (48 h), compared with its parent compound genistein with an IC_{50} of 192.4 ± 2.28 μM at 48 h (Figure 2(b)). Combined with LPS treatment, 1, 5, and 10 μM of GEN-27 exerted no effect on the survival and proliferation of THP-1 cells. However, GEN-27 at 20 μM obviously reduced the cell viability of THP-1 cells (Figure 2(c)). Therefore, 1, 5, and 10 μM of GEN-27 were used for all subsequent experiments.

3.2. GEN-27 Inhibits Proliferation of Human Colorectal Carcinoma HCT116 Cells. As shown in Figure 3(a), GEN-27 dramatically reduced the cell viability in HCT116 cells with IC_{50} values of 37.98 ± 0.13 μM (24 h) and 15.11 ± 0.80 μM (48 h), respectively. However, genistein exhibited relatively weak inhibitory effect on the proliferation of HCT116 cells, with IC_{50} of 189.3 ± 2.27 μM (24 h) and 151 ± 2.13 μM (48 h) (Figure 3(c)). Consistent with what we found in THP-1 cells, 1, 5, and 10 μM GEN-27 plus LPS did not induce evident cell death in HCT116 cells (Figure 3(b)). Moreover, different from genistein (100 μM), which showed a dramatic G2/M phase arrest, GEN-27 dose-dependently increased the G0/G1 population in HCT116 cells (Figure 3(d)). The apoptosis-induced cell death rate was significantly elevated by GEN-27 treatment, as determined by Annexin-V/PI assay. The data from Western blot demonstrated that GEN-27 dose-dependently reduced the expression levels of proliferating cell nuclear antigen (PCNA), apoptosis-associated protein bcl-2, and cell cycle regulation protein cyclin D1 (Figures 3(f) and 3(g)). Taken together, GEN-27 inhibits HCT116 cell proliferation through inducing G0/G1 cell cycle arrest and cell apoptosis.

3.3. GEN-27 Suppresses the Proliferation of HCT116 Cells in Response to THP-1-Conditioned Medium Induced by LPS. The interaction between tumor cells and multiple components of the tumor microenvironment, including B and T cells, macrophages, mast cells, fibroblasts, and extracellular matrix, could promote tumor progression [9]. These components can regulate cell growth, differentiation, and survival of tumor cells and thus contribute to tumor promotion and progression via producing soluble factors such as chemokines, cytokines, and growth factors. THP-1 cells have a uniform genetic background with peripheral blood mononuclear cells (PBMC). In response to stimulation with LPS, THP-1 cells

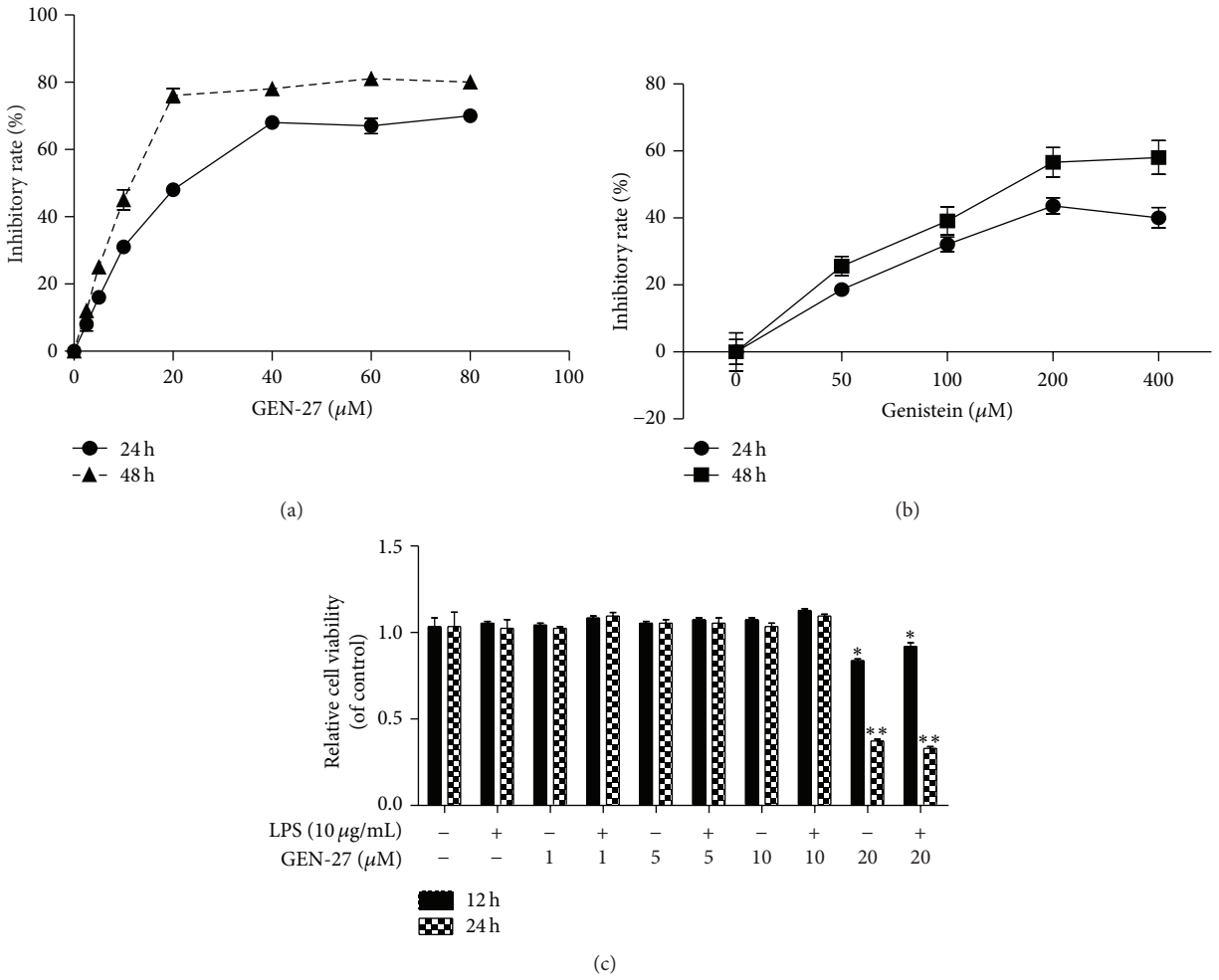
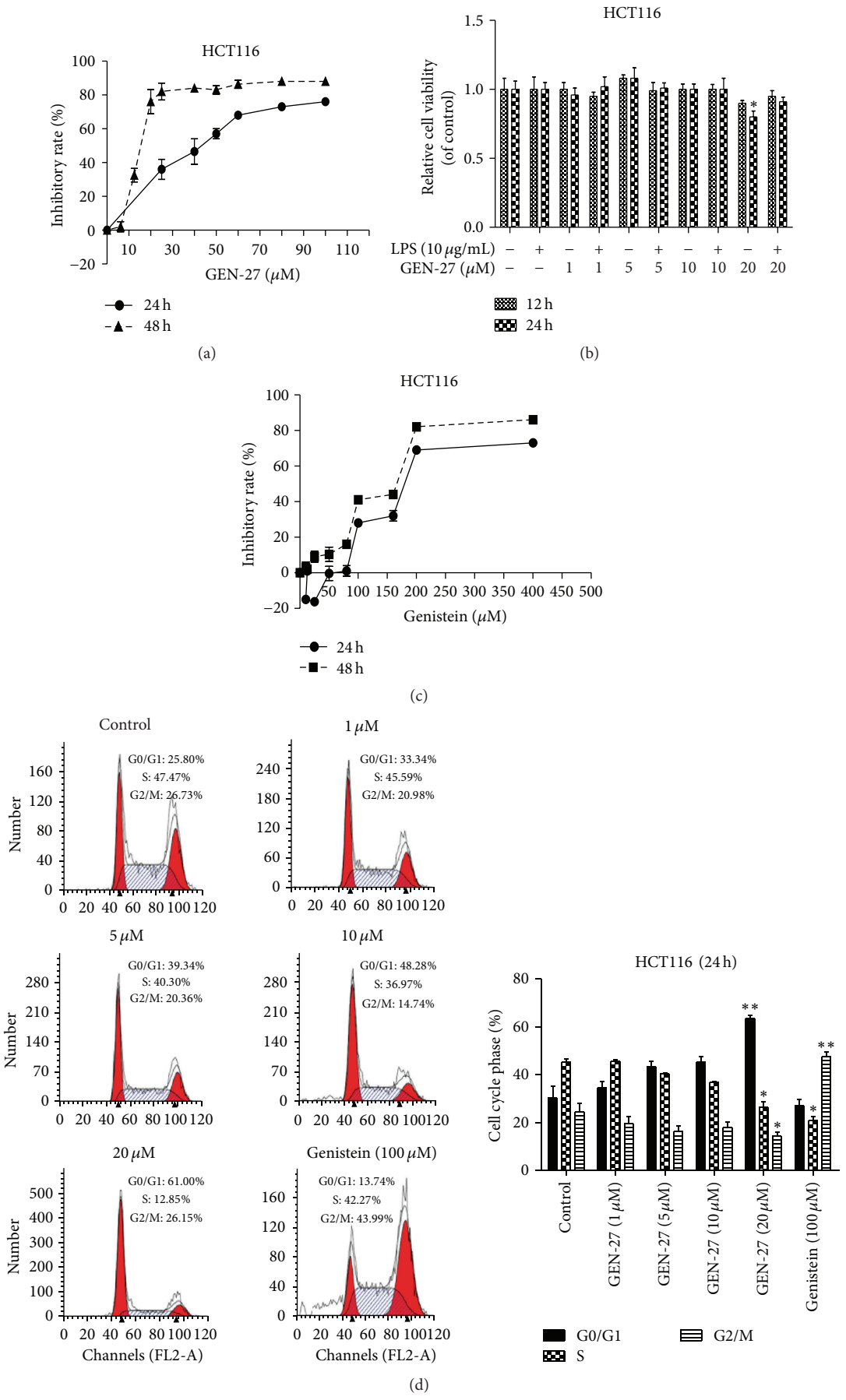


Figure 2: Effects of GEN-27 and LPS on the cell viability of THP-1 cells. (a) THP-1 cells were treated with the different concentrations of GEN-27 alone or (b) the different concentrations of genistein. (c) THP-1 cells were exposed to GEN-27 at different concentrations (1, 5, 10, and 20 μM) in the presence or absence of LPS (10 μg/mL). After treatments, cell viability was determined by MTT assay. Each value indicates the means ± SDs and is representative of the results obtained from three independent experiments. Asterisks ($^{*}P < 0.05$ versus control group; $^{**}P < 0.01$ versus control group, and $^{\#}P < 0.05$ versus LPS group) indicate significant difference compared with the appropriate control cells.

exhibit a similar transcriptional pattern with PBMC-derived macrophages [17]. Thus THP-1 cells are widely used to mimic monocytes in cell culture models. Proinflammatory factors IL-6 and IL-1β are secreted by many cell types, such as immune cells and tumor, stromal, and endothelial cells, which play an important role in inflammation-associated carcinogenesis [3]. Figure 4(b) showed that LPS (10 μg/mL) treatment stimulated the secretion of the IL-6 and IL-1β from THP-1 cells, and GEN-27 dramatically reduced this increase stimulated by LPS in a dose-dependent manner. Consistently, real-time PCR data revealed that the mRNA levels of IL-6 and IL-1β increased by LPS were significantly downregulated by the treatment of GEN-27 in a dose-dependent manner (Figure 4(a)).

To determine the effects of inflammatory cells on tumor cells, HCT116 cells were cultured with the culture supernatant of THP-1 cells stimulated by LPS for 24 h. As shown in Figure 4(c), THP-1 cell-derived factors enhanced the proliferation of HCT116 cells and this effect was suppressed by GEN-27 treatment in a dose-dependent manner, as verified by the reduction of the expressions of PCNA, cyclin D1, and bcl-2 proteins using Western blot (Figures 4(e) and 4(f)). As shown in Figure 4(d), 3 μg/mL IL-1 receptor antagonists (IL-1RA) or 10 μM GEN-27 treatment alone significantly suppressed the proliferation of HCT116 cells induced by conditioned medium, while the suppression induced by GEN-27 was not affected by IL-1RA. Compared with the IL-1RA treatment alone, cotreatment of GEN-27 plus IL-1RA exhibited a significant additive effect on the reduction of HCT116 cell proliferation, which confirmed the vital role of IL-1β in the anticancer effect of GEN-27. Taken together, GEN-27 significantly inhibited HCT116 cells proliferation stimulated by THP-1-derived conditioned medium via reducing the secretion of IL-6 and IL-1β from THP-1 cells.



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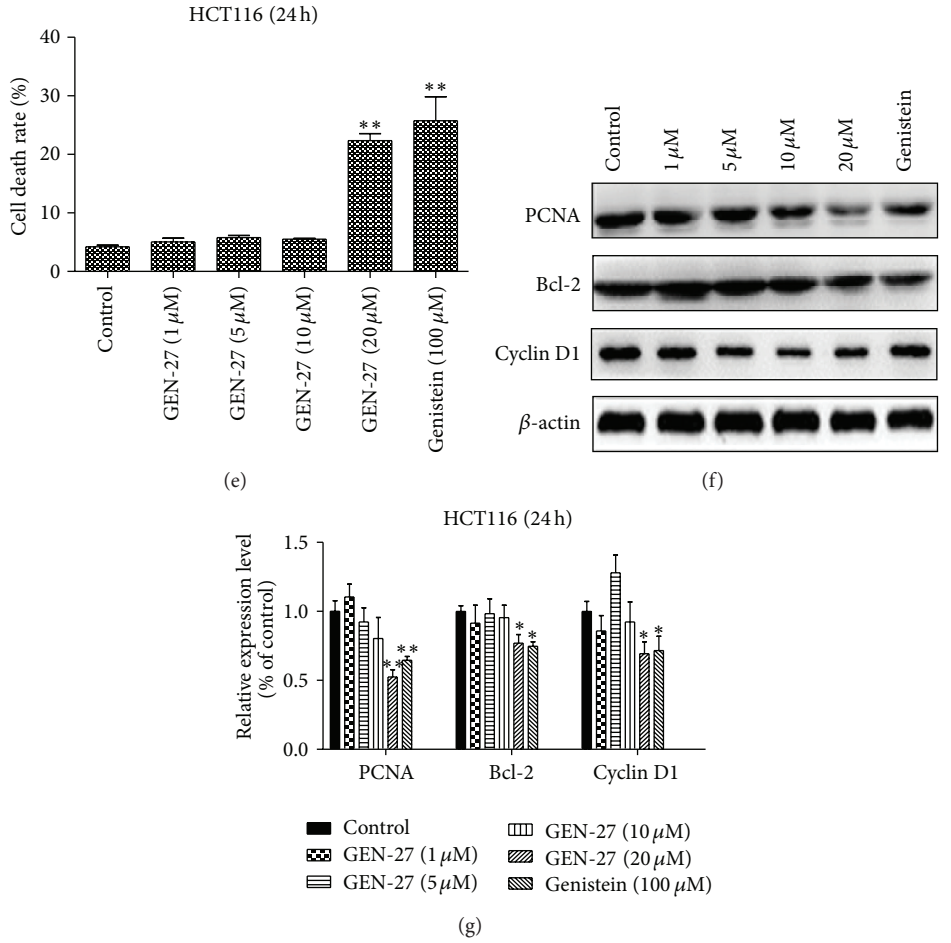


Figure 3: Effects of GEN-27 and LPS on the cell viability of HCT116 cells. (a) Cells were treated with GEN-27 for 24 h and 48 h, (b) exposed to GEN-27 in the presence or absence of LPS (10 μg/mL), or (c) exposed to different concentrations of genistein; then cell viability was determined by the MTT assay. (d) Cell cycle was detected by flow cytometry following PI staining. Cells were treated with different concentrations of GEN-27 and 100 μM genistein for 24 h. Different percentages of three cell phases (G0/G1, S, and G2/M) were shown. (e) Annexin-V/PI double-staining assay of HCT116 cells. Cells were treated with the indicated doses of GEN-27 for 24 h; histograms of death rates were quantitated, containing the early and late apoptosis. (f) The expressions of total proteins PCNA, bcl-2, and cyclin D1 were assessed by Western blot. (g) The relative expressions of total proteins PCNA, bcl-2, and cyclin D1 were normalized to β-actin. Each value indicates the means ± SDs and is representative of the results obtained from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control.

3.4. GEN-27 Inhibits LPS-Induced NF-κB Pathway in THP-1 Cells. Previous studies have shown that NF-κB is a crucial transcription factor that regulates the production of proinflammatory cytokines IL-6 and IL-1β [18, 19]. Translocation of p65, the functional active subunit of NF-κB, is a hallmark of molecular inflammatory phenomenon. The results in Figures 5(a)–5(f) showed that LPS treatment caused a rapid translocation of NF-κB p65 into nuclear fraction, which was markedly inhibited by GEN-27 in dose- and time-dependent manner. Meanwhile, the increase in total NF-κB p65 expression induced by LPS was time- and dose-dependently decreased by GEN-27 in THP-1 cells (Figures 5(a) and 5(c)). One of the main mechanisms involved in the activation of NF-κB is the phosphorylation of IκBα and IKKα/β, which causes the accumulation of NF-κB p65 and its translocation into the nucleus. As shown in Figures 5(c) and 5(f), LPS-treated THP-1 cells exhibited increased phosphorylation of IκBα and IKKα/β and this induction was inhibited by GEN-27. To further identify the inhibitory effect of GEN-27 on NF-κB signaling, BAY 11-7082, an NF-κB inhibitor, was used to inhibit IKKα/β activation, which leads to the translocation of p65 into nucleus. LPS increased p65 level in nuclear fraction, which was repressed by GEN-27 or BAY 11-7082. The reduction induced by GEN-27 was not effected by BAY 11-7082, as verified by the expression of downstream target genes IL-6 and IL-1β at mRNA level (Figures 5(g)–5(i)). These findings suggested that GEN-27 suppressed NF-κB activation by inhibiting nuclear translocation of NF-κB p65 and the phosphorylation of IKKα/β and IκBα.

3.5. GEN-27 Blocks LPS-Induced NF-κB Pathway in HCT116 Cells. To further corroborate the inhibitory effect of GEN-27 on NF-κB pathway in HCT116 cells, we evaluated GEN-27's effects on NF-κB activation induced by LPS. As

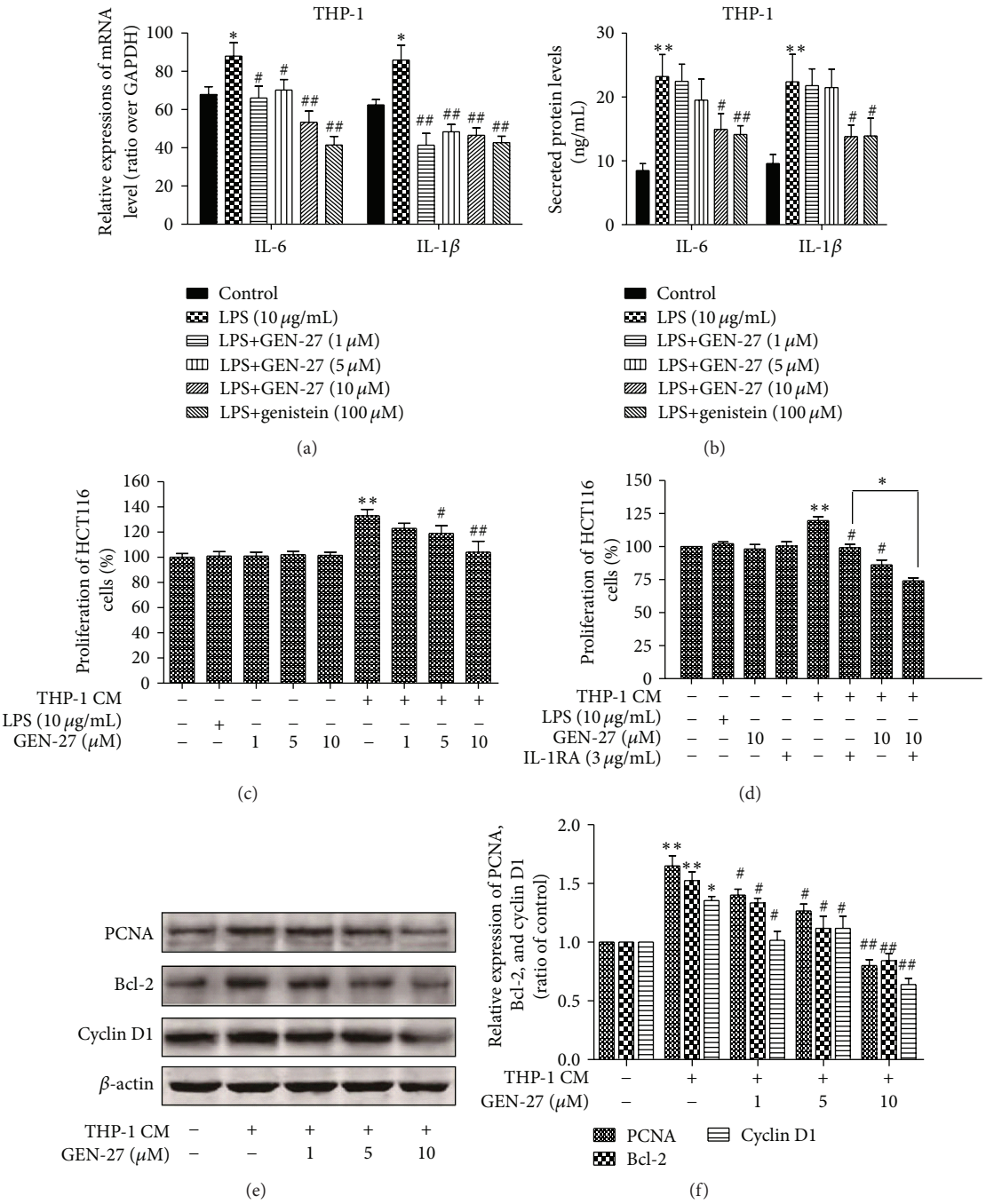
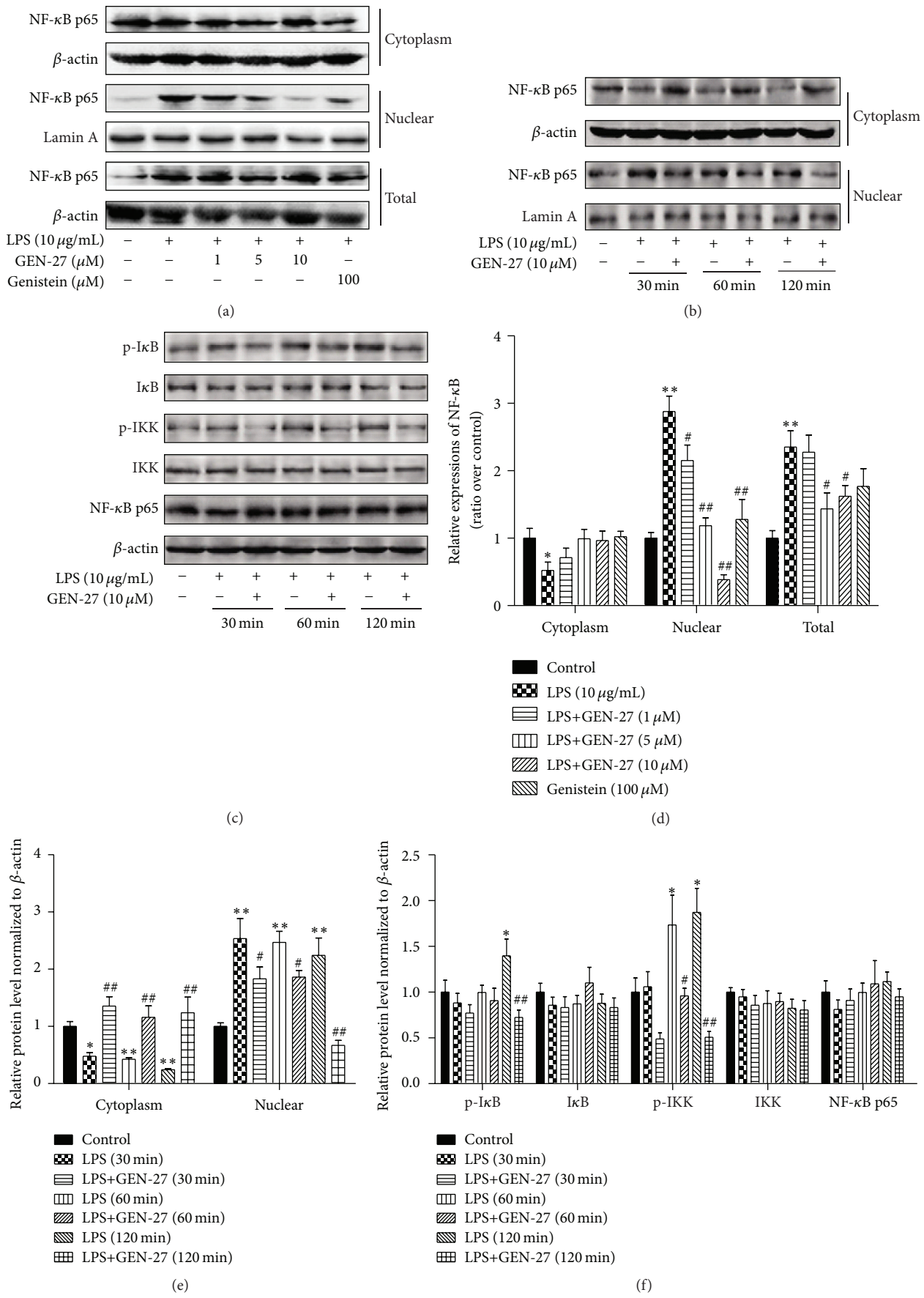


Figure 4: Effects of GEN-27 on LPS-induced production of proinflammatory cytokines in THP-1 cells and the proliferation of HCT116 cells in response to the stimulation by THP-1-conditioned medium. (a) Human THP-1 cells were incubated with 10 μg/mL LPS for 24 h in the absence or presence of 1, 5, and 10 μM GEN-27 and 100 μM genistein. The expression of IL-6 and IL-1β mRNA in THP-1 cells was assessed by real-time PCR. GAPDH was used as an endogenous housekeeping gene. (b) The secretion levels of IL-6 and IL-1β in THP-1 cells after treatments of indicated doses of GEN-27 and genistein were assessed by ELISA. (c and d) HCT116 cells were either left untreated or treated with 10 μg/mL LPS, or THP-1-conditioned medium and indicated dose of GEN-27 together, or combination of 3 μg/mL IL-1RA, THP-1-conditioned medium, and indicated dose of GEN-27 for 24 h. Cell viability was assessed using an MTT assay and the results are expressed as the percentage of surviving cells over control cells. (e and f) The expressions of total proteins PCNA, bcl-2, and cyclin D1 were assessed by Western blot. The relative expressions of total proteins PCNA, bcl-2, and cyclin D1 were normalized to β-actin. Each value indicates the means ± SDs and is representative of the results obtained from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus LPS alone.



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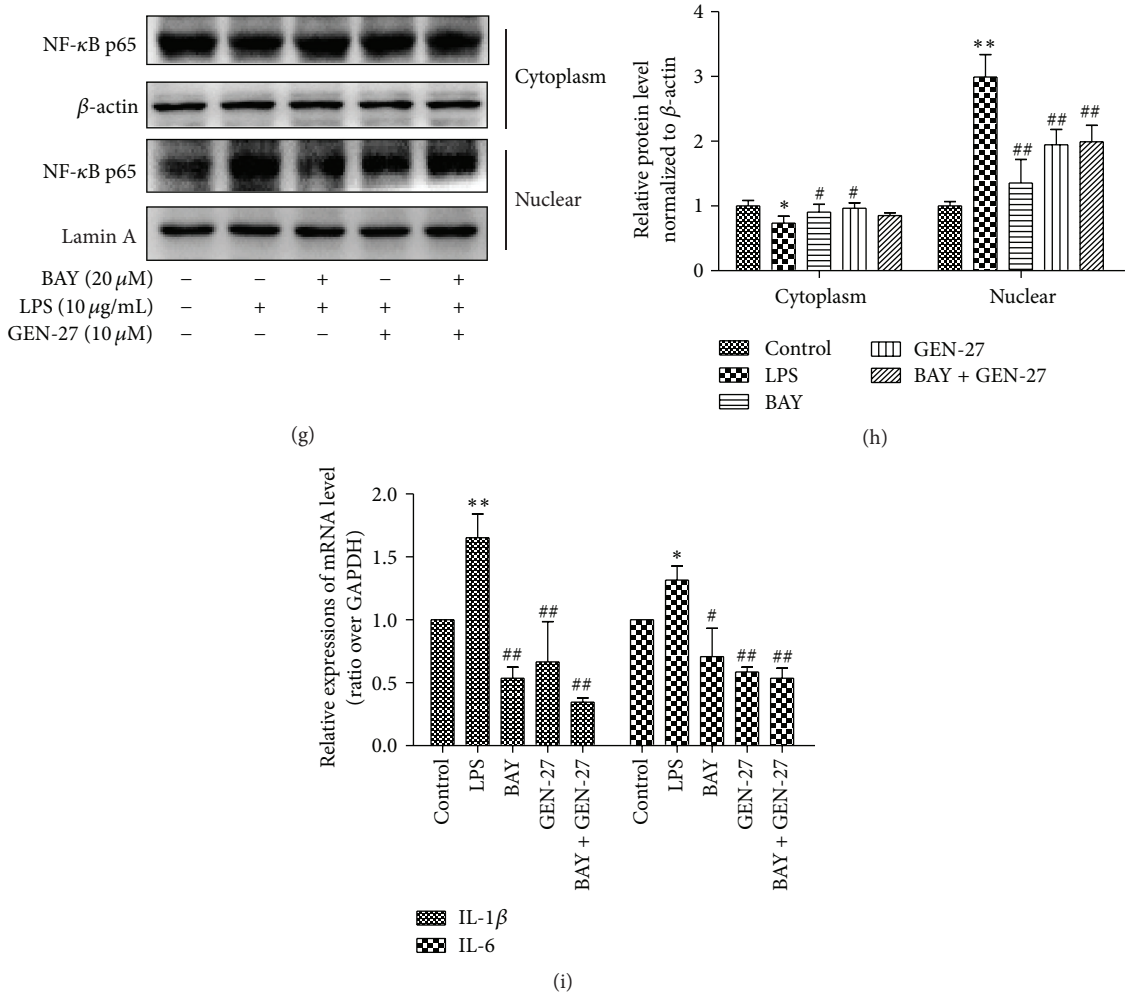
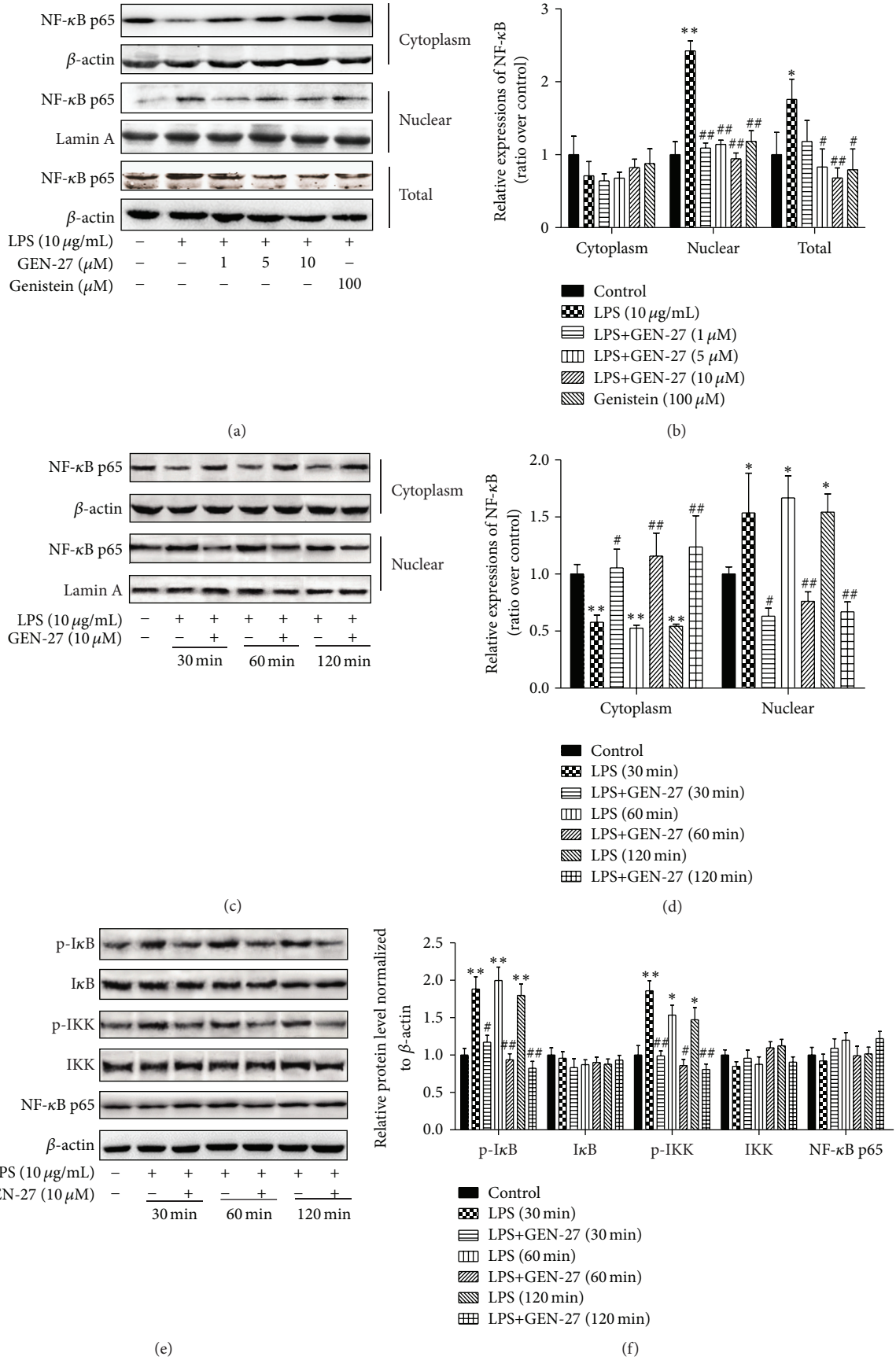


Figure 5: GEN-27 inhibited LPS-induced NF-κB p65 activation in THP-1 cells. THP-1 cells were either left untreated or treated with 10 μg/mL LPS, or 10 μg/mL LPS, and indicated concentrations of GEN-27 together, or combination of 10 μg/mL LPS, 10 μM GEN-27, and 20 μM Bay 11-7082. (a, b, and g) The expressions of NF-κB p65 in the cytoplasm and nucleus were determined by Western blot analysis. (c) The expressions of total NF-κB p65, p-IκBα, IκBα, p-IKKα/β, and IKKα/β were determined by Western blot analysis, respectively. (d, e, f, and h) The relative expressions of NF-κB p65, p-IκBα, IκBα, p-IKKα/β, and IKKα/β were normalized to β-actin. (i) The mRNA expressions of IL-6 and IL-1β in THP-1 cells from each group were determined by real-time PCR. Data (means ± SDs) were representative of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus LPS alone or corresponding LPS group at indicated time.

shown in Figures 6(a)–6(d), the amount of NF-κB p65 in the nucleus was markedly increased after exposure to LPS, and this response was significantly inhibited by GEN-27, which was validated by the reduction of the phosphorylation of IκBα and IKKα/β (Figures 6(e) and 6(f)). The inhibition on the nuclear translocation of p65 by GEN-27 was further verified by immunofluorescence confocal microscopy (Figure 6(g)).

3.6. GEN-27 Inhibits IL-1β-Induced Cell Proliferation in HCT116 Cells. IL-1β is a pleiotropic proinflammatory cytokine and can be secreted by immune, stromal, and tumor cells. The interaction between colon cancer cells and inflammatory cells promotes secretion of the release of IL-1β from immune cells [20]. Elevated IL-1β levels have been shown to be associated with increased colon tumor growth and invasion [21]. As shown in Figure 7(a), IL-1β treatment caused the proliferation of HCT116 cells, which was blocked by GEN-27 or BAY 11-7082. GEN-27-mediated attenuation of cell proliferation was not changed by the cotreatment of BAY 11-7082, which was verified by the reduction in the expression levels of PCNA, bcl-2, and cyclin D1 (Figures 7(b) and 7(c)). Moreover, GEN-27 significantly repressed LPS-induced p65 nuclear localization and phosphorylation levels of IκBα and IKKα/β (Figures 7(d) and 7(e)), which demonstrated that the antiproliferation effect of GEN-27 is dependent on the downregulation of NF-κB pathway. To further corroborate this effect, HCT116 cells were transfected with p65 overexpression plasmid. The reduction of p65 nuclear localization induced by GEN-27 was remarkably reversed by p65 overexpression (Figure 7(f)). Moreover, overexpressed p65 did not influence the proliferation of HCT116



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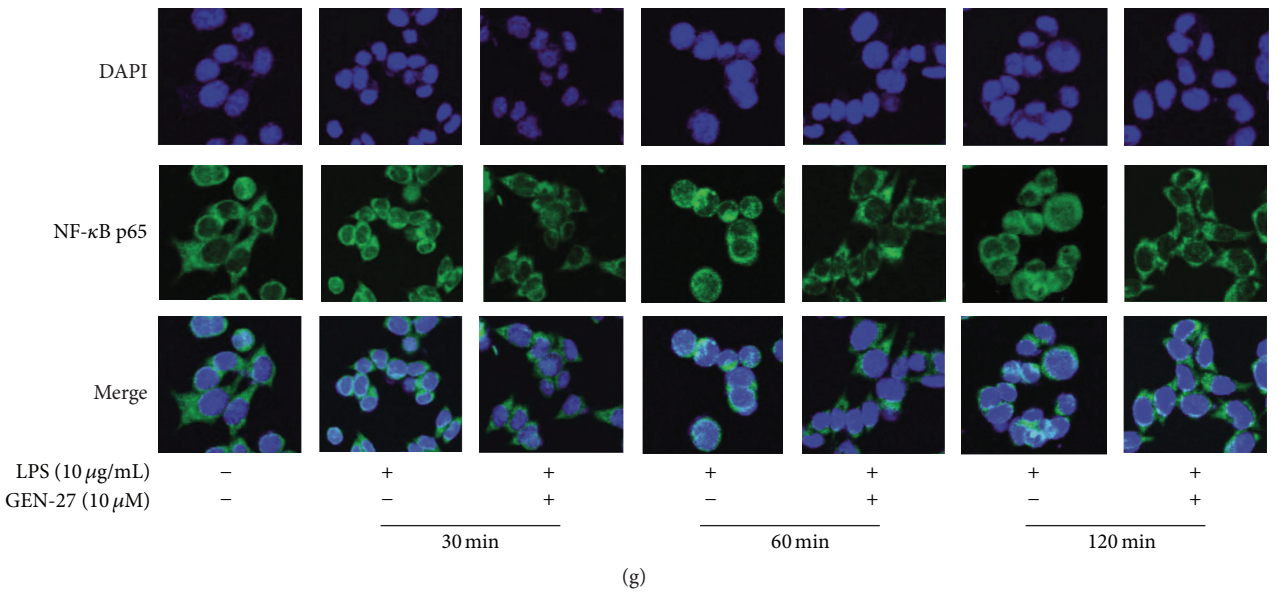

Figure 6: GEN-27 suppressed LPS-induced NF-κB p65 activation in HCT116 cells. HCT116 cells were treated with LPS (10 μg/mL) together with indicated concentrations of GEN-27 and genistein (100 μM) for the indicated times. (a–f) The nuclear translocation and protein levels of NF-κB/p65, p-IκBα, IκBα, p-IKKα/β, and IKKα/β were determined by Western blot. Data shown are representative of three experiments. The relative expressions of total proteins p-IκBα, IκBα, p-IKKα/β, and IKKα/β were normalized to β-actin. (i) The localization of NF-κB p65 was visualized using fluorescence microscopy after immunofluorescence staining with NF-κB p65 antibody (green). Cells were also stained with DAPI for visualization of the nuclei (blue). Data (means ± SDs) were representative of at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus LPS alone or corresponding LPS group at indicated time.

cells. Compared with p65 overexpression cells, the combined treatment with p65 plasmid and GEN-27 showed no effect on HCT116 cell proliferation (Figure 7(i)). However, when HCT116 were simultaneously treated by IL-1β, overexpressed p65 increased the cells proliferation and this effect was partly reversed by GEN-27 (Figure 7(i)), which were verified by the expression levels of PCNA, bcl-2, and cyclin D1 (Figures 7(j) and 7(l)). Taken together, these results indicated that GEN-27 inhibited IL-1β-induced proliferation of human colon cancer cells through blocking NF-κB pathway.

4. Discussion

It is generally accepted that at least 15% of cancer is caused by chronic inflammation [22]. Chronic inflammation has been proposed to be a major contributor to CRC, which is the third leading cause of cancer-related death in developed countries. In fact, the CRC incidence is relatively low in Asian countries compared with Western countries. Lower incidence and mortality rates of CRC have been thought to be due to high consumption of soybeans and their products in Asian countries [23–25]. Genistein is one of the major bioactive constituents of soybeans and exerts antioxidant, anti-inflammatory, anticancer, antiviral, and neuroprotective activities. In this study, we initially investigated the inhibitory effect of GEN-27, a genistein derivative, on human monocyte THP-1 cells and colon cancer HCT116 cells and found that GEN-27 inhibited cell proliferation and induced G0/G1 cell cycle arrest and cell apoptosis with higher potency than its parent compound genistein. The main aim of this study was to determine the effects of GEN-27 on the proliferation of colon cancer cells in inflammatory microenvironment. We utilized LPS-stimulated THP-1 cells to mimic the inflammatory cells in microenvironment of solid tumors. Since GEN-27-mediated reduction of cell growth would influence the observation of its anti-inflammatory effect, we chose relatively low concentrations that were nontoxic to cells in subsequent experiments.

Previous studies have reported that genistein could suppress cell growth and proliferation in multiple cancer cell lines by an accumulation of cells at the G2/M phase. This effect was related to the inhibition of insulin-like growth factor-1 (IGF-1) receptor signaling and the PI3k/AKT pathway, also including the upregulation of p53 and CDK inhibitor p21 waf1/cip1 [12, 26–30]. However, several reports found that genistein induces G0/G1 arrest in MCF-7 cells, HB4a cells, BALB/c 3T3 cells, and B16-F1 cells mediated through induction of p21 and suppression of cyclin D1 and cyclin E, key protein regulators of G1/S transition of cell cycle [31–33]. In our study, 100 μM genistein delayed HCT116 cells at G2/M phase, but its derivative GEN-27 (10 μM) induced G0/G1 arrest through inhibition of cyclin D1 expression and NF-κB nuclear translation. Activated NF-κB can upregulate the transcription of cell cycle regulator cyclin D1 via binding to multiple sites within the promoter region, which promotes the G1/S-phase transition. During cell cycle progression, cyclin D1 activates cyclin-dependent kinases CDK4 and CDK6 and then forms cyclin D1-CDK4 and D1-CDK6 complexes, which can phosphorylate the retinoblastoma protein, such as pRB and pRB-related p107 and p130 proteins. Phosphorylation of

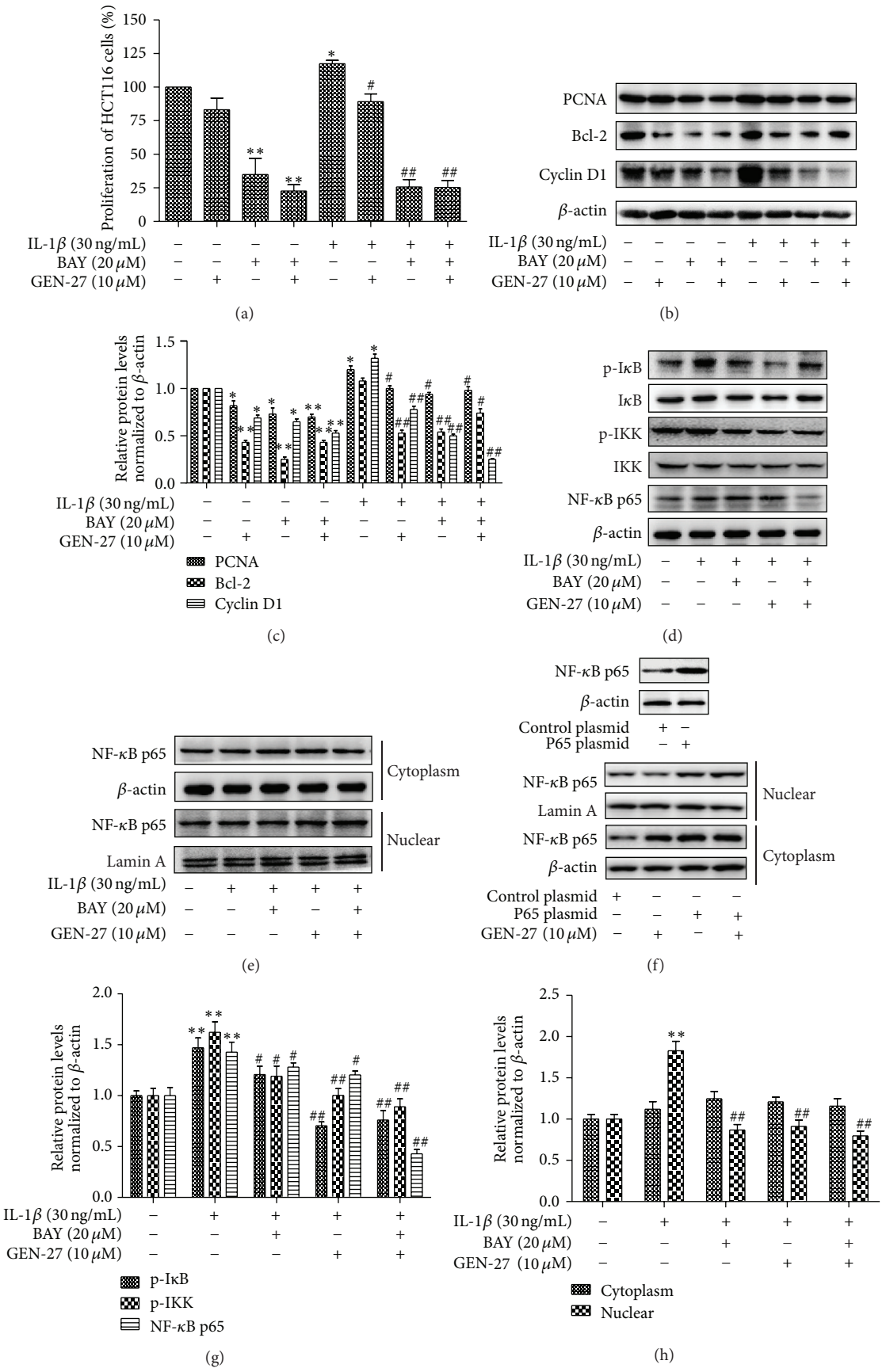

Figure 7: Continued.

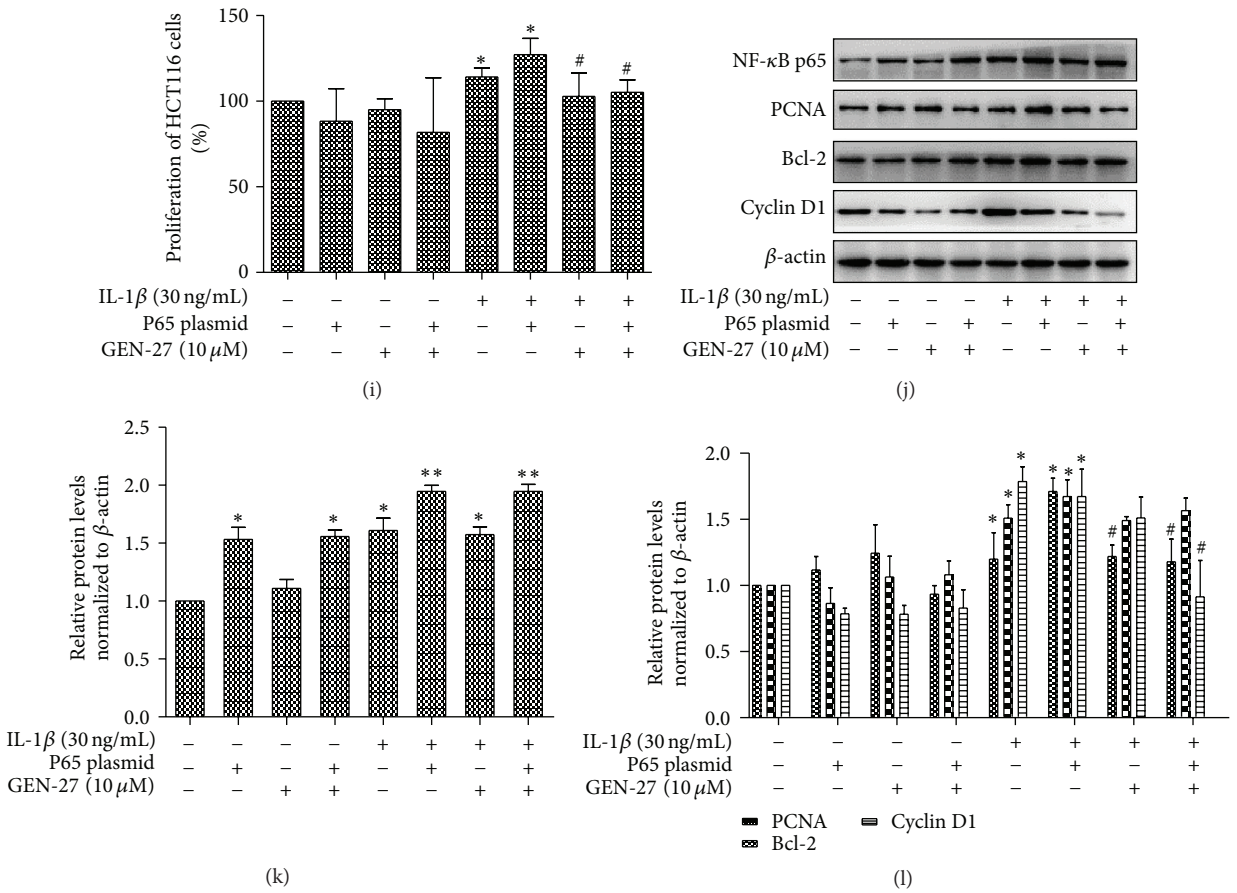

FIGURE 7: GEN-27 inhibited IL-1β-induced proliferation of human colon cancer cells. HCT116 cells were either left untreated or treated with 30 ng/mL IL-1β, or 30 ng/mL IL-1β and 10 μM GEN-27 together, or combination of 30 ng/mL IL-1β, 10 μM GEN-27, and 20 μM Bay 11-7082 for 24 h. (a and i) Cell viability was assessed using an MTT assay and the results are expressed as the percentage of surviving cells over control cells. (b, d, and e) NF-κB/p65 nuclear translocation and protein levels of total NF-κB/p65, p-IκBα, IκBα, p-IKKα/β, IKKα/β, PCNA, bcl-2, and cyclin D1 were determined by Western blot. (c, g, and h) The quantitation of those proteins expression levels relative to β-actin expression according to (b, d, and e). (f) NF-κB/p65 nuclear translocation and total protein levels of p65 with or without p65 overexpression. (j, k, and l) Protein levels of total NF-κB/p65, PCNA, bcl-2, and cyclin D1 were determined by Western blot. The relative expressions of those proteins were normalized to β-actin. All graphic data shown are the means ± SDs. Results are representative of those obtained from three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus IL-1β alone.

pRB, p107, and p130 derepresses the transcriptional activity of E2F transcription factors, thereby allowing the G1 to S-phase transition [34]. In addition, cyclin D1-CDK4/6 complexes sequester the cell cycle inhibitors p27Kip1 and p21Cip1 away from cyclin E-CDK2, thereby contributing to activation of cyclin E-CDK2 kinase. Therefore, the reduction of cyclin D1 could explain, by inhibiting NF-κB nuclear translocation and IκB phosphorylation, at least in part, the increase of cells in the G1/G0 phase by GEN-27 treatment in our experiments.

Many clinical studies depicted that most solid tumors infiltrated with immune cells, which promoted tumor progression. It had been shown that coculture of cancer cells with fibroblasts could generate an activated microenvironment, rich in inflammatory mediators and growth factors [35], or with macrophages could promote the release of IL-1β, which induced the activation of WNT signaling and supported the growth of tumor cells [20, 36]. In present study, similar results were observed where THP-1-derived conditional medium stimulated by LPS could promote the growth of HCT116 cells, and this process was suppressed by GEN-27 via inhibiting the secretion of proinflammatory cytokines IL-1β and IL-6 (Figure 3).

Inflammatory cytokines, growth factors, and chemokines, which are produced by inflammatory cells including macrophages, lymphocytes, or dendritic cells or, more often, by the tumor cells themselves, can regulate preneoplastic growth and the initiation of tumor, and they also play vital roles in two stages of tumor development: promotion and progression. For example, TNF plays a dual role in tumorigenesis. Low concentration of TNF can promote the development of inflammation-related cancers. On the other hand, TNF can disrupt tumor vasculature and induce cell apoptosis [3]. IL-6, as a multifunctional NF-κB-regulated cytokine, is a critical tumor promoter during early CRC tumorigenesis via enhancing proliferation of tumor-initiating cells. IL-6 produced by lamina propria myeloid cells protects normal and

premalignant intestinal epithelial cells (IECs) from apoptosis mediated by the transcription factor STAT3 [37]. Our previous study reported that oroxylin A, a natural flavonoid, inhibited colitis-associated carcinogenesis through modulating IL-6/STAT3 pathway in AOM/DSS mouse model and in HCT116 cells [38]. In AOM/DSS mice model, IL-1β levels in the colonic tissues are mainly produced by infiltrating neutrophils, prompt colon carcinogenesis by eliciting IL-17 response in intestinal myeloid cells [39]. These results indicated that inflammatory cytokines played an important role in inflammation-associated carcinogenesis. In this study, we found that GEN-27 treatment significantly decreased the excessive production of IL-6 and IL-1β in LPS-stimulated THP-1 cells in a dose-dependent manner without causing any cytotoxicity. The proliferation of HCT116 cells caused by conditional medium was significantly blocked by IL-1RA treatment, and the reduction caused by IL-1RA was further reduced by GEN-27, which suggested a vital role of IL-1β in GEN-27-mediated inhibitory action on cancer cell proliferation. These results also indicated that GEN-27 could potentially have preventive effect on colitis-associated CRC tumorigenesis.

Lipopolysaccharide (LPS), an endotoxin and the outer cell wall component of gram-negative bacteria, can trigger host inflammatory responses which are critical for host defense against bacterial infections [40]. LPS is specifically recognized by TLR4, a transmembrane receptor expressed in normal and malignant cells [41, 42]. The binding of LPS to TLR4 induces MyD88-dependent intracellular signaling. MyD88 recruits IL-1 receptor-associated kinases (IRAKs) and tumor-necrosis factor receptor-associated factor 6 (TRAF6) upon ligand stimulation, and then TRAF6 activates the transforming growth factor-β-activated kinase 1 (TAK1) complex. TAK1 then activates the IKK complex that mediates NF-κB activation. Simultaneously, TAK1 activates the MAP kinase family, such as p38 MAPK, c-Jun NH_2-termina kinase (JNK), and extracellular signal-regulated kinase (ERK) [43]. NF-κB is an important transcription factor that controls cell survival by regulating programmed cell death, proliferation, and growth arrest, which is mediated by the downstream target genes. Activation of NF-κB transcription factor is one of the main links between inflammation and tumorigenesis. Sustained activation of NF-κB is found to be related to poor clinical prognosis of cancer. NF-κB-driven cytokine production by myeloid cells is instrumental in CAC tumor growth, whereas NF-κB activation in intestinal epithelial cells (IECs) promotes the survival of newly emerging premalignant cells [37]. Sustained activation of NF-κB promotes growth of CRC by upregulating the antiapoptotic pathway and potentiating tumor cell survival [44]. It also enhances angiogenesis and invasiveness by mediating the production of cyclooxygenase 2 (COX-2), vascular cell adhesion molecule (VCAM), and matrix metalloproteinases (MMPs). Recent studies have shown that LPS-induced metastatic growth response depends on both TNFα production by host hematopoietic cells and NF-κB activation in tumor cells. NF-κB inhibition in colon cancer cells converts the LPS-induced growth response to tumor regression [45]. Genistein is reported to have significant inhibitory effect on NF-κB pathway in many cancer cell lines [46–48]. Our data suggested that, similar with genistein, GEN-27 impaired the activation of NF-κB pathway induced by LPS, which is demonstrated by the reduction in the translocation of NF-κB p65 into nucleus and phosphorylation of IκBα and IKKα/β both in THP-1 and in HCT116 cells (Figures 5 and 6).

IL-1β is a major proinflammatory cytokine with numerous roles in various physiological and pathological states. It also functions as a pleiotropic cytokine involved in tumor generation, growth, and metastasis in multiple types of cancers [49]. Recent studies have shown that IL-1β can promote sphere-forming capacity and EMT transformation concomitant with upregulated expression of stemness markers Bmi1 and nestin in colon cancer cells, suggesting that IL-1β may promote colon tumor growth and invasion through activation of CSC self-renewal and EMT [21, 50]. IL-1β was released from tumor-associated macrophages to activate WNT signaling and to promote the growth of tumor cells [20]. Our data showed that IL-1β significantly promoted cell growth in HCT116 cells, while this response could be inhibited by GEN-27 treatment, which is ascribed to the inactivation of NF-κB pathway by GEN-27. It is suggested that GEN-27 could prevent IL-1β-induced cancer cell growth and could potentially be used as a chemopreventive agent against inflammation-related colon cancer. In fact, several anti-IL-1β agents have been tested in clinical trials in patients with diverse inflammatory diseases [51]. A better understanding of the intricate roles of IL-1β signaling in the malignant process will facilitate the application of novel IL-1β modulator in cancer patients.

In conclusion, we found that proinflammatory cytokines IL-6 and IL-1β were produced by LPS-stimulated THP-1 cells, which in turn promoted the proliferation of HCT116 cells. GEN-27 alone at low concentrations had no effect on the apoptosis or proliferation of HCT116 cells, but it significantly inhibited the growth of cancer cells in response to THP-1-conditioned medium through blocking NF-κB signaling. In addition, GEN-27 remarkably suppressed IL-1β-mediated HCT116 cells proliferation, which confirmed the major role of IL-1β in promoting cancer cell growth. Our findings established that GEN-27 might serve as a potential chemopreventive agent against inflammation-associated colon cancer.

Conflict of Interests

The authors declare no competing financial interests.

Authors' Contribution

Yajing Wang and Ping Lu contributed equally to this work.

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Research Article

IL-6 Inhibition Reduces STAT3 Activation and Enhances the Antitumor Effect of Carboplatin

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Recent studies suggest that tumor-associated macrophage-produced IL-6 is an important mediator within the tumor microenvironment that promotes tumor growth. The activation of IL-6/STAT3 axis has been associated with chemoresistance and poor prognosis of a variety of cancers including colorectal carcinoma and thus serves as a potential immunotherapeutic target for cancer treatment. However, it is not fully understood whether anticytokine therapy could reverse chemosensitivity and enhance the suppressive effect of chemotherapy on tumor growth. In this study, we aimed to investigate the effect of IL-6 inhibition therapy on the antitumor effect of carboplatin. Enhanced expression of IL-6 and activation of STAT3 were observed in human colorectal carcinoma samples compared to normal colorectal tissue, with higher levels of IL-6/STAT3 in low grade carcinomas. Treatment of carboplatin (CBP) dose-dependently increased IL-6 production and STAT3 activation in human colorectal LoVo cells. Blockade of IL-6 with neutralizing antibody enhanced chemosensitivity of LoVo cells to carboplatin as evidenced by increased cell apoptosis. IL-6 blockade abolished carboplatin-induced STAT3 activation. IL-6 blockade and carboplatin synergistically reduced cyclin D1 expression and enhanced caspase-3 activity in LoVo cells. Our results suggest that inhibition of IL-6 may enhance chemosensitivity of colon cancers with overactive STAT3 to platinum agents.

1. Introduction

IL-6, produced by tumor-associated macrophage, is an important mediator that promotes tumor growth [1, 2]. Although there was evidence supporting a role in T-cell activation and trafficking [3], IL-6 within the tumor microenvironment is generally considered as a malevolent player that promotes tumor progression. By activating downstream Janus kinase (JAK) signal transducer and activator of transcription-3 (STAT3) signaling, IL-6 promotes cancer cell proliferation, survival, and metastatic dissemination. In addition, IL-6 may also act on other cell types within the tumor microenvironment to enhance tumor growth by supporting angiogenesis [4] and immune escape [5, 6].

Platinum drugs such as cisplatin, carboplatin, and oxaliplatin are a class of chemotherapy agents that trigger apoptosis of tumor cells by binding to and causing DNA cross-linking. They are widely used in cancer chemotherapy due to their broad spectrum of activities against several solid tumors [7]. However, the drug resistance is a major problem in platinum-based therapy, with 75% relapse for cisplatin [8]. Enhanced activation of STAT3 has been suggested as a major contributor to platinum resistance [9, 10]. In this investigation, we examine the effect of carboplatin (CBP) and

IL-6 blockade combination therapy on the growth of LoVo, a human colon carcinoma cell line.

2. Materials and Methods

2.1. Human Colorectal Carcinoma Tissue Collection. Colorectal tumor and nontumor colon tissue samples were collected at the time of surgical resection at Dongguan 6th Hospital. All procedures involving human participants were approved by the Research Ethics Board and the Institutional Review Board (IRB) at the Guangdong Medical College and Dongguan 6th Hospital. Written informed consent was obtained before tissue collection.

2.2. Cell Culture and Reagents. The human colorectal cancer LoVo cells were purchased from ATCC (Manassas, VA, USA). LoVo cells were cultured in F12K medium supplemented with 10% fetal bovine serum, 100 g/mL streptomycin, and 100 U/mL penicillin, at 37°C, 5% CO_2, and high humidity. The sources of antibodies (Abs) were as follows: IL-6 was purchased from R&D (Minneapolis, MN, USA), p-STAT3 was purchased from Abcam (Cambridge, MA, USA), cleaved caspase-3 was purchased from Cell Signaling (Beverly, MA, USA), and STAT3, cyclin D1, GAPDH, and the HRP-labeled secondary antibodies were purchased from EnoGene (Nanjing, China). Carboplatin was purchased from Melone-Pharma (Dalian, Liaoning, China). Annexin-V-FITC apoptosis detection kit, DAB Substrate Kit, and Cell Counting Kit-8 (CCK-8) were purchased from Beyotime (Beyotime, Shanghai, China). IL-6 ELISA kit was from NeoBioscience (Shenzhen, Guangdong, China).

2.3. Immunohistochemistry Detection. All human colorectal tumor and nontumor specimens were fixed in 10% neutral-buffered formalin, dehydrated in ascending series of ethanol, and routinely embedded in paraplast. Sections were cut at 10 μm and overnight stained with indicated primary antibodies after deparaffinization, rehydration, antigen recovery, and blocking. After washing, sections were incubated with HRP-labeled corresponding secondary antibodies and the signal was developed with a DAB Substrate Kit (Beyotime, Shanghai, China).

2.4. ELISA Detection of IL-6. LoVo cells were treated with indicated concentration of CBP or vehicle for 48 h. Culture supernatant was collected for the detection of IL-6 using an ELISA kit (NeoBioscience) according to the manufacturer's instruction.

2.5. Apoptosis Detection. Cell apoptosis was measured by an Annexin-V-FITC apoptosis detection kit (Beyotime, Shanghai, China) following the manufacturer's instruction. Briefly, cells were incubated with 5 μMol/L Annexin-V and 1 μg/mL propidium iodide (PI) at room temperature for 15 min. Cells were then analyzed on a BD FACSCalibur cytometer within 1 h.

2.6. Western Blot. Cells were lyzed after 24 hours of indicated treatment and subjected to western blot detection of p-STAT3, STAT3, cyclin D1, cleaved caspase-3, and GAPDH as described [11]. Briefly, the blots were probed with an indicated primary Ab followed by an HRP-conjugated secondary antibody. The reactive bands were visualized using an ECL western blot kit.

3. Results

3.1. IL-6 Expression and STAT3 Activation in Human Colorectal Carcinoma. Increased expression of IL-6 has been detected and associated with an unfavorable prognosis in patients with various types of cancers including breast cancer, colorectal carcinoma, and ovarian cancer [12–17]. To confirm whether IL-6-STAT3 axis is activated in colon cancer, human colorectal carcinoma and matched nontumor colon tissue samples were used for the immunohistochemistry detection of IL-6, phosphor-STAT3 (p-STAT3), and STAT3. As shown in Figure 1, IL-6 expression increased in colorectal carcinoma especially in low grade carcinoma. Consistent with this, both the activation of STAT3 and expression of total STAT3 were upregulated in colorectal carcinoma and higher levels were seen in low grade carcinoma (Figure 1(a)). These results were further confirmed by western blot detection. Levels of IL-6, STAT3, and p-STAT3 were all increased in the human colorectal carcinoma samples, with a higher increase in low grade carcinoma (Figure 1(b)).

3.2. Effect of Carboplatin (CBP) Treatment on IL-6 Production by LoVo Cells. Enhanced activation of STAT3 has been suggested to associate with the chemoresistance of platinum agents [9, 10]. To examine whether CBP treatment could affect IL-6 production in tumor, LoVo cells were treated with different concentrations of CBP for 48 h. Treatment with 1 μg/mL CBP did not affect IL-6 level in the culture supernatant, while both 5 and 20 μg/mL CBP treatments increased IL-6 production with a dose-dependent effect (Figure 2). Treatment of 20 μg/mL CBP resulted in an over 2-fold increase in IL-6 secretion. Since IL-6 has been suggested to promote tumor survival [1, 2], CBP-induced IL-6 production might contribute to drug resistance to platinum.

3.3. Synergistic Effect of CBP and IL-6 Blockade on Colorectal Cancer Cell Apoptosis. Increased production of IL-6 and enhanced activation of STAT3 have been suggested to associate with platinum resistance [9, 10, 18]. To test the effect of IL-6 blockade on CBP chemosensitivity, cell viability and apoptosis of LoVo cells were examined 72 hours after treatment of IL-6 neutralizing antibody (Ab) and/or CBP. As shown in Figure 3(a), a large amount of CBP-treated or IL-6-Ab-treated cells changed their shape from a flat adherent to a rounded morphology, indicating an early stage of apoptosis. Combined treatment of IL-6 blockade and CBP significantly enhanced this change with most of the cells showing apoptotic morphological changes. Consistent with that, cell viability assay by CCK-8 confirmed that CBP treatment induced cell death/CCK-8 release and IL-6 blockade further promoted cell death in CBP-treated LoVo cells (Figure 3(b)).

To further confirm the apoptosis induced by CBP and IL-6 blockade, LoVo cells after indicated treatments were

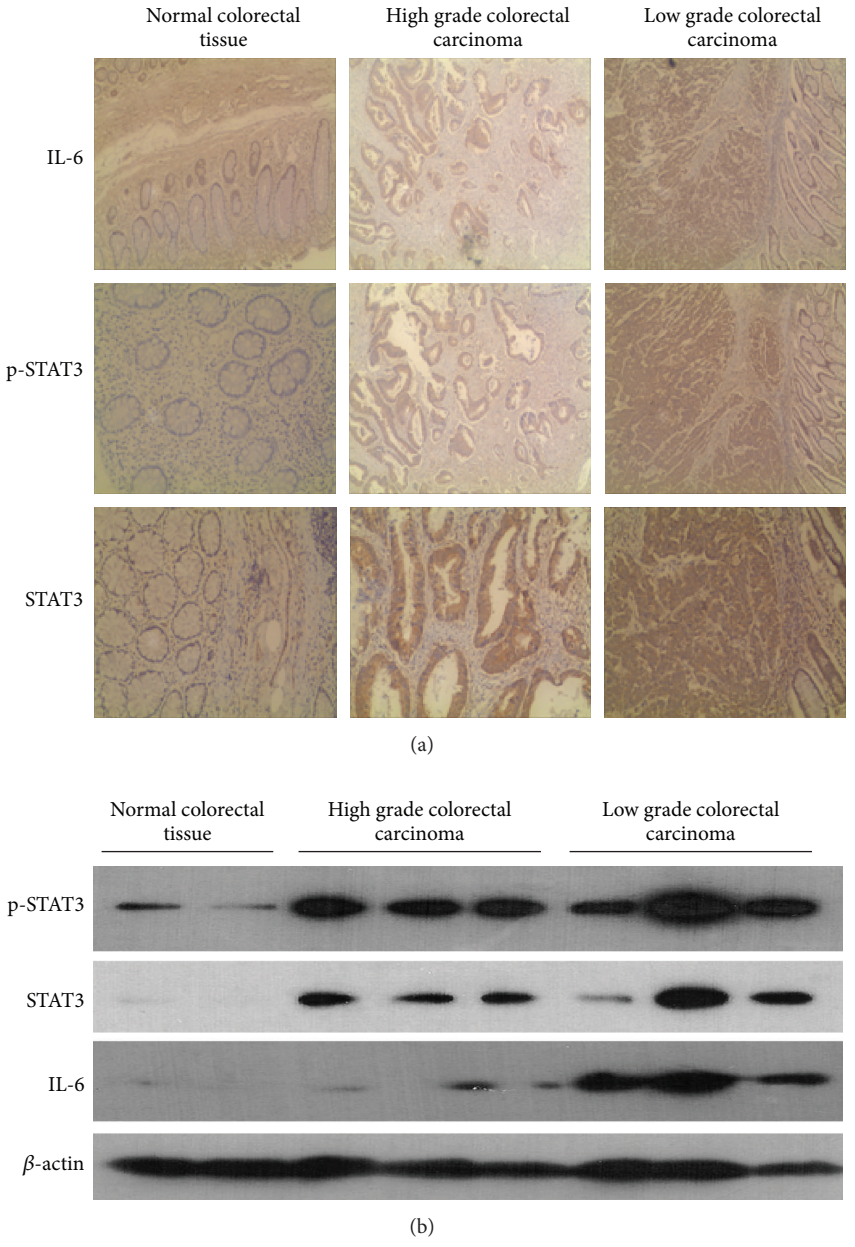

Figure 1: IL-6 expression and STAT3 activation in human colorectal carcinoma: (a) human colorectal carcinoma and nontumor samples were collected at surgery and paraffin-embedded sections were used for the immunohistochemistry detection of IL-6, p-STAT3, and STAT3. Representative images (200x magnification) were shown. (b) Western blot showing the expression of p-STAT3, STAT3, IL-6, and β-actin (internal control) in normal colorectal tissue, high grade colorectal carcinoma, and low grade colorectal carcinoma.

stained with Annexin-V and PI and analyzed on a flow cytometer. Results showed that both IL-6 Ab and CBP treatment increased apoptotic and necrotic cell number. Although frequencies of dead cells (cells in quadrants Q1, Q2, and Q4) were at a similar level in IL-6 Ab- and CBP-treated groups, the CBP-treated cells showed higher number of necrotic cells (quadrant Q1: 1.2% versus 5.4% versus 11.5% for cells treated with PBS versus IL-6 Ab versus CBP, resp.) while IL-6-treated cells displayed higher number of apoptotic cells (quadrant Q4: 2.4% versus 7.0% versus 3.6% for cells treated with PBS versus IL-6 Ab versus CBP, resp.). Combined treatment of IL-6 Ab + CBP dramatically enhanced both apoptosis (1.2% versus 15.5% for PBS versus IL-6 Ab + CBP, Figure 4) and necrosis (2.4% versus 13.7% for PBS versus IL-6 Ab + CBP, Figure 4).

3.4. Effect of IL-6 Blockade and CBP on Pro- and Antisurvival Molecules. Western blot was then used to confirm

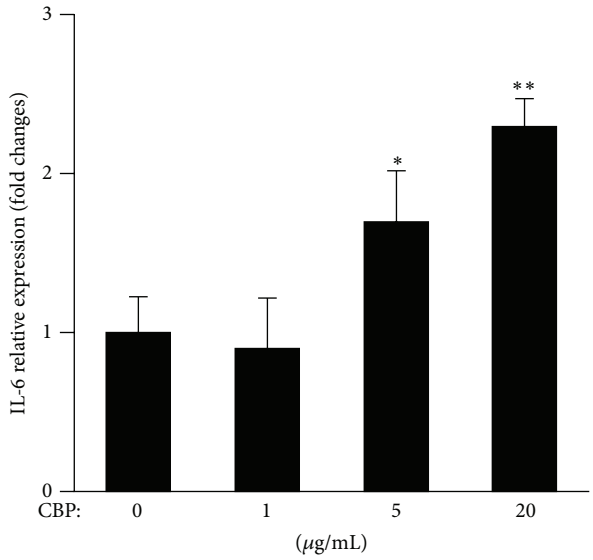

Figure 2: CBP treatment increased IL-6 production by LoVo cells: LoVo cells were treated with CBP at indicated doses for 48 h. Culture supernatant was collected for ELISA detection of IL-6. $^{*}P < 0.05$; $^{**}P < 0.01$.

the effect of CBP and IL-6 blockade on STAT3 activation. Consistent with the increase of IL-6 after CBP treatment, CBP enhanced the activation of STAT3 as evidenced by increased p-STAT3 level, while IL-6 blockade suppressed STAT3 activation (Figure 5). Cyclin D1, a well-described downstream target for STAT3 [19], was also increased by CBP and reduced by IL-6 neutralization (Figure 5). Cleaved caspase-3 is an apoptosis marker and is indispensable for cell apoptosis [20]. Both CBP and IL-6 blockade increased the activation of caspase-3, while combined treatment of IL-6 blockade and CBP further increased the activation of caspase-3 (Figure 5).

4. Discussion

Combination therapy is the future direction for cancer treatment. More and more clinical evidence showed that monotherapy such as surgery, chemotherapy, or radiation therapy alone does not provide a satisfactory result for cancers. Chemotherapy is one of the most commonly used traditional therapies for cancer. However, studies indicate that most chemotherapy agents are detrimental to immunity. Therefore, immunotherapy or biologic therapy is increasingly used in combination with chemotherapy, a strategy referred to as "biochemotherapy" or "chemoimmunotherapy" [21–25]. Of all the currently employed combination therapeutic strategies, platin-based regimen combined with biologic agents has attained the highest attention [26]. In the current study, we investigate the effect of anti-IL-6 therapy on CBP therapy.

IL-6 belongs to a cytokine family signaling through a common receptor gp130. The binding of IL-6 to its receptor results in the activation of the associated Janus kinases (JAKs), followed by the recruitment and activation of STAT3 and STAT1 [27]. Although an in vitro study suggests that IL-6 suppresses in vitro growth of some cancer cells [28], IL-6/STAT3 has been shown to promote tumor progression and immune escape in a variety of in vivo models [4–6]. Deficiency in STAT3 has been shown to protect against colitis-associated colorectal cancer in mice [29]. Therefore, IL-6/STAT3 has been suggested as a potential immunotherapeutic target for malignant diseases [6, 30]. By using immunohistochemistry, western blot, and ELISA, we found in this study that IL-6 is upregulated in colorectal carcinoma especially in low grade carcinoma, accompanied with enhanced STAT3 activation. In addition to active STAT3 (p-STAT3), total STAT3 protein level was also increased in both high grade and low grade colorectal carcinomas. This is probably because long term activation of STAT3 by IL-6 enhances the transcription and expression of STAT3. This result is consistent with the findings by other groups that IL-6 increased during tumorigenesis [31, 32].

Carboplatin, also known as cis-diammine(1,1-cyclobutanedicarboxylato)platinum(II), is used as an anticancer chemotherapy drug for a variety of cancer types due to its broad spectrum of activities against several solid tumors [7]. It triggers tumor cell apoptosis by causing DNA cross-linking. However, its use in clinic is largely limited by the high incidence of drug resistance. Studies suggested that the activation of STAT3 is enhanced in platinum therapy and is responsible for platinum resistance [9, 10]. We therefore hypothesize that IL-6 inhibition could improve platinum resistance. We first confirmed that CBP dose-dependently increased IL-6 production by LoVo cells. IL-6 blockade had a synergistic effect on promoting tumor cell apoptosis when combined with CBP.

CBP has been shown to induce cancer cell apoptosis via activation of caspases [33]. We verified this effect by detecting the viability and apoptosis rate. We also examined the effect of IL-6 blockade on tumor cell apoptosis with or without the presence of CBP. Our results suggest that IL-6 blockade alone reduces tumor cell viability and promotes apoptosis. Furthermore, IL-6 blockade and CBP have synergistic effect on promoting apoptosis.

As a transcription factor, STAT3 mediates the expression of various genes including Bcl-XL, survivin, and cyclin D1 [34, 35]. In our study, we confirmed that both IL-6 blockade and CBP activate caspase-3. Combined treatment of IL-6 blockade and CBP had a synergistic effect on caspase-3 activation. CBP therapy enhanced STAT3 activation while IL-6 blockade eliminated STAT3 activation in both CBP-treated LoVo cells and vehicle control. In consistency with that, cyclin D1 slightly increased in CBP-treated cells, while IL-6 blockade dramatically diminished cyclin D1 expression in both CBP-treated LoVo cells and vehicle control. These suggest that IL-6 blockade may block the adverse effect of CBP-induced IL-6 upregulation and it has a synergistic effect on enhancing proapoptotic signaling.

In summary, our results indicate that IL-6 blockade may enhance the antitumor effect of CBP and eliminate the adverse effects caused by CBP-induced IL-6 upregulation.

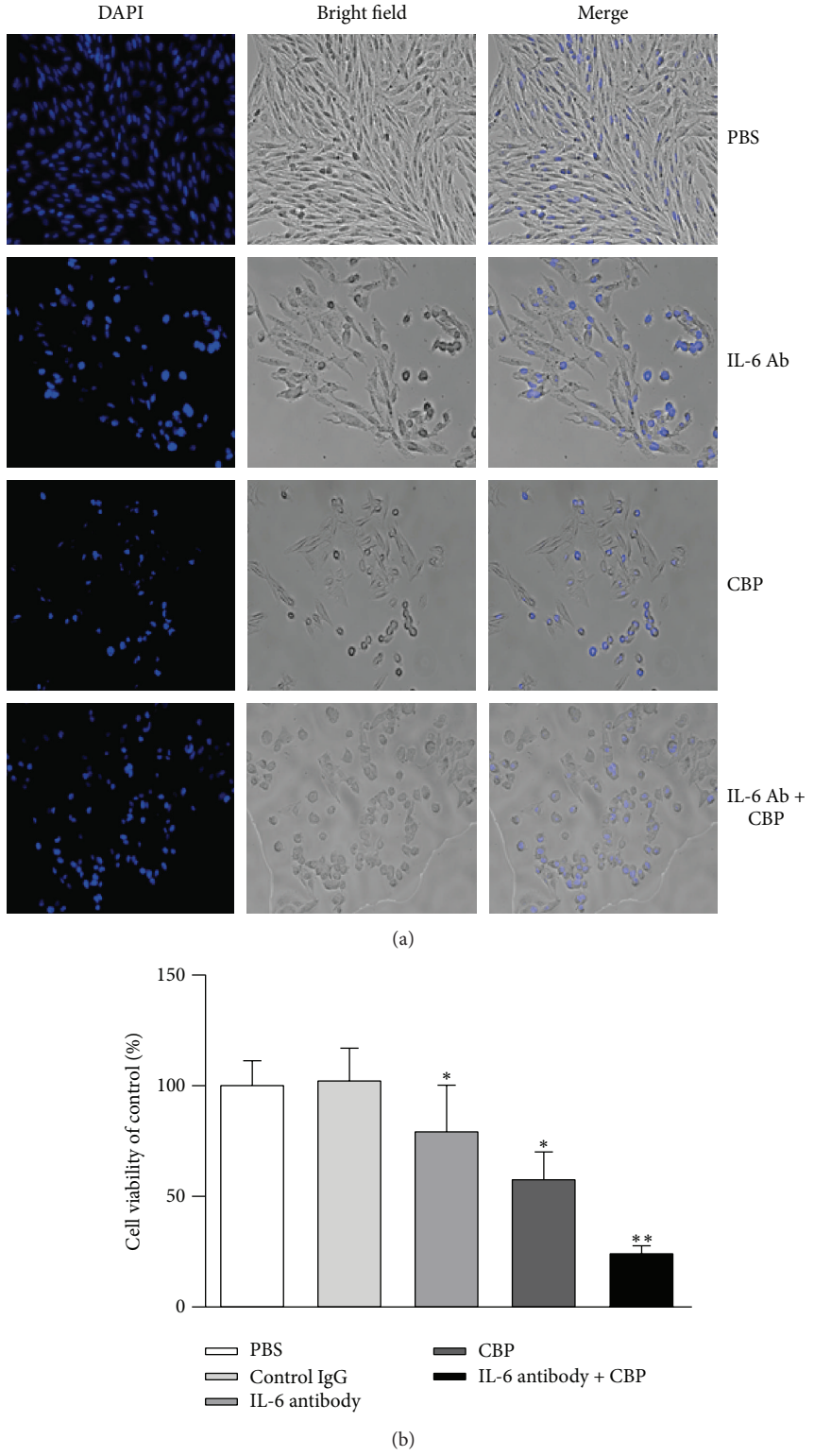

Figure 3: Synergistic effect of CBP and IL-6 blockade on LoVo cell survival: (a) LoVo cells were treated with 20 μg/mL CBP and/or 500 μg/mL IL-6 neutralizing antibody (Ab) or PBS. Cells were stained with DAPI (0.2 μg/mL) and morphological change was examined after 72 h under microscope (100x). (b) LoVo cells were treated with 20 μg/mL CBP and/or 500 μg/mL IL-6 neutralizing antibody (Ab) or PBS. Cells were collected for the detection of viability using CCK-8 kit. $^{*}P < 0.05$; $^{**}P < 0.01$.

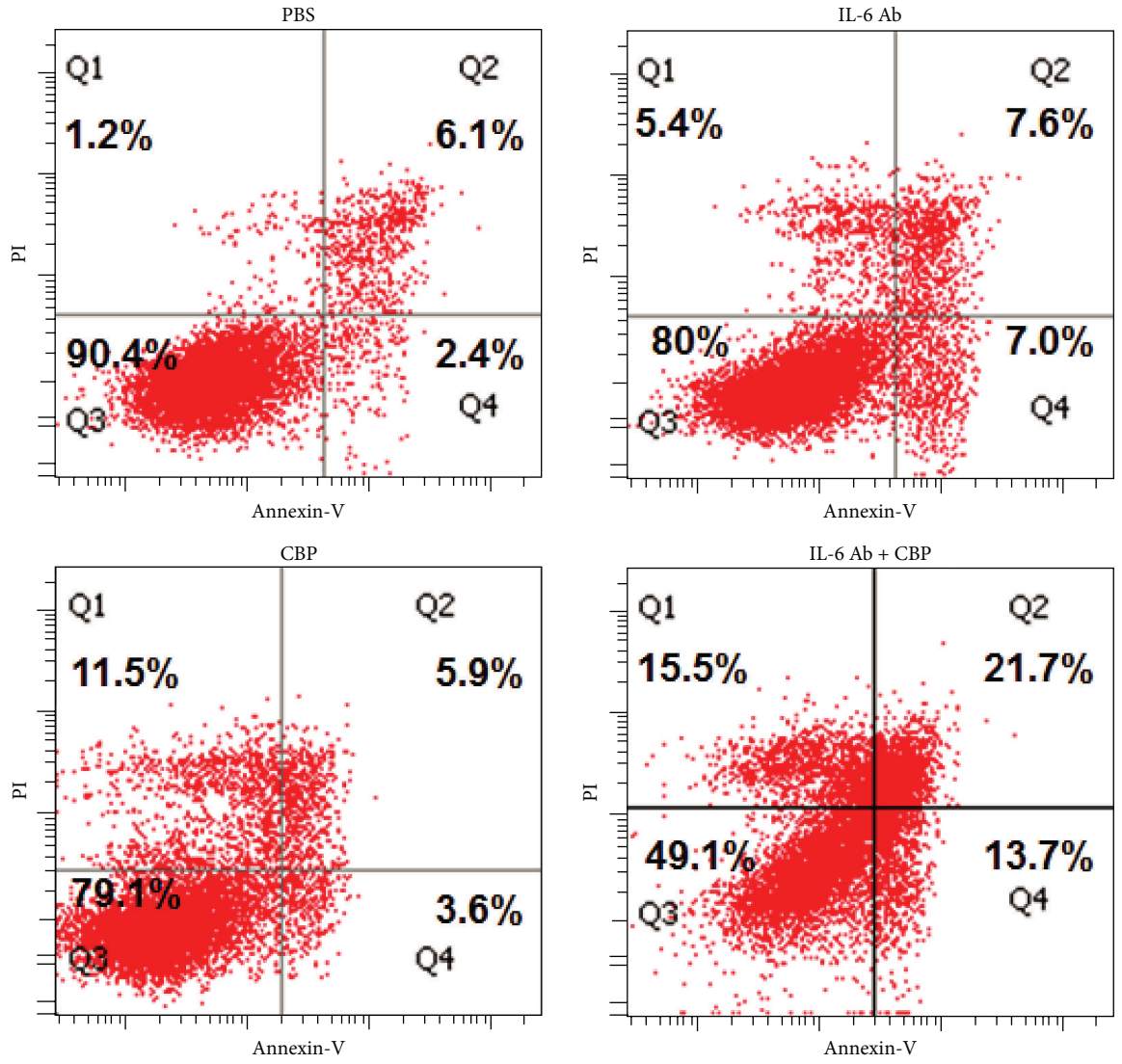

Figure 4: Effect of CBP and IL-6 blockade on LoVo cell apoptosis: LoVo cells were treated with 20 μg/mL CBP, 500 μg/mL IL-6 neutralizing antibody (Ab), or both combined or PBS for 24 h. After staining with Annexin-V-FITC and PI, cells were analyzed on a flow cytometer.

However, further studies are required to confirm this effect in vivo.

Conflict of Interests

The authors report no conflict of interests in this work.

Authors' Contribution

Zhi-Yong Wang, Jun-Ai Zhang, and Yan-Fang Liang performed experiments and acquisition of data. Zhi-Yong Wang, Yuan-Bin Lu, Yu-Chi Gao, You-Chao Dai, Shi-Yan Yu, Yan Jia, and Xiao-Xia Fu interpreted the results and analyzed the data. Jun-Fa Xu and Jixin Zhong contributed to the study design. The paper was written by Xiaoquan Rao, Jun-Fa Xu, and Jixin Zhong. All authors have read and approved the paper. Zhi-Yong Wang, Jun-Ai Zhang, and Xian-Jin Wu contributed equally to this work.

Acknowledgments

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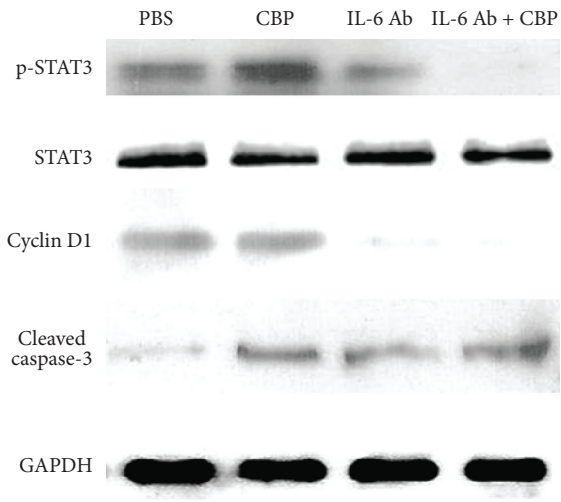

Figure 5: Western blot analysis of STAT3 and downstream molecules: LoVo cells were treated with 20 μg/mL CBP, 500 μg/mL IL-6 neutralizing antibody (Ab), or both combined or PBS control for 24 h. Total proteins were then isolated for the western blot detection of p-STAT3, total STAT3, cyclin D1, cleaved caspase-3, and GAPDH.

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Review Article

Parallel Aspects of the Microenvironment in Cancer and Autoimmune Disease

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Cancer and autoimmune diseases are fundamentally different pathological conditions. In cancer, the immune response is suppressed and unable to eradicate the transformed self-cells, while in autoimmune diseases it is hyperactivated against a self-antigen, leading to tissue injury. Yet, mechanistically, similarities in the triggering of the immune responses can be observed. In this review, we highlight some parallel aspects of the microenvironment in cancer and autoimmune diseases, especially hypoxia, and the role of macrophages, neutrophils, and their interaction. Macrophages, owing to their plastic mode of activation, can generate a pro- or antitumoral microenvironment. Similarly, in autoimmune diseases, macrophages tip the Th1/Th2 balance via various effector cytokines. The contribution of neutrophils, an additional plastic innate immune cell population, to the microenvironment and disease progression is recently gaining more prominence in both cancer and autoimmune diseases, as they can secrete cytokines, chemokines, and reactive oxygen species (ROS), as well as acquire an enhanced ability to produce neutrophil extracellular traps (NETs) that are now considered important initiators of autoimmune diseases. Understanding the contribution of macrophages and neutrophils to the cancerous or autoimmune microenvironment, as well as the role their interaction and cooperation play, may help identify new targets and improve therapeutic strategies.

1. Introduction

The immune/inflammatory response is mostly beneficial to the host and is designed to combat and eradicate invading pathogens and then reestablish homeostasis. This universal response can also be activated in sterile inflammation, without any obvious infection, to repair excessive damage. The immune response is broadly categorized either as proinflammatory (consisting of Th1 and Th17 cells, M1-activated macrophages, and proinflammatory mediators designed to kill pathogens or tumor cells) or as anti-inflammatory (dominated by Th2 cells, M2-activated macrophages, and anti-inflammatory cytokines, designed to repair tissue damage). Of course, this approach is over simplistic, as more types of cell activation, including different types of regulatory T cells, macrophages, and B cells, are constantly being revealed.

In both cancer and autoimmune diseases an aberrant activation of the immune/inflammatory response leads to chronic diseases and accumulation of tissue damage. However, from an immunological standpoint, these two families of diseases are fundamentally different and even represent two opposite ways in which the immune system can go wrong. In cancer, the tumor cells are mostly unrecognized as antigens because a dominant anti-inflammatory response driven by the tumor cells suppresses any antitumoral immune response and promotes tumor progression and dissemination (immunosuppression). In fact, tumors are called wounds that do not heal, because the tumor hijacks the wound healing machinery and uses it to promote itself [1, 2]. In contrast, in autoimmune diseases, self-tolerance is broken and the inflammatory response is activated in excess against the host tissue cells, which express autoantigens that are misrecognized and attacked by the immune system, gradually leading to permanent tissue damage.

Differences between cancer and autoimmunity are evident even at the cellular levels. In solid cancers, the immune infiltrate is composed mostly of macrophages, as well as T regulatory cells (Tregs), some T effector cells (CD8 cytotoxic T cells), and NK cells, whereas other cell types, such as neutrophils, dendritic cells, and fibroblasts, remain mostly

at the tumor rims. In contrast, autoimmune diseases are usually dominated by Th1 and Th17 cells and their cytokine products IL-2, IFNγ, and IL-17 (in Th1 autoimmune diseases such as rheumatoid arthritis, RA, multiple sclerosis, MS, and Hashimoto thyroiditis, HT) or by Th2 cells and their anti-inflammatory cytokines IL-4, TGFβ, and IL-10 (in Th2 autoimmune diseases such as systemic lupus erythematosus, SLE, systemic or local sclerosis, SSc, or scleroderma). Relative to healthy individuals, Tregs are partially impaired in autoimmune patients, partly explaining the broken tolerance which characterizes autoimmunity [3, 4].

Multiple factors play a role in determining the outcome of the aberrant inflammatory process, including the type of inflicted tissue or organ, the degree of tissue injury sustained, the type of cells activated, the amounts of protein and lipid mediators that are locally and systemically secreted by those cells, and the extent to which immune regulatory checkpoints are activated. Collectively, these comprise the microenvironment.

Despite the many differences and the opposite activation of the inflammatory process as a whole, some interesting similarities exist between cancer and autoimmunity, particularly in the way phagocytes are activated and in shared processes like angiogenesis. In this review we attempt to highlight some similarities in microenvironmental elements between cancerous and autoimmune diseases, focusing specifically on the roles macrophages and neutrophils play in these diseases and how these similarities provide potential new avenues for their treatment.

2. A Causal Relationship between Cancer and Autoimmunity

In recent years the paradigm that chronic inflammation contributes to carcinogenesis has gained much support, but the reciprocal idea that cancer may invoke autoimmunity remains controversial. The fact that cancer and autoimmune diseases may sometimes occur in the same individual suggests a possible link between these two different clinical conditions. In such people, it is likely that the inflammatory process drives both autoimmunity and malignancy. However, it is unclear whether the autoimmune disease preexists and its chronic inflammatory process leads to malignancy in some of the cases ("inflammation-induced cancer") or whether immune responses directed against tumor antigens eventually lead to autoimmune diseases ("tumor-induced autoimmunity").

2.1. Can an Autoimmune Disease Cause Cancer? Chronic inflammation has long been associated with increased risk of cancer. For example, patients with inflammatory bowel diseases (IBD, ulcerative colitis and Crohn's disease) have a 4–7-fold increased risk of developing colorectal cancer [13]. Autoimmune diseases are characterized as low-grade chronic inflammatory diseases that demonstrate leukocyte infiltration to the tissue, mostly by lymphocytes, and elevated levels of local and/or systemic inflammatory mediators, including cytokines, chemokines, and growth factors (e.g., IL-1β, TNFα, IL-6, CCL2/MCP-1, CXCL8/IL-8, and VEGF), reactive oxygen and nitrogen species (ROS, RNS), and autoantibodies [14]. The accumulation of these mediators results in slow and gradual tissue damage accompanied by somewhat increased angiogenesis and tissue remodeling, which is also called "smoldering inflammation" [13, 15]. This creates the "extrinsic pathway" linking inflammation and cancer [13]. Mechanisms that explain the extrinsic pathway include the generation of ROS/RNS that can cause DNA damage, the induction of the activation-induced cytidine deaminase (AID) by proinflammatory cytokines that results in accumulation of nucleotide alterations and increased genetic instability, and the role that key inflammatory transcription factors (e.g., NF-κB and STAT3) play by inducing inflammatory cytokines and chemokines (e.g., IFNγ, IFNα, TNFα, and IL-17), as well as key cell cycle and survival proteins (e.g., Bcl2 family members, cyclin D, cIAPs, and c-FLIP) [reviewed in [13, 16, 17]]. Thus, chronic autoimmune diseases may indeed predispose patients to cancer over time. Many of these mediators (but not all) are products of phagocytes, especially neutrophils and macrophages, which affect tissue cells and drive their genetic instability.

2.2. Can Cancer Lead to an Autoimmune Disease? An "intrinsic pathway" that takes place within tissue cells links cancer to inflammation, whereby genetic events that activate oncogenes or inhibit tumor suppressor genes may also lead to induction of inflammatory proteins. For example, EGFR activation may activate COX-2 through the activation of the transcription factors Sp1 and p38-mitogen-activated protein kinase (MAPK); the oncogene *ras* is involved in the induction of the chemokine IL-8/CXCL8; and PTEN mutations cause an upregulation of the transcription factor HIF-1, which, in turn, upregulates the chemokine receptor CXCR4 [summarized in [13]]. Mutated genes in tumors that elicit an immune response may also lead to initiation of an autoimmune disease; if the response is cross-reactive with the normal protein, the appropriate MHC haplotype is expressed, and the tissue specificity is correct. One example was found in patients with systemic lupus erythematosus (SLE) or Wegener's granulomatosis (WG) that were also diagnosed with cancer around the same time [18, 19], raising the question of which occurred first. A more detailed example is a group of scleroderma patients with increased risk of cancer that were shown to have developed autoantibodies to RNA polymerase III subunit (RPCI, encoded by the *POL3RA* locus), as opposed to other scleroderma patients with no cancer that had autoantibodies only to centromere B protein (CENTB) or topoisomerase-I [20, 21]. In these patients, both humoral and cellular specific immune responses were observed, suggesting that the mutations in the *POLR3A* gene, which are rare in human tumors, were the initiator event triggering an immune response.

3. The Microenvironment in Cancer and Autoimmunity

The microenvironment of inflamed tissues includes different cell types that secrete a myriad of mediators, including cytokines, chemokines, growth factors, lipid mediators, ROS

and RNS, remodeling enzymes, and neuropeptides. These are derived from both tissue and stroma cells and orchestrate the recruitment of new cells into the inflammatory site, their interactions with each other, and their functions within the site. Although this occurs mostly locally within the tissue, these mediators may also exert systemic influences on remote organs, for example, at the premetastatic site in cancer or when autoimmunity spreads to several remote organs. Below, we discuss some aspects of the cancerous and autoimmune microenvironments that are common to both.

3.1. The Hypoxic Microenvironment. Low oxygen tensions (hypoxia) are observed in all inflamed tissues. Because different tissues exhibit a wide range of oxygen tensions, even under normal conditions, a functional definition determines that hypoxia results when the oxygen supply does not meet the oxygen demand of the cells [47]. Hypoxia stabilizes the hypoxia-inducible factors (HIFs), which are the master regulatory transcription factors that carry out the adaptation response of cells to hypoxia, including the shift to glycolysis, induction of angiogenesis, increased invasion of leukocytes, and immune suppression [reviewed in [48–52]]. Upregulation of HIF-1α induces angiogenesis and the shift to glycolysis, as HIF-1α binds to the hypoxia response element (HRE) found in the promoters of genes such as VEGF and the glycolytic enzymes. The switch to anaerobic glycolysis increases lactate levels, causing cellular acidosis and increased production of ROS, and leading to lipid peroxidation, membranal damage, impaired activity of ion channels, and increased membrane permeability. This increases spillage of cellular content and causes tissue acidosis and damage [52–54], which, in turn, recruit more leukocytes into the site and trigger inflammation. Hence, hypoxia and inflammation are interdependent, as chronic inflammation is accompanied by hypoxia and prolonged hypoxia leads to inflammation [55].

In cancer, the uncontrolled proliferation of tumor cells increases tumor mass, which becomes depleted of oxygen and nutrient supply as the tumor reaches a diameter of 2-3 mm, because of the increased distance from blood vessels. Since hypoxia is a major drive for angiogenesis, new blood vessels are produced to increase reoxygenation, and so different oxygen tensions can be measured in different regions within the tumor (Table 1) [56]. Partial pressure of oxygen values below 5 mmHg is measured in more than 50% of advanced solid tumors [57, 58]. Tumor cells are characterized by enhanced glycolysis, even in normoxic conditions (the Warburg effect), and hypoxia further enhances the anaerobic metabolism. The byproduct of glycolysis is lactic acid, which is transported out of the tumor cell to the microenvironment to prevent cell death by intracellular acidosis. Thus, neighboring stroma cells, particularly macrophages, are exposed to increased levels of lactate, which is actively transported into them. Lactate contributes to macrophage polarization by stabilizing HIF-1α and inducing expression of typical M2-phenotype markers like VEGF and arginase-I (ARG-I) [59], so that tumor-associated macrophages (TAMs) sense the metabolic changes in tumor cells and respond to them in a proangiogenic manner [1].

In autoimmune diseases, the increased infiltration of leukocytes into the inflamed site increases the demand for oxygen beyond the available supply. Low oxygen tensions were reported in organs with an ongoing inflammatory autoimmune process, such as the synovia in RA patients [10] and the pancreas in diabetes [60]. Thus, many macrophages infiltrate the synovium of RA patients, where they encounter a profound hypoxic microenvironment, upregulate HIF-1α, and mediate an angiogenic process that is necessary for the formation of the inflammatory pannus and leukocyte infiltration [51]. Likewise, migration of T cells and macrophages into the sclerotic lesions of MS patients generates a hypoxic microenvironment that drives secretion of proangiogenic factors, including VEGF, angiopoietins, and MMPs, and induces angiogenesis around the demyelinating plaques [61]. Increased serum lactate concentrations in MS patients correlate with disease activity score and reflect the hypoxic microenvironment [62]. The role of hypoxia and angiogenesis in diseases like systemic lupus erythematosus (SLE) is not clear, but it is known that about 50% of the patients suffer from anemia, which leads to tissue hypoxia and reduced oxygen delivery, especially, but not limited to, the pulmonary vascular beds [8]. Accordingly, elevated levels of proangiogenic factors, such as VEGF, FGF, PIGF, TNFα, TGFβ, and HGF, were found in the serum of SLE patients [63]. Vascular disease, chronic tissue hypoxia, and excessive fibrosis that affects the skin and internal organs are the hallmarks of systemic sclerosis (SSc, scleroderma). An imbalance between proangiogenic factors (e.g., VEGF, PDGF) and antiangiogenic factors (e.g., angiostatin, thrombospondin-1) leads to increased serum levels of VEGF in early stages of the disease and increased serum levels of angiostatin in the late stage of the disease [64, 65].

Thus, in both diseases local hypoxia initiates a change in cell metabolism and elevates tissue acidosis, contributing to macrophage polarization and most importantly promoting the angiogenic switch, which is necessary for both cell survival and disease progression. Therefore, hypoxia and angiogenesis, although in different measures (Table 1), are two features of the microenvironment common to both cancer and autoimmunity.

3.2. Macrophages. Monocytes migrate into tissues and differentiate into macrophages that perform multiple, sometimes opposing functions that are needed in tissues, such as patrolling and maintaining homeostasis, eradicating tumor cells and pathogens, initiating wound healing and tissue repair, and resolving inflammation. These tasks are carried out by secreting inflammatory mediators (e.g., cytokines, chemokines, lipid mediators, and ROS/RNS) or anti-inflammatory mediators (e.g., IL-10, TGFβ, and PGE_2), presenting antigens to T cells and eliciting an adaptive immune response, scavenging apoptotic cells or necrotic debris, and depositing matrix proteins. Macrophages cannot perform all these tasks simultaneously, but they exhibit enormous plasticity, as they can be activated in different ways and constantly shift between them, according to the conditions in the changing microenvironment [66, 67]. This concept has been thoroughly reviewed before [68–74]. One

Table 1: Hypoxia in the microenvironment.

	Cancer tissue	RA (SF)	MS/EAE	SLE
Hypoxia	≤5 mmHg (70–80 μm from vessel); ≤0.5 mmHg (≥150 μm from vessel); [5] 0–2.5 mmHg (breast cancer) [6]	18–24 mmHg	9–20 mmHg in EAE [7]	Not directly measured; anemic hypoxia reported [8]
Normal tissue	Range: 25–72 mmHg (depending on tissue) [9]	40–70 mmHg [10–12]	35 mmHg [7]	

extreme activation mode is the classically or M1-activated macrophages, which are activated to kill pathogens and tumor cells and accordingly express MHC class II and costimulatory molecules, Fc receptors to enhance phagocytosis, and proinflammatory and cytotoxic mediators (e.g., NO, TNFα). On the opposite extreme are the alternatively or M2-activated macrophages, which enhance wound healing and angiogenesis by expressing scavenger receptors (e.g., MARCO, CD206) and anti-inflammatory mediators (e.g., IL-10, TGFβ, and PGE_2), growth factors (e.g., VEGF), and matrix proteins. The hallmark of this type of activation is the high expression of arginase-I (ARG-I), which produces L-ornithine, the precursor for collagen synthesis, and polyamines that act as proliferative signals of cells. Another more refined approach further distinguishes between M2a, M2b, and M2c macrophages, where M2a are fibrotic, M2b are immune regulators and produce IL-1β, IL-6, and TNFα, and M2c are anti-inflammatory and are involved in tissue repair and remodeling [74]. In the continuum between the M1 and M2 options, macrophages can be activated in many forms of activation, which are very difficult to isolate and characterize. For example, regulatory macrophages are responsible for suppressing the Th1/M1 inflammatory response. Some of these cells are activated by Toll-like receptors (TLRs) ligands in combination with immune complexes, and some are activated by anti-inflammatory signals, such as adenosine or phagocytosed apoptotic cells [68, 72]. Immature monocytes/macrophages, which compose the monocytic myeloid-derived suppressor cells (M-MDSCs) population, also belong to regulatory macrophages and secrete IL-10 and TGFβ to help suppress Th1 and $CD8^+$ T cells and recruit regulatory T cells [75–77]. MDSCs inhibit T effector cells by expressing both inducible nitric oxide synthase (iNOS) and ARG-I that compete for their mutual substrate L-arginine, leading to its depletion and reduced production of CD3ξ chain in the TCR receptor, and therefore decreased antigen-specific T cell responses and proliferation [78].

In cancer, macrophages play a dual role. The concept of immunoediting [79] suggests that, in early stages of tumor development, the immune system successfully surveys and eradicates tumor cells. Tumor cells that survive remain constantly under immune pressure, which helps to "sculpt" their phenotype into a more aggressive one, until finally, at the third stage, they escape immune recognition and become established. This concept describes a close relationship between tumor and immune cells, which is crucial for the determination of tumor fate and progression [80]. Furthermore, it suggests that the regulation of the immune response is critical to the fate of the tumor: if the response is mostly proinflammatory, the immune cells will turn against the tumor and eradicate its cells, whereas if the response is anti-inflammatory, the immune cells will provide mediators that are necessary for tumor growth and promote tumor progression. Much progress has been made in recent years in our understanding of how tumor cells actively tip the balance and maintain a favorable, anti-inflammatory, and immunosuppressive response through their interactions with macrophages.

The majority of the macrophages found in the tumor originate from monocytes that were recruited to the site. Circulating monocytes are heterogeneous and are generally divided into at least two subsets: a major subset of classical monocytes ($Ly6C^+$ in murine and $CD14^{++}CD16^-$ in human) and a minor subset of nonclassical monocytes ($Ly6C^-$ in murine and $CD14^+CD16^+$ in human). There is currently controversy as to the role of different monocytes subsets in tumor progression. It has been suggested that nonclassical monocytes are preferentially recruited into the primary tumor, and classical monocytes are recruited more to the metastatic sites [81]. In contrast, other studies show that nonclassical patrolling monocytes have a role in preventing metastatic spread [82]. Furthermore, other methods of monocytes classification, based on different markers, are possible, although not yet common. For example, classifying monocytes according to their Tie-2 expression may be very relevant in cancer, as those monocytes are recruited into the tumor and have a profound and strong proangiogenic activity that is critical for tumor progression [83, 84]. This remains for now a subject of great interest.

Macrophages make up the major inflammatory cell population within tumors (Table 2), as they can infiltrate deep into the hypoxic microenvironment, unlike other leukocytes [85]. Several macrophage subsets have been found located in different regions of the tumor [86]. Tumor-associated macrophages (TAMs) are responsible for supporting tumor growth and dissemination. This is achieved by secreting IL-10 and TGFβ which inhibit adaptive immune responses, VEGF, and other proangiogenic factors that promote angiogenesis, growth factors such as EGF that are necessary for the tumor cell viability, and matrix remodeling enzymes such as matrix metalloproteinases (MMPs) that enable cellular motility. TAMs are activated in a manner approximating M2-activation, and thus express ARG-I, produce matrix proteins, and secrete elevated levels of IL-10 and TGFβ. Additional

Table 2: Examples for the distribution of macrophages and neutrophils in different types of cancer and autoimmune diseases.

Type of carcinoma	Localization	Percentage (%)	Mice/human	Ref
	Macrophages in cancer			
Mammary gland	Macrophages are found infiltrating all areas of the tumors (including the perinecroptic areas)	>40%	Mice	[22]
Gastrointestinal tumors		<30%	Human	[23]
Diffuse large B-cell lymphoma	Many macrophages are found in the stroma, in close contact with the cancer cells	20%	Human	[24]
Non-small-cell lung cancer (NSCLC)		15%–30%	Human	[25, 26]
Prostate cancer	In stroma and in close contact with cancer cells	10%–15%	Human	[27]
Pancreatic cancer	Intratumoral and in the invasive front	30–50%	Human	[28]
Colon cancer	Intratumoral, numbers increase with tumor stage and grade	25%–50%	Human	[29]
L929 Fibrosarcoma, B16 melanoma, LLC lung carcinoma cells	Intratumoral	23–51%	Mice	[30]
	Neutrophils in cancer			
Lung cancer	Infiltrating the tumor	8%	Human	[31]
Clear cell renal cell carcinoma (RCC)	Intratumoral or near vessels	14%	Human	[32]
Mesothelioma, lung cancer	Intratumoral	0.7–2.5%	Mice	[33]
L929 Fibrosarcoma, B16 melanoma, LLC lung carcinoma cells	Intratumoral or near vessels	3–8%	Mice	[30]
	Macrophages in autoimmune diseases			
Rheumatoid arthritis (RA)	Lining the synovial membrane	41%	Human	[34]
	Lining and infiltrating the synovium	35–46%	Human	[35]
	Lining and infiltrating the synovium	17–36%	Human	[36]
	Infiltrating the synovium	26%	Human	[37]
Multiple sclerosis (MS)	Infiltrating and at the rim of the lesion	15–30%	Human	[38]
Systemic lupus (SLE)	Kidney: infiltrating all parenchyma, found surrounding glomeruli and around perivascular aggregate	26%	Mice	[39]
Systemic lupus (SLE)	Throughout the nephritic kidney	4%	Mice	[40]
Scleroderma	Skin	23%	Rat	[41]
Systemic sclerosis (SSc)	Superficial and deep dermis at early stages	13%	Human	[42]
	Neutrophils in autoimmune diseases			
Rheumatoid arthritis (RA)	Lining and infiltrating the synovium	8–15%	Human	[35]
	Infiltrating the synovium	4.5–7%	Human	[37]
Experimental autoimmune encephalomyelitis (EAE)	Within brain lesions In the spinal cord	0.4–3% 8%	Mice	[43–45]
Systemic lupus (SLE, juvenile)	$CD15^+$ low density granulocytes in circulation	10%	Human	[46]

forms of macrophage activation in tumors include the Tie-expressing monocytes (TEMs), which are strongly proangiogenic and reside close to blood vessels [87] and MDSCs. MDSCs infiltrate the tumors and expand proportionally to the tumor burden [70, 72, 84]. TAMs, TEMs, and MDSCs are all obligatory components of the tumor microenvironment and share many similar markers and functions (especially TAMs and MDSCs), so it is very difficult to distinguish between them or to isolate them for *in vitro* studies.

Several microenvironmental conditions ensure that macrophages in tumors are activated in a way approximating M2-activation. First, the tumor cells secrete soluble mediators, such as M-CSF/CSF-1, VEGF, and TGFβ, which recruit macrophages to the tumor and maintain their viability, while polarizing them towards M2-activation [88–90]. Second, the hypoxic microenvironment can shift even M1-activated macrophages towards M2-activation, utilizing multiple transcriptional and posttranscriptional mechanisms [48, 91–93]. Lastly, in a process called efferocytosis, macrophages engulf apoptotic cells, particularly apoptotic neutrophils (that were recruited to the tumor, secreted their content, and died by apoptosis; see Section 3.3), and

this triggers M2-activation to promote angiogenesis, wound healing, and tissue remodeling [94]. Once M2 activated, these macrophages enhance their secretion of TGFβ and IL-10, thus further immunosuppressing M1-activated macrophages in their vicinity. In contrast, macrophages that phagocytose tumor cells undergoing secondary necrosis, which release danger-associated molecular patterns (DAMPs) such as HMGB1, are M1-activated, lead to increased secretion of inflammatory cytokines (TNFα, IL-1β, and IL-12), and promote Th1 responses [95]. Thus, because of their plasticity, it is likely that, in the same tumor, some macrophages will be M1-triggered and most will be M2-activated, depending on their relative location within the tumor mass. This plasticity is now used in the treatment of cancer, as immunotherapy using monoclonal antibodies (e.g., anti-OX40, anti-EMMPRIN) was shown to modulate the microenvironment, reduce the levels of anti-inflammatory cytokines (e.g., TGFβ), change the T cell infiltrate, and repolarize macrophages to become M1-activated, capable of killing tumor cells [96, 97]. Furthermore, drugs that alter TAMs activation were shown to enhance the effect of different immunotherapy approaches by changing the microenvironment [98]. However, the mechanisms that allow such manipulations are not entirely elucidated.

In contrast to cancer, the polarization state of macrophages in autoimmune diseases is poorly defined. Following the Th1/Th2 paradigm and extending it to the M1/M2 paradigm, one would expect to find M1 macrophages in Th1 autoimmune diseases such as RA, MS, and HT and M2 macrophages in Th2 autoimmune diseases such as SLE and scleroderma. However, the data is controversial. In one study, macrophages from the synovial fluid of RA patients expressed proinflammatory polarization markers (e.g., MMP12, CCR2), consistent with the elevated levels of proinflammatory cytokines detected in these patients' synovial fluids [123]. However, in another study, synovial fibroblasts were induced by TNF to secrete soluble factors that suppressed macrophage production of IFNβ and limited macrophage ability to respond to IFNβ by inhibiting Jak-STAT signaling, leading to decreased levels of M1-chemokines such as CXCL9 and CXCL10 [124]. In MS patients, activated microglia in preactive and remyelinating lesions expressed a mixed phenotype with both M1 markers (CD74, CD40, and CD86) and M2 markers (CCL22 and CD209, but not CD206) [125], whereas, in a mouse model of experimental autoimmune encephalitis (EAE), inhibition of the Aurora kinase blocked disease development and shifted macrophage phenotype from M1 to M2 [126]. In SLE, the contribution of macrophages to disease pathogenesis was hardly investigated. In a mouse model of SLE, generated by immunization with activated lymphocyte-derived DNA, macrophages infiltrating the nephritic tissues exhibited activation markers of M2b polarization ($MHCII^{high}CD86^{+}IL\text{-}10^{high}IL\text{-}12^{low}$) [127]. However, much evidence points to a possible mixed activation of macrophages in SLE, which includes both M1 and M2b polarized macrophages. For example, high levels of proinflammatory cytokines (e.g., TNFα, GM-CSF, IFNγ, CCL2, and CXCL10) are found in serum of SLE patients, alongside high levels of IL-10 and IL-6 [128]. Both systemic and localized sclerosis (scleroderma) are autoimmune diseases manifested by vascular injury and progressive fibrosis of the skin, lung, and internal organs. The cytokine balance in these conditions is shifted towards Th2 cytokines, such as TGFβ, PDGF, IL-4, and IL-13. Accordingly, macrophages are M2-polarized with high expression of the CD206 marker [129]. Interestingly, this shift towards M2 was shown to be mediated by the enzyme N-acetylglucosaminyltransferase-V (GnT-V) that glycosylates surface proteins, as mice with deficiency in the gene ($MGAT5^{-/-}$) were resistant to bleomycin-induced scleroderma and showed decreased M2-activation of cutaneous macrophages, with a similar total count of macrophages as the wild type mice [130].

The role of macrophages in cancer diseases has been investigated in depth, whereas their role in autoimmune diseases merits more research. The plasticity of macrophages and their ability to respond to changing conditions suggest that their polarization *in vivo* is difficult to assess. Unlike the defined *in vitro* stimulus, mixed signals in the complex microenvironment *in vivo* may result in different subpopulations of macrophages exhibiting different polarization and different functions. It is, therefore, important to precisely define the conditions in the microenvironment in each disease and to understand how these change over time in different parts of a tumor, in different organs, and in different stages of disease development. Furthermore, the mixed polarization of macrophages that is observed *in vivo* can be the result of intermediate transitioning from one polarization to another, or a result of a complex tissue structure that includes niches or even microniches that exhibit small nuances in the microenvironment. It is also important to remember that although most macrophages are recruited from the circulation during inflammation, some macrophages are resident in the tissue. At present, the specific role of tissue resident macrophages within the tumoral or autoimmune microenvironment is not well understood, mostly because of our current inability to distinguish them from recruited monocytes and due to their scarcity within the microenvironment. This is further complicated by the fact that, in some tissues, such as the intestine and heart, resident macrophages are gradually replaced by monocyte-derived macrophages [131, 132], whereas, in the brain, resident microglia are long-lived and can proliferate to maintain their numbers independently of monocyte infiltration [133]. The question whether these resident macrophages have different roles than the infiltrating monocyte-derive macrophages remains unresolved, but at least, in the murine model of EAE, microglia seem to be activated in early stages of disease development, supporting this premise [133]. Lastly, a new field of study of the trained innate immunity now demonstrates how innate immune cells may acquire a memory through epigenetic reprograming [134]. The significance of this subject to the activation of macrophages awaits further investigation and raises the question of how the history of the macrophages affects their ability to respond to the changing microenvironment and polarize correctly.

3.3. Neutrophils. Neutrophils were viewed as cells that terminally differentiate in the circulation, migrate into tissue in response to inflammatory signals, degranulate in response to triggering, and die of apoptosis immediately after. However, recent findings challenge this concept and place neutrophils, together with macrophages, as cells that secrete a myriad of regulatory mediators that shape their immediate microenvironment, all depending on the diverse cell types they meet.

In cancer, and using an analogy to the M1- and M2-activation modes of macrophages, neutrophils are now also categorized as antitumoral N1 and protumoral N2 tumor-associated neutrophils (TANs) [33]. Neutrophils make up a relatively small percentage of the tumor mass and are primarily found at the tumor rims and in nonnecrotic areas. They can infiltrate the tumor in small numbers (Table 1) and then are often found near blood vessels or in compact aggregations. However, changing the tumor microenvironment by blocking TGFβ signaling increases neutrophil infiltration and reduces tumor size [33]. TANs within the primary tumor are protumoral, as they secrete the proangiogenic factor Bv8, which is also responsible for myeloid cells recruitment, especially at early stages of malignancy [135], as well as the proangiogenic matrix metalloproteinase MMP-9, both in larger amounts than their cognate TAMs [30]. Furthermore, once TGFβ is blocked, a collaboration between TAMs and TANs is demonstrated, as TAMs produce neutrophil chemoattractants that recruit $CD11b^+Ly6G^+$ neutrophils into the tumors [33]. Note that mature neutrophils and immature granulocytic MDSCs are practically indistinguishable, as they both express the same surface markers ($CD11b^+Ly6G^+$), and it is yet unclear whether mature neutrophils arrive at the tumor from the circulation or whether immature MDSCs mature to N2 TANs within the tumor [33].

Neutrophils make up a much smaller fraction of the immune infiltrate in the tumors compared to macrophages, but their relative contribution is still unclear. For example, in some tumors, they may be the main producers of MMP-9 and not the more abundant macrophages [30]. It is clear that the contribution of $CD11b^+Gr1^+$ granulocytic MDSCs to the formation of the premetastatic niche is significant. These granulocytic MDSCs (and not monocytic MDSCs) infiltrate the lung premetastatic niche well before tumor cells arrive there and secrete *in situ* large amounts of MMP-9, resulting in aberrant and leaky vasculature in the premetastatic lung. In addition, these G-MDSCs inhibit the secretion of IFNγ by lung macrophages and increase the secretion of anti-inflammatory cytokines, such as IL-4, IL-5, and IL-10, indicating an immune suppression of the lung [136]. They can also secrete the neutrophil chemoattractants S100A9 and S100A8, as well as the proangiogenic Bv8. Interestingly, Bv8 also induces the migration of metastatic cells, suggesting that G-MDSCs direct the homing of metastatic tumor cells into the lung [137]. In contrast, other studies demonstrate that depletion of $Ly6G^+$ neutrophils does not change the size of the primary tumor but increases the lung metastatic burden, suggesting that tumor entrained neutrophils (TENs) at the premetastatic niche inhibit, rather than promote, metastasis [138]. Furthermore, adoptive transfer of such TENs significantly inhibited formation of lung metastatic foci, as they were highly cytotoxic to tumor cells. This cytotoxicity is triggered by the tumoral secretion of CCL2. However, neutrophils become cytotoxic only at the premetastatic lung and not at the primary tumor site where they are subjected to high levels of the local inhibitory effects of TGFβ [138]. This antitumoral effect of TENs was only temporary, and eventually they failed to inhibit metastasis. Thus, neutrophils display different functions at the primary tumor site and at the premetastatic niche, and within the metastatic niche their role changes over time.

Unlike macrophages, neutrophils can produce the proinflammatory cytokine IL-17, to mediate their involvement in cancer. IL-17 is mainly produced by either neutrophils or Th17 lymphocytic cells. High IL-17 levels or high frequency of cells producing IL-17 correlates with poor prognosis, whereas high Th17 cell frequencies were correlated with improved prognosis [139], suggesting that neutrophils might be the culprits. In fact, in different tumor types (e.g., head and neck, ovarian, endometrial, prostate, breast, lung, and colon carcinomas), IL-17 was mostly produced by neutrophils (66% of the IL-17 producing cells in the tumor mass), whereas Th17 cells constituted only a small fraction of the immune infiltrate producing IL-17 (4%) [140]. In contrast, other studies suggested that IL-17 was secreted by Th17 or $\gamma\delta$ T cells, which were responsible for neutrophil recruitment into the tumors. The recruited neutrophils, in turn, immunosuppressed $CD8^+$ cytotoxic T cells and promoted angiogenesis and metastasis [141, 142]. Thus, although IL-17 is considered proinflammatory, its correlation with poor prognosis suggests that it also has protumoral roles. For example, IL-17 can increase tumor cell growth and migration [140, 141], induce IL-6 and CCL20 that recruit Th17 to the tumor site, and modulate gene expression of nontumor cells (including enhanced production of cytokines and chemokines, transcription factors, and antiapoptotic proteins), suggesting that neutrophils play an important role in tumors at an early stage [141].

In some autoimmune diseases, neutrophils are a major component of the immune infiltrate. For example, in RA, 90% of all leukocytes in the joint may be neutrophils [143], suggesting that they have a significant contribution to the pathogenesis of the disease. Although generally the role of neutrophils in autoimmune diseases has not been thoroughly investigated, their importance is now gradually gaining acceptance. Several possible mechanisms of action for neutrophils in autoimmune diseases have been suggested, as follows.

Neutrophils are phagocytes with a strong cytotoxic potential, and when activated in a proinflammatory manner (N1) they can enhance their secretion of proteases and ROS and, in an autoimmune context, inflict tissue damage. They also secrete chemokines that attract more neutrophils, macrophages, and other stroma cells into the inflamed site, thus amplifying the destructive effect in this context. In RA, migration of neutrophils to the joint is regulated by their enhanced expression of chemokine receptors (e.g., CCR2) that lead them towards elevated levels of CCL2 found in the synovial fluid (SF) [144]. Furthermore, IL-17 that is produced by neutrophils is an important mediator in arthritis, as IL-17 KO mice exhibit a clinical score less

severe than wild type mice in the K/BxN serum-induced arthritis model [145]. In MS patients, circulating neutrophils are primed compared to healthy controls and exhibit reduced apoptosis and enhanced expression of surface markers (e.g., TLR2, IL-8 receptor) [146]. Disruption of the blood brain barrier (BBB) in MS patients or in mice with EAE allows entry of leukocytes, including neutrophils, into the brain. Secretion of IL-17 by both Th17 and neutrophils helps to further disrupt the BBB and attract even more neutrophils and macrophages to the site of inflammation, especially at the preclinical stage before disease onset [43]. In patients with type I diabetes (T1D), the role of neutrophils remains controversial; however several observations indicate that circulating neutrophils are slightly reduced during the early stages of the disease and that they are accumulating at the exocrine pancreas in very small blood vessels or adjacent to acinar cells [147]. Neutrophils that are triggered by immune complexes are found in SF of RA patients, along with elevated levels of ROS [143]. In fact, neutrophils carrying the R620W polymorphism in the tyrosine phosphatase *Lyp*, which is highly expressed in neutrophils, exhibit enhanced migration and extravasation through endothelial cells, increased Ca^{2+} influx, and increased ROS production upon stimulation [148], demonstrating the importance of this polymorphism in the susceptibility to autoimmune diseases.

In another possible mechanism of action in autoimmune diseases, neutrophils have the ability to produce the enzyme peptidyl arginase deaminase-4 (PAD-4), which modifies the amino acid L-arginine into L-citrulline and is therefore involved in the generation of autoantibodies against citrullinated proteins found in both RA and MS patients in early stages [149, 150]. Moreover, neutrophils can release chromatin extracellular traps (neutrophil extracellular traps, NETs) in a process termed "NETosis" (or "ETosis" when other cell types, such as mast cells, eosinophils, or macrophages, perform it, although less efficiently). These NETs are composed of chromatin fibrils, a combination of DNA and proteins, including histones (70% of the proteins), HMGB1, neutrophil elastase (NE), myeloperoxidase (MPO), the peptide LL-37, and the hCAP18 fragment of cathelicidin. These proteins are recognized by immune cells (e.g., dendritic cells) as alarmins or danger-associated molecular pattern (DAMP) molecules when they are bound to DNA and spilled out of the cells. Citrulline is uncharged in neutral pH, as opposed to arginine, and can change protein folding, structure, and function. Some proteins may naturally include citrulline (e.g., myelin basic protein, MBP, several histone proteins), whereas others undergo citrullination in the inflammatory site (e.g., fibrin and fibrinogen in RA joints). When these proteins are posttranslationally modified by citrullination, neoepitopes may be revealed that are no longer tolerated, leading to the production of proinflammatory cytokines such as TNFα, IL-6, and IFNα [reviewed in [151]]. Low density granulocytes (LDG), a subset of immature neutrophils whose numbers increase in SLE patients, are particularly susceptible to NETosis, as they secrete IFNα [152]. NETosis is associated with the finding of antineutrophil cytoplasmic antibodies (ANCA) found in many SLE patients [153, 154] and is consistent with the finding of IFNα in the pancreas of T1D patients and the finding of IFNα and NETs in nonobese diabetic (NOD) mice that spontaneously develop T1D [reviewed in [147]]. In MS patients, higher serum levels of NETs were found [146].

Thus, neutrophils secrete many proinflammatory and cytotoxic mediators leading to the aggravation of the inflammatory response and culminating in gradually accumulating tissue damage, and they can cast NETs that lead to generation of autoantibodies, thus providing a hint to the etiology of autoimmune diseases. Both these mechanisms highlight neutrophils as significant and important cells in the generation of autoimmune diseases.

Neutrophils clearly play a large role in the microenvironment of both cancer and autoimmunity, but they are not as well understood as their "sibling" macrophages. Evidence suggests that they play a crucial role during early stages of diseases, but their role in later stages requires more investigation.

3.4. Macrophage-Neutrophil Cooperation. Macrophages and neutrophils show a high degree of overlap or redundancy as they secrete similar mediators, such as ROS, MMPs, cytokines, and chemokines. However, there are differences in the quantities produced and in gene expression. For example, both cell types secrete MMP-9 but in different quantities, and neutrophils, but not macrophages, can also secrete MMP-8; both phagocytes produce ROS, but neutrophils produce more hypochlorous acid; macrophages are by far better antigen presenting cells, whereas neutrophils excel in casting NETs. Both cell types are of myeloid origin and, therefore, have similar surface markers. Both types of cells exhibit similar plasticity, where the M1/N1 activation is geared to perform killing functions, whereas M2/N2 activation is directed towards healing wounds and promoting angiogenesis.

There is now some evidence of cooperation between macrophages and neutrophils. Both cell types secrete cytokines and chemokines that recruit each other and enhance each other's proinflammatory activities, thus enhancing resolution of inflammation [155] (see Table 3 for details of some cytokines and chemokines in the microenvironment). Macrophages secrete the macrophage migration inhibitory factor (MIF) to enhance neutrophil survival and secretion of MMP-9, in the context of both cancer [156] and autoimmunity [157]. The manner by which neutrophils die profoundly affects macrophage polarization, and, therefore, the subsequent course of disease. In cancer, in the absence of activating signals, neutrophils have a short half-life of 6–18 hours in the circulation, before dying by apoptosis, and the process of their engulfment and processing by macrophages (efferocytosis) results in macrophage polarization towards M2-like activation and enhances immunosuppression [158]. Furthermore, neutrophils secretion of IL-17 helps to shift macrophage activation towards the M2b regulatory phenotype [159]. In contrast, in autoimmune diseases, presence of GM-CSF and hypoxia can delay neutrophil apoptosis and increase their survival [143]. Moreover, in early RA patients, antiapoptotic cytokines (e.g., IL-4, GM-CSF, and G-CSF) that are found in their SF may lead to defects and low levels

Table 3: Example concentrations of cytokines and chemokines in the microenvironment.

		Cancer (breast, pg/mL/mg)[a]	RA (SF[b], pg/mL)	MS/EAE (CSF[c], pg/mL)	SLE (serum, pg/mL)
IL-1β	Disease	2.7–3.5 [99, 100]	2.6 [36] 9.26 [101]	0.02 [102] 44.1 [103]	0.24 [104] 11 [105]
	Healthy/remission	0 [100]	0 [36] 7.7 [101]	0 [102]	0.1 [104] 5 [105]
TNFα	Disease	7.2 [100]	14.0 [106]	1.85 [102] 5.34 [107] 9.0 [108] 39.4 [103]	0.34 [104] 1.24 [109] 7.8–8.0 [110, 111] 44.76 [105]
	Healthy/remission	1.6 [100]	3.5 [106]	0.93 [102] 1.95 [107]	0.1–2.2 [104, 109, 111] 20 [105]
IFNγ	Disease	27.6 [100]	0 [36]	3.27 [102] 5.7 [107] 11.6 [108]	0.64 [104] 6.5–7.05 [109, 110]
	Healthy/remission	16.6 [100]	0–3.5 [36, 106]	0.2–0.52 [102, 108] 3.7 [107]	1.3–11.7 [104, 109, 110]
IL-17A	Disease	0 [100]	0 [36] 12 [112]	6.93 [102] 16.53 [107]	97.42 [109]
	Healthy/remission	0 [100]	0 [36] 4 [112]	3.36 [102] 13.7 [107]	3.30 [109]
IL-6	Disease	17.2 [100]	1,253 [36] 355 [101]	2.86 [102] 6.02 [107] 13.2 [103, 108]	10.02 [109] 20.8 [110] 70.45 [105]
	Healthy/remission	1.2 [100]	1,170 [36] 87 [101]	2.5–12 [102, 108] 6.24 [107]	0.5–2.18 [109, 110] 20 [105]
TGFβ	Disease	86.7 [113]	768 [36]	74.6 [107]	42,990 [109]
	Healthy/remission		0 [36]	64 [107]	82,710 [109]
IL-10	Disease	0.3 [100]	16.2 [36]	0.95 [102] 4.34 [107]	1.2 [111] 2.82 [31, 104] 9.78 [109]
	Healthy/remission	0 [100]	0 [36]	0–0.63 1.13 [102] 0.38 [107]	0.54 [104, 109, 111]
CCL2/MCP-1	Disease	121 [100]	25,000 [114]	116.3 [108] 574.4	136 [115]
	Healthy/remission	1.9 [100]	920–2900 [114]	163–526 [108, 116]	71 [115]
VEGF	Disease	1,148 [117]	1,100 [118] 1,800 [119]	Below the level of detection [120]	300.8 [121]
	Healthy/remission	163 [117]	700 [119]	Below the level of detection [120]	124 [121]
IL-4	Disease	1.7–3.1 [99, 100]	0 [36]	0.17 [102] 3.3 [107] 8.6 [116]	0.1–0.2 [104, 110]
	Healthy/remission	0 [100]	0 [36]	0.03–0.1 [102, 116] 1.74 [107]	0 1–0.3 [104, 110]
IL-8	Disease	68 [100]	584 [101]	30–35 [102, 122]	358 [111]
	Healthy/remission	1 [100]	451 [101]	28–31 [102, 122]	150 [111]
IL-12	Disease	2.3 [100]	10.5 [106]	1.44 [102] 4.9 [116]	1.0 [104]
	Healthy/remission	1.4 [100]	6.1 [106]	0.56–1.4 [102, 116]	0.18 [104]

[a]Measured in tumor extracts.
[b]Measured in the synovial fluid (SF).
[c]Measured in the cerebrospinal fluid (CSF).

of apoptotic death in neutrophils, suggesting that their engulfment by macrophages after secondary necrosis elicits a proinflammatory response.

This evidence suggests that macrophages and neutrophils communicate with each other and cooperate to regulate the microenvironment, explaining why both cell types seem to play similar roles in clinical settings. It has even been shown that when macrophages are depleted, or even change their activation mode, neutrophils gain the ability to infiltrate a tumor instead [98]. Therefore, myeloid cells and molecules that mediate their cooperation become new attractive targets for cancer immunotherapy. However, many questions that merit further investigation remain unanswered. For example, what factor(s) direct the tumor microenvironment, so that M2-TAMs become the dominant cellular component, rather than N2 TANs? Can macrophages compensate for the lack of neutrophils, or are neutrophils necessary for tumor growth, despite their being such a small percentage of the tumor mass? To what extent is this cooperation between macrophages and neutrophils necessary for tumor progression or for the development of the metastatic niche? And finally, is there a direct interaction between these two cell types, and if so, what protein(s) mediate it? Similarly, various interesting possibilities exist for the study of the role macrophages-neutrophils interactions play in autoimmune diseases.

3.5. Autoantibodies. Antibodies are effector molecules that specifically bind to their antigens and thus tag the cell for destruction either via complement fixation or via other effector cells (e.g., macrophages, NK cells) that have the appropriate Fc receptor. The binding of antibodies can also promote or inhibit cell signaling and activation. During early stages of an autoimmune disease, the process of NETosis exposes many citrullinated self-proteins to the immune system, and since the modification renders these proteins neoantigens, tolerance is broken and the immune system can generate autoantibodies and enhance epitope spreading, resulting in autoimmune responses [149, 151]. Other posttranslational modifications (PTM), such as carbamylation and oxidation, can also generate neoantigens and autoantibodies [160]. The binding of these autoantibodies to their modified targets may drive tissue damage through their effector functions and contribute to the generation of autoimmune diseases [161], suggesting a causative role for the autoantibodies. However, it should be remembered that NETosis is a physiological and protective process (e.g., limiting invading pathogens) that does not necessarily lead to an autoimmune response. Additional factors (e.g., specific genetic background of an individual, specific polymorphism in genes related to NETosis, and defects in the mechanisms responsible for the clearance of NETs) must also exist to allow an autoimmune disease to develop [154].

In many autoimmune diseases autoantibodies can be found in the serum of patients and these may have critical role in the pathogenesis of these diseases through aberrant signaling of cells or through their destruction. In fact, autoantibodies can be considered a hallmark of autoimmune diseases and are therefore often used as biomarkers for disease progression. For example, presence of autoantibodies against insulin, GAD65, and IA-2 can confirm the diagnosis of type I diabetes (T1D) [147], and anti-dsDNA antibodies bind to resident kidney cells and trigger signaling that promotes inflammation and fibrosis in SLE [162]. Antinuclear antibodies (ANAs) are widely used as diagnostic biomarkers, and they have been shown to be involved in the pathogenesis of several autoimmune diseases, particularly systemic autoimmune diseases, as they form immune complexes with their target proteins and generate inflammation in many organs, like the kidney, lung, skin, brain, joints, and others [163]. Some ANAs are associated with specific diseases. For example, autoantibodies to double-stranded DNA and antihistones are associated with SLE, whereas anti-DNA-topoisomerase-I and anti-centromere protein B (CENTB) are linked to scleroderma [163].

Autoantibodies can be found in patients with inflammatory diseases that may ultimately progress into cancer, such as chronic hepatitis and liver cirrhosis, even in early, precancerous stages. Once cancer progresses, many autoantibodies can be found in different types of solid cancers, directed against over 100 tumor-associated antigens (TAAs), including autoantibodies to CA-125, chromogranin A, and plasminogen [164–166]. However, some of these autoantibodies overlap with autoantibodies found in patients with autoimmune diseases, such as different ANAs (e.g., anti-Sm, anti-CENTB), autoantibodies to double-stranded DNA, p53, and c-Myc [167, 168]. Autoantibodies to citrullinated proteins were found significantly more frequently in the sera of diffuse large B-cell non-Hodgkin lymphoma patients than in healthy controls [169]. The presence of such autoantibodies in cancer may be explained by the increased necrotic death of tumor cells, combined with neutrophil-derived NETosis and proteolysis of spilled proteins that may reveal cryptic epitopes. However, the role these autoantibodies play in cancer is still undetermined. It is possible that such autoantibodies may confer partial protection from cancer by promoting tumor cell death through complement-dependent cytotoxicity (CDC) or macrophage-mediated antibody-mediated cell cytotoxicity (ADCC), at least in early stages of cancer development. This has been shown for anti-TPO and anti-Tg autoantibodies in patients with both Hashimoto's thyroiditis and papillary thyroid cancer [14]. Other protective effects, such as inhibition of protein activity or induction of cell cycle arrest, should also be investigated. However, it is likely that, in later stages of tumor growth, the immunosuppressive microenvironment hampers those effects. Clearly, the relevance of autoantibodies to tumor pathogenesis merits more investigation.

Antibodies are, therefore, components in the microenvironment of both autoimmune and cancerous diseases. Although they are known to be very powerful effector molecules, the pathogenic role of antibodies in these diseases, especially in cancer, remains not fully elucidated, and it is possible that lessons learnt in one clinical scenario will improve our understanding of the other.

4. Concluding Remarks

We reviewed here several aspects of the microenvironment in two clinically and immunologically opposing diseases and showed that, despite their fundamental differences, there are some instructive parallels between them. For example, hypoxia and angiogenesis are a common denominator in both diseases, although oxygen tensions may be variable and not comparable *per se*. Likewise, the presence of autoantibodies is a similar feature, especially when autoantibodies against the same self-antigens are involved. In this respect, it is likely that research of these elements in the context of one disease will shed light on their role in a different disease.

Innate immunity, and specifically myeloid cells, has long been recognized as crucial for tumor progression and metastasis, whereas its role in autoimmune diseases is only beginning to be unfolded. The paradigm that autoimmune diseases are mediated exclusively by B and T cells of adaptive immunity is gradually shifting to one recognizing the vital role that myeloid cells play as drivers and regulators of the microenvironment and of autoimmune responses. The adaptive immune cells (T and B lymphocytes) must be activated by antigen presenting cells, a process requiring the prolonged activation of both macrophages and neutrophils. In particular, after macrophages were recognized as cells with enormous plasticity that respond to and regulate a changing microenvironment, this concept has extended to recognize similar properties in neutrophils in both cancer and autoimmune diseases. In view of the chronicity of both cancer and autoimmune diseases, the paradigm that neutrophils are short-lived and fully differentiated cells now shifts to include the understanding that neutrophils can extend their survival according to conditions in the microenvironment. Indeed, the newly discovered involvement of neutrophils in both cancer and autoimmunity and the importance of the interactions between neutrophils and macrophages present a novel field of study, which will probably expand in the future.

Lastly, identifying the parallels in these two clinically opposing diseases may provide us with new targets and tools for therapy. For example, the ability of macrophages to home in on the hypoxic regions in tumors leads us to use these cells as vehicles to deliver gene therapy [170]. Amazing progress has been made in immunotherapy during the last few years, where different regulatory checkpoints and "go signals" are targeted in an attempt to change the microenvironment. In autoimmune diseases such as RA, anti-TNF biologics are now routinely administered and improve life quality for many patients, and, in cancer, we have recently witnessed the success of combined anti-CTLA4 and anti-PD-L1/PD-1 in the treatment of melanoma [171]. Targeting the process of leukocytes recruitment into inflamed sites is now gaining more success. Using CCR2 antagonists inhibited tumor growth and prevents metastasis [81, 172], as well as reducing inflammation and joint destruction in a murine model of adjuvant-induced arthritis [173]. Additional targets, such as the CSF-1 receptor kinase or CX3CL1, lead to macrophage depletion and greatly improved kidney pathologies in mouse models of nephritic lupus [174, 175]. Neutrophil recruitment can also be targeted by blocking CXCL8 or CXCL6 signaling with antibodies, and this approach has produced similar benefits in inhibiting tumor growth and metastasis [176, 177]. Other strategies that target the immunosuppressive microenvironment, specifically by targeting different steps in TGFβ signaling pathway, also show efficacy in reducing invasiveness, migration, and tumor size in murine models of breast [178, 179], glioma [180], and colon cancer [181]. This targeting of TGFβ pathway ameliorated immunosuppression and shifted the cellular composition within tumor microenvironment towards increased $CD8^+$ T cells, macrophages, and NK cells [180].

These novel and promising immunotherapies can be further extended with novel targets, like anti-IL-6 receptor, anti-CD20, and many others that are already in the pipeline. By studying the parallels and differences between cancer and autoimmunity, other potential targets could be identified and appropriate strategies developed to achieve the desired outcome of treatment for cancer and autoimmune diseases.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Michal A. Rahat drafted and wrote the paper. Jivan Shakya collected data and helped organize the paper. Both authors have given approval to the final version of the paper.

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Review Article

Cancer Stem Cells and Macrophages: Implications in Tumor Biology and Therapeutic Strategies

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Cancer stem cells (CSCs) are a unique subset of cells within tumors with stemlike properties that have been proposed to be key drivers of tumor initiation and progression. CSCs are functionally defined by their unlimited self-renewal capacity and their ability to initiate tumor formation *in vivo*. Like normal stem cells, CSCs exist in a cellular niche comprised of numerous cell types including tumor-associated macrophages (TAMs) which provides a unique microenvironment to protect and promote CSC functions. TAMs provide pivotal signals to promote CSC survival, self-renewal, maintenance, and migratory ability, and in turn, CSCs deliver tumor-promoting cues to TAMs that further enhance tumorigenesis. Studies in the last decade have aimed to understand the molecular mediators of CSCs and TAMs, and recent advances have begun to elucidate the complex cross talk that occurs between these two cell types. In this review, we discuss the molecular interactions that define CSC-TAM cross talk at each stage of tumor progression and examine the clinical implications of targeting these interactions.

1. Introduction

Cancer stem cells (CSCs), also known as tumor-initiating cells or tumor-propagating cells, constitute a biologically unique subset of stemlike cells within the bulk tumor cell population. These cells are hypothesized to be key drivers of the multistep process of oncogenesis, giving rise to the clonogenic core of tumor tissues. Thus, according to the CSC model of tumor heterogeneity [1], malignancies have a hierarchical developmental structure with the CSC at the top of the hierarchy (Figure 1). This idea that tumor initiation and progression are driven by stemlike cells was first proposed >150 years ago by Virchow [2] and has long been debated. While their existence has been confirmed across numerous different tumor entities, including acute myeloid leukaemia [3], pancreatic cancer [4, 5], breast cancer [6], lung cancer [7], hepatocellular carcinoma [8], head and neck cancer [9], colon cancer [10, 11], prostate cancer [12], melanoma [13, 14], and glioblastoma [15], the origin of CSCs is not fully understood. This review does not aim to discuss the origin of CSCs, except to point out that whether CSCs arise from normal stem/progenitor/differentiated cells or acquire mutations that confer stem cell-like properties, CSCs should not be confused with normal stem cells becoming cancerous ("cancerous stem cells") [16]. Rather CSCs are believed to have acquired, over time, phenotypes and characteristics of normal stem cells such as unlimited self-renewal and the capacity to divide indefinitely and at the same time maintain the ability to generate multiple cell lineages, including differentiated progenies [17, 18]. Thus, CSCs are functionally defined by their self-renewal capacity, their multipotency, and their exclusive ability to initiate tumors in mice upon serial passage [1, 16].

The clinical implication of the CSC model suggests that only elimination of the CSC will result in eradication of the tumor, while failure to do so will inevitably lead to tumor relapse. This concept is supported by data demonstrating that primary tumors with a clear stem cell signature are consistently associated with poor response rates and relapse [19–22], and CSCs are more resistant to chemotherapy and

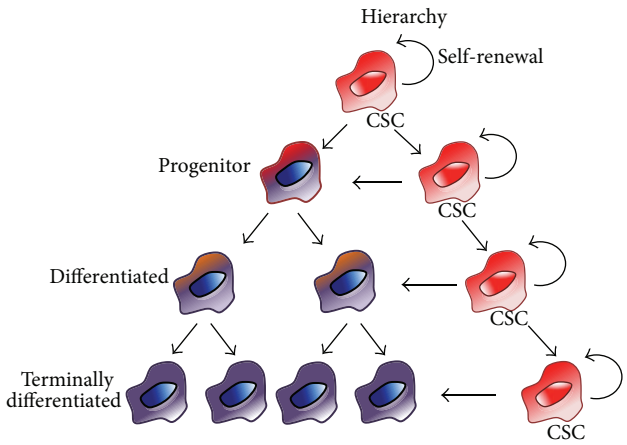

Figure 1: The CSC model. Over time, CSCs acquire phenotypes and characteristics of normal stem cells such as unlimited self-renewal and the capacity to divide indefinitely and at the same time maintain the ability to generate multiple cell lineages, including differentiated progenies. A CSC can thus divide (1) asymmetrically (differentiation) giving rise to one CSC and a specialized differentiated cell or (2) symmetrically (self-renewal) giving rise to two identical CSCs. In both cases, the capacity of self-renewal remains intact and ensures the survival of the CSC pool and supports the hierarchical model of tumor cell heterogeneity.

radiotherapy than "differentiated" tumor cells [22, 23], likely due to cellular defense mechanisms shared with normal stem cells [24–26]. Consequently, the idea of eliminating CSCs as a therapeutic strategy is already beginning to revolutionize how we foresee cancer treatment in the immediate future, with CSC-specific compounds expected to lead the battle. However, we are far from achieving this goal, as our understanding of the CSC niche and the cellular determinants that CSCs need for survival is in its infancy.

Like somatic stem cells, CSCs exist in a cellular niche that provides key signals for self-renewal and tumorigenesis [27, 28] (Figure 2). More specifically, the tumor microenvironment protects CSCs from immune surveillance, apoptosis, and chemotherapeutics and above all, the niche provides CSCs with factors that maintain, drive, and promote their "stemness." In general, developing tumors promote the creation of a unique cellular microenvironment containing extracellular matrix proteins (e.g., collagen, elastin) and a diverse collection of cells, including cancer-associated fibroblasts; stellate cells [in pancreatic cancer or hepatocellular carcinoma (HCC)]; immune cells such as myeloid-derived suppressor cells, monocytes, macrophages, and T-cells; and endothelial cells [29–31]. While each cell or environmental component has a particular function on its own, together they create a dynamic niche replete with secreted factors that synergize and cooperate to develop a complex communication network known as cross talk, with the CSC at center stage.

The importance of the tumor microenvironment in promoting cancer initiation and tumor growth has been increasingly recognized over the past decade [31–35]. In addition to providing structural support for tumor development, the tumor-associated microenvironment of many solid tumors provides cues to CSCs that regulate their self-renewal and metastatic potential as well as their resistance to conventional chemotherapeutic agents [33, 36]. For example, in human breast cancers, recruited mesenchymal stem cells (MSC) interact with breast CSCs through cytokine loops involving interleukin- (IL-) 6, CXCL7, prostaglandin E2, IL-8, or Gro-α stimulating their self-renewal capacity [37, 38]. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth through elevated SDF-1/CXCL12 secretion [37], and lung stromal fibroblast-derived periostin creates a metastatic niche for breast CSCs [39]. In pancreatic cancer, tumor-associated pancreatic stellate cells create a paracrine niche for pancreatic CSCs via Nodal/Activin secretion [33]. Likewise, hepatic stellate cells in HCC contribute to liver CSC chemoresistance by secreting hepatocyte growth factor (HGF) [40]. These studies provide further evidence that the tumor microenvironment is essential for CSC functions.

An area of great interest is the role of inflammatory cells in the CSC niche. The tumor microenvironment is characterized by chronic inflammation, which, instead of inhibiting tumor growth, favors tumor formation by stimulating cell proliferation, activating CSCs, and promoting metastasis [28, 41]. Leading the tumor inflammatory response are tumor-associated macrophages (TAMs) [42]. A correlation between high numbers of TAMs and rapid disease progression and poor patient outcome has been observed for decades [32, 43, 44]; however, only recently was this paradoxical phenotype explained. We now understand that this correlation is due to TAM-mediated paracrine signaling, in which macrophage-derived factors activate the CSC compartment and promote stemlike features of CSCs, exacerbating tumor progression, metastasis, and even CSC chemoresistance. In this review, we focus on the role of TAMs in CSC biology and pathogenesis in solid tumors. We critically discuss the contribution of TAMs on premalignancy, primary tumor CSCs, circulating CSCs, and the initiation of premetastatic niches in distant organs. We also examine the prospects of directly targeting TAMs or disrupting TAM-CSC cross talk for cancer therapy.

2. Tumor-Associated Macrophages

Macrophages, a heterogeneous population of innate myeloid cells, originate from monocytic precursors and can undergo specific differentiation/polarization in the blood or within tissues [45, 46]. In addition to monocytes, the yolk sac and fetal liver represent two additional sources for colony-stimulating factor-1 receptor- (CSF-1R-) dependent macrophages during early development [47, 48]. Macrophages are not static but rather are extremely plastic and can assume multiple phenotypes in response to constantly changing environmental cues (e.g., bacterial infection, wounds, and cancer). From a simplistic point of view, macrophages are polarized towards a classically activated or "M1" phenotype via type I helper T (Th1) cytokines [e.g., interferon- (IFN-) γ] and/or activation of Toll-like receptors upon engagement with bacterial components (e.g., lipopolysaccharides). M1 macrophages are therefore involved in Th1 responses to pathogens and microbes and are characterized by elevated proinflammatory

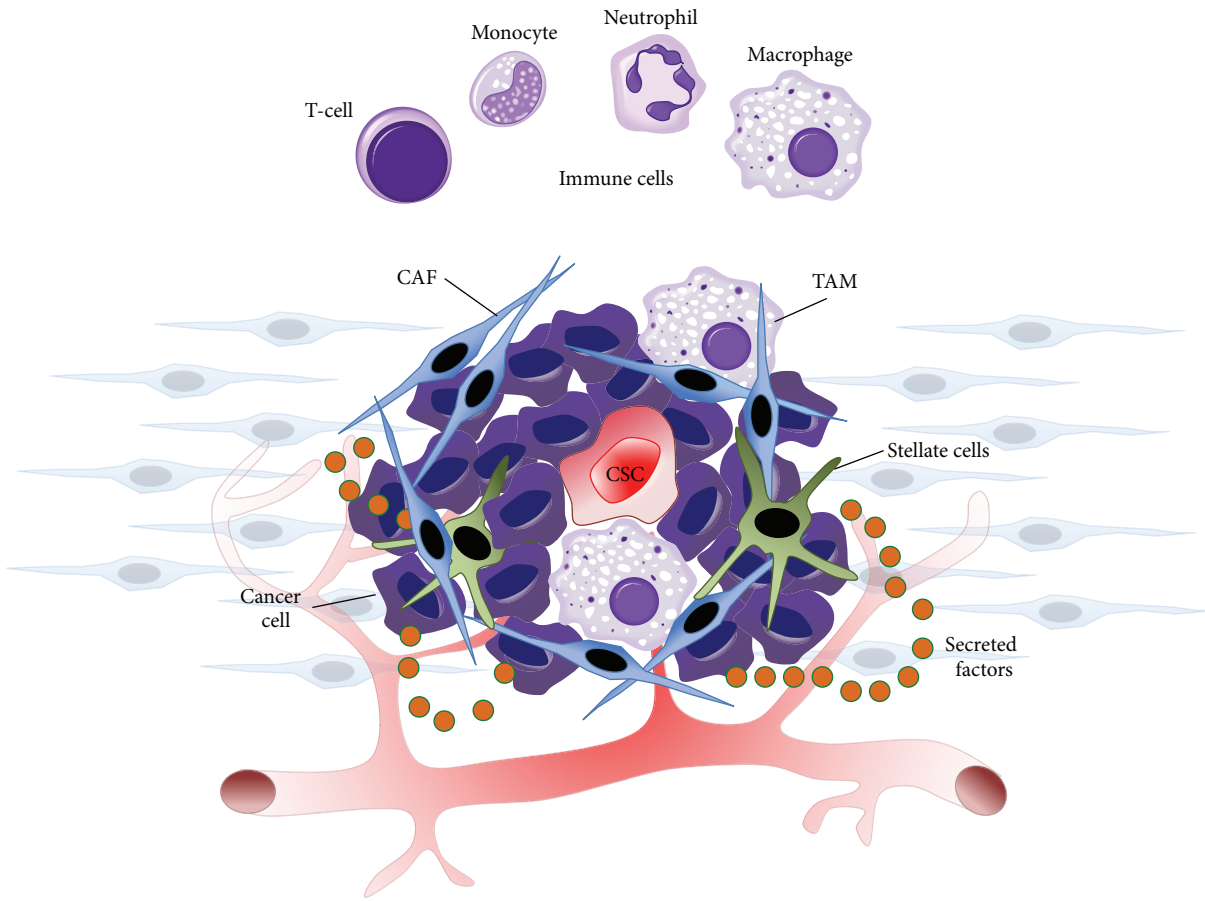

FIGURE 2: The CSC niche and tumor microenvironment. At center stage is the CSC, in contact with a complex and dynamic cellular network, including daughter cancer cells, stellate cells (in the case of HCC and PDAC), cancer-associated fibroblasts (CAFs), and immune cells, which include T-cells, monocytes, neutrophils, and tumor-associated macrophages (TAMs). Nourished by the circulatory system, these cells communicate with one another and directly with the CSC via secreted factors, forming a positive feedback loop that promotes CSC tumorigenicity and metastasis.

cytokines such as IL-12, IL-1β, IL-6, and tumor necrosis factor α (TNF-α), increased expression of major histocompatibility complex (MHC) class II, generation of reactive oxygen and nitrogen intermediates, and enhanced cell killing [49]. In response to IL-4, IL-10, and IL-13, however, macrophages can polarize towards an alternatively activated "M2" phenotype participating in Th2-type responses including humoral immunity, wound healing, and tissue remodeling [50]. They are characterized by high expression of scavenging molecules, mannose and galactose receptors, activation of the arginase pathway, production of IL-10, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), and efficient phagocytic activity [49, 50] (Figure 3).

Monocyte infiltration into a tumor is mediated by chemokines (e.g., CCL2, CCL5, and CXCL12), CSF-1, and components of the complement cascade [51, 52]. Once they are within the tumor, the tumor environment rapidly promotes their differentiation into tumor-conditioned macrophages. TAMs were initially believed to be biased away from an M1 phenotype, expressing M2 protumor markers [53]. We now understand that while they do share greater similarity with alternatively activated M2 macrophages, tumor macrophages are composed of several distinct populations that share features of both M1 and M2 macrophages. Thus, merely classifying tumor macrophages as M1 or M2 does not accurately reflect the differentiated or biological state of TAMs. Rather, the classification of TAMs should be related to the function of the macrophage subpopulation within the tumor (e.g., metastasis-promoting macrophage, angiogenic macrophage, and immunosuppressive macrophage) as has been proposed by others [44, 50, 53, 54]. For such classification purposes, researchers have relied primarily on the analysis of cell surface markers, none of which are entirely restricted to a specific subpopulation or lineage. In the murine setting, the absence of Gr1 (Ly6G) and the expression of the canonical markers CD11b, F4/80, and CSF-1R in combination with mRNA analysis of additional markers (Figure 3) are routinely used to classify macrophage subtypes [44]. In the human setting, antibodies to the glycoprotein CD68, the LPS-coreceptor CD14, CD312, CD115, HLA-DR, or FcγRIII (CD16) have been used to identify macrophages, but with mixed and oftentimes contradictory results [46]. Combinations of these markers provide higher specificity and should be used when possible to discriminate macrophages

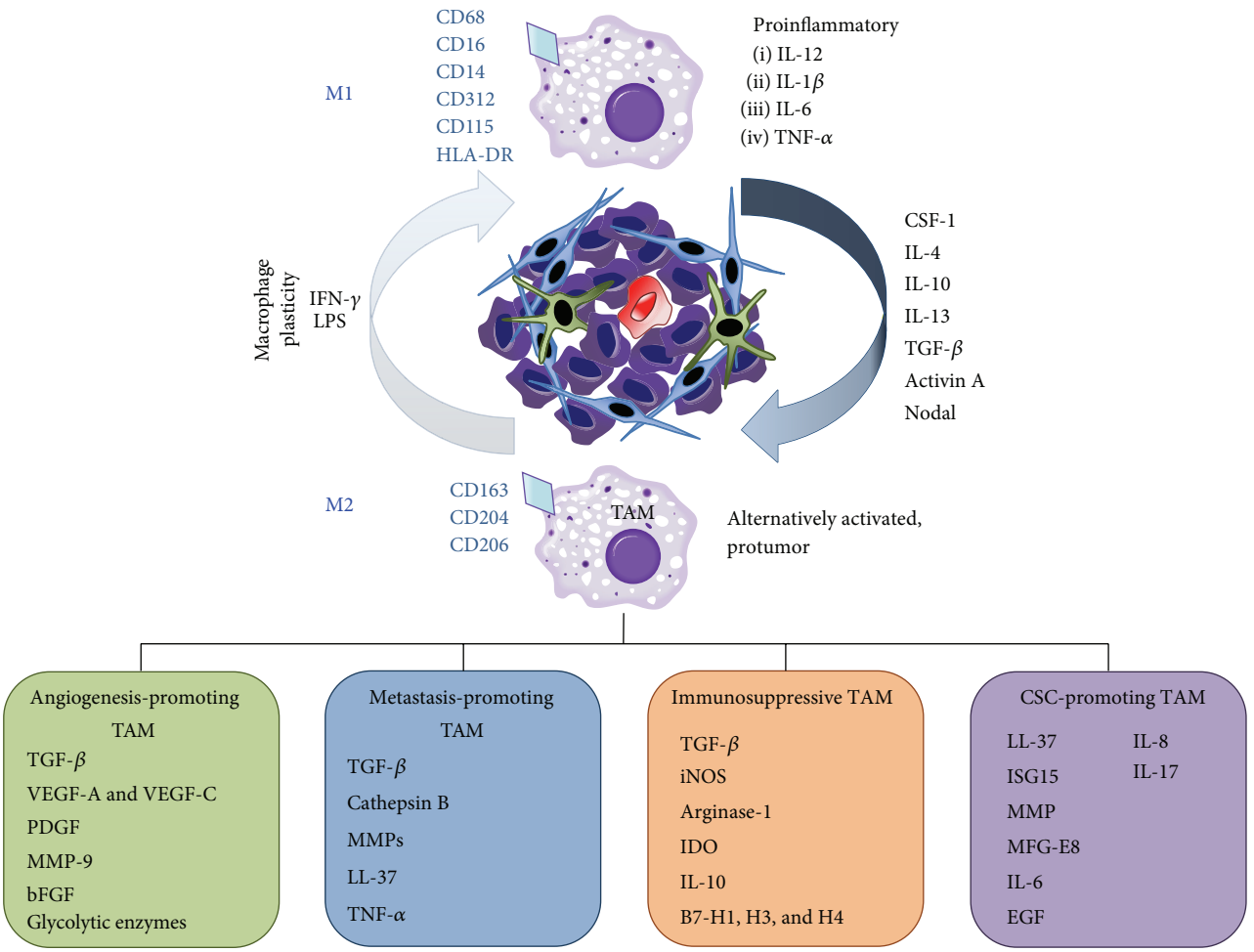

Figure 3: Macrophage plasticity and characterization. The binary M1/M2 classification of macrophages suggests that human macrophages exist as either proinflammatory M1 macrophages or protumor M2 TAMs, which can be identified based on the expression of cell surface cell membrane markers. This concept has been challenged by the identification of numerous TAM subtypes (angiogenesis-promoting TAM, metastasis-promoting TAM, immunosuppressive TAM, and CSC-promoting TAM) that exist within the primary tumor and metastatic sites. The existence of a specific TAM subtype is driven by the interaction of macrophages with factors secreted by the tumor microenvironment, leading to transcriptional rewiring of TAMs with a specific gene signature profile. TAMs are highly plastic and can shift between subtypes based on tumor-specific signals and stimuli.

from other myeloid-derived cells, such as polymorphonuclear neutrophils and eosinophils. To more specifically identify M2-like TAMs and subsets, the hemoglobin-scavenger receptor CD163 [55, 56], the macrophage scavenger receptor 1 CD204 [53, 57, 58], the mannose receptor CD206 [59], and more recently the T-cell immunoglobulin and mucin-domain containing protein-3 (Tim-3) [60] have been used with great success. Ultimately, however, there remains considerable controversy regarding how to properly classify and identify TAMs. While classifications based on TAM functions, such as the promotion of angiogenesis or immunosuppression, are now being used to better categorize TAMs (Figure 3), it is important to note that macrophages are dynamic, plastic cells capable of performing many functions simultaneously. Thus, this approach may be self-limiting and underscore the multifunctional capabilities of TAMs. Since the scientific community has yet to come to a consensus regarding what markers to use and how to refer to macrophages, the binary M1/M2 classification remains commonly used [47].

TAMs directly participate in tumor initiation, progression, and metastasis via numerous mechanisms including (1) the secretion of proteolytic molecules such as MMPs to facilitate ECM remodeling [61–64], (2) the expression of nonproteolytic proteins like chemokines [65, 66], TGF-β1 [67, 68], and hCAP/LL-37 [69, 70] to facilitate tumor cell proliferation, migration, and invasiveness, (3) the expression of angiogenic mediators such as TGF-β, VEGF-A, VEGF-C, platelet-derived growth factor (PDGF), and MMP-9 to sustain the growth of the tumor stroma and promote *de novo* tumor blood vessel formation [44, 65, 71, 72], or (4) the expression of immunosuppressive factors including TGF-β, inducible nitric oxide synthase (iNOS), arginase-1, IDO (indoleamine 2,3-dioxygenase), and IL-10 to facilitate T-cell proliferation and activity [73–75]. While the mechanisms

underlying the protumor effects of TAMs on bulk tumors have been extensively studied, there is now growing clinical and experimental evidence to support that TAMs also enhance tumor progression by directly communicating with CSCs to promote their stemness and/or subsequent oncogenic properties [76].

3. The Premalignant Niche

Normal adult stem cells occupy protective niches in various tissues where they function in tissue homeostasis and repair. The activity of stem cells in their tissue-specific niche is regulated by their own intrinsic molecular activity and the signals that they receive from neighboring differentiated cells [77, 78]. Increasing evidence, discussed below, suggests that macrophages interact with stem cells within their tissue-specific niche to modulate self-renewal and tissue remodeling in both normal and preinvasive tissues.

Alterations in tissue organization and homeostasis can precede tumor initiation, as exemplified by the increased cancer risk associated with chronic inflammation and wound healing. Moreover, epidemiological studies have shown that the administration of nonsteroidal anti-inflammatory drugs (NSAIDs) in low doses results in a significant decreased risk of developing colon, breast, esophageal, Hodgkin's lymphoma, pancreatic, and stomach cancer [79]. Thus, even before cancer begins, chronic inflammation or prolonged inflammatory episodes can set the stage for oncogenesis. The transcription factor nuclear factor-kappa B (NFκB) is at the heart of cancer-related inflammation. In inflammatory cells, the NFκB pathway results in the induction of numerous tumor-promoting chemokines and cytokines such as IL-6, TNF-α, IL-1β, CXCL8, VEGF, and CSF-1 [80]. In a mouse model of colitis-associated cancer, suppression of NFκB in myeloid cells was shown to significantly decrease the incidence and size of tumors [81]. Subsequent studies showed that activation of NFκB in macrophages leads to production of IL-6 and signal transducer and activator of transcription 3 (STAT3) signaling in neighboring cells, which promotes premalignant intestinal epithelial cell survival and CSC proliferation *in vivo* [82–84]. CCAAT/enhancer binding protein beta (C/EBPβ) transcriptionally activates IL-6 in epithelial cells and is a direct target of IL-6 in macrophages. Interestingly, C/EBPβ was shown to regulate stem cell self-renewal and maintenance in the normal mouse mammary gland [85], and C/EBPβ, IL-6, and STAT3 are all overexpressed in preinvasive mammary hyperplasia as compared to normal mammary gland (H. Machado, unpublished data).

Interestingly, the effect of NFκB activation on tumor initiation seems to be cell type-specific. In a diethylnitrosamine-(DEN-) induced model of HCC, mice with IκB kinase beta-(IKKβ-) deficient hepatocytes alone showed a significant increase in tumor number and size, which were characterized by increased reactive oxygen species (ROS), JNK signaling, and hepatocyte death. This cell death stimulated myeloid cells to produce mitogens such as IL-6, TNF-α, and HGF, which stimulated proliferation of the surviving hepatocytes. This effect was mitigated either when an antioxidant was administered to these mice or by conditional deletion of IKKβ in hepatocytes and Kupffer cells [86]. While the role of CSCs in this model is unknown, studies using the normal mammary epithelial cell line, MCF10A, showed that activation of NFκB leads to Lin28-mediated repression of Let7, resulting in a biphasic increase in IL-6 and ultimately self-renewal of CSCs [87]. NFκB activation in infiltrating macrophages has also been tightly linked to pancreatitis and the development of pancreatic intraepithelial neoplasia (PanIN). During pancreatitis, acinar cells can undergo a transdifferentiation process known as acinar-to-ductal metaplasia (ADM) where their phenotype changes to a duct-like progenitor cell [88]. This process is driven by NFκB-stimulated macrophage secretion of TNF-α, CCL5, and MMP-9 [89]. Once these duct-like progenitors are formed they can progress to PanINs if an oncogenic mutation is acquired, such as in KRAS [90]. Interestingly, a recent study showed that oncogenic KRAS signaling induces intracellular adhesion molecule-1 (ICAM-1) expression and the attraction of M1 polarized macrophages. Once recruited, these M1 macrophages promote ADM by secreting TNF-α and MMP-9 [91]. While M1 macrophages are generally believed to be "antitumor," they may also contribute to oncogenic mutations by releasing reactive nitrogen and oxygen intermediates in premalignancy.

During inflammation, macrophages and other infiltrating leukocytes generate high levels of ROS and nitric oxide intermediates that generate DNA damage and genetic instability in epithelial cells. In addition, inflammatory cytokines and ROS deregulate DNA repair enzymes and p53 transcriptional activity leading to microsatellite and chromosome instability [83]. In mouse models with high levels of ROS, hematopoietic stem cells and oligodendrocyte/type 2 astrocyte progenitor cells have dramatically reduced self-renewal capacity due to the expression of senescence related proteins $p16^{INK4a}$ and $p19^{Arf}$ [92]. In tumors, CSCs upregulate cellular antioxidants to quench ROS [93, 94]. While the effect of ROS on CSCs in the preinvasive niche is not known, ROS scavenger proteins in CSCs may help select for their survival in premalignant lesions.

4. Primary Tumors

While TAMs in the preinvasive niche contribute to oncogenic transformation and survival, a growing body of evidence suggests that they are critical for the self-renewal and maintenance of CSCs in established tumors. STAT3 and NFκB are key regulators of these processes. Once infiltrated into tumors, TAMs contribute to chronic inflammation by secreting inflammatory cytokines, such as IL-1β, IL-6, and IL-8 (CXCL8) [66, 95–97]. In breast cancer xenografts, IL-6 activates STAT3 by binding to its receptor (gp130) and directly stimulates breast CSC self-renewal [87]. Similarly, binding of IL-8 to the receptor CXCR1 promotes breast CSC expansion and prevents apoptosis [98]. Both of these cytokines are activated by the NFκB pathway and, in a positive feedback loop mechanism, maintain and activate NFκB [99]. In HCC, TAMs promote the expansion of $CD44^+$ stemlike HCC cells in an *in vitro* coculture system. Furthermore, TAM-derived IL-6 induced $CD44^+$ stemlike cell expansion by activating

STAT3, and blocking IL-6 with tocilizumab ablated $CD44^+$ sphere formation *in vitro* and tumor growth in patient-derived HCC xenografts [100]. Mitchem et al. showed that ablation of CCR2 or CSF-1R signaling significantly blocked TAM infiltration into pancreatic ductal adenocarcinoma (PDAC), decreased the number of $CD44^+ALDH1^+$ CSCs, and improved response to chemotherapy. Infiltrating TAMs also enhanced tumor-initiating properties of $CD44^+ALDH1^+$ pancreatic CSCs by activating STAT3 signaling [101].

IL-17 is another proinflammatory cytokine produced by macrophages and T-cells and has been shown to contribute to cancer-associated inflammation in numerous cancers [102–105]. Xiang et al. demonstrated that IL-17 promotes the self-renewal of ovarian $CD133^+$ cancer stemlike cells through a mechanism involving NFκB and p38 MAPK [106]. Using several different ER^+ breast cancer cell lines, Ward et al. showed that coculture of M2 macrophages, but not M1 macrophages, increased tumor sphere formation *in vitro*, although the mechanism by which these macrophages promoted CSC expansion was not tested. Treatment of CSC spheres with zoledronate, a bisphosphonate currently used to treat osteoporosis and bone metastasis, reduced M2 macrophage-mediated sphere formation and migration [107].

The Sox family of transcription factors has also been shown to regulate CSCs in breast cancer. It is well known that a positive feedback loop exists between TAMs and tumor cells, involving epidermal growth factor (EGF) and CSF-1 [108]. Tumor cells secrete CSF-1 that promotes TAM production of EGF, and TAM-derived EGF stimulates tumor cell CSF-1 secretion. In mouse mammary tumor models, TAMs upregulate Sox 2 expression, which increases numerous stem cell genes including Sox-2, Oct-4, Nanog, and Sca-1. Inhibition of the EGF receptor (EGFR1) or STAT3 activation reduced Sox2 expression and CSC-associated phenotypes, suggesting a unique paracrine signaling pathway between TAMs and CSCs [109]. Overexpression of Sox-2 was also shown to increase breast CSC self-renewal by increasing tumor sphere-forming ability *in vitro* [110]. Sox-4, another pluripotency-associated gene, induced Ezh2 expression [111], which promoted breast CSC expansion by activating Raf-1 and β-catenin [112].

In addition to mediating CSC self-renewal and expansion, TAMs have been shown to be responsible for the maintenance of the CSC niche. A recent study by Lu and colleagues demonstrated juxtacrine signaling by TAMs and tumor-associated monocytes with mouse mammary CSCs to support the maintenance of a stemlike state [113]. EphH4 binding to its receptor on tumor cells resulted in the activation of Src and NFκB, the latter of which caused the secretion of numerous cytokines that function in CSC maintenance. The IL-6/STAT3 pathway was also shown to increase tumor-initiating activities in murine colon and lung cancer cell lines by milk fat globulin epidermal growth factor-8 (MFGE-8). TAMs produced large amounts of both MFGE-8 and IL-6, which coordinately induced tumor potential and CSC chemoresistance through STAT3 and Hedgehog signaling, the latter of which regulates normal stem cell self-renewal. Interestingly, the MFGE-8 receptor, α_v-integrin, was expressed in much higher levels on CSCs as compared to non-CSCs, further supporting a role for MFGE-8 in CSC maintenance [114].

While numerous studies have demonstrated that TAMs directly regulate CSC self-renewal and maintenance, there is a growing body of research that suggests that, in turn, CSCs recruit macrophages to solid tumors and enhance a protumor phenotype in TAMs. Zhou et al. recently showed that the extracellular matrix protein periostin is preferentially expressed on $CD133^+CD15^+$ glioma stem cells and recruits macrophages through integrin $\alpha_v\beta_3$ from the peripheral blood to the brain. Deletion of periostin in glioma stem cells resulted in decreased M2 TAM density, reduced tumor growth, and consequently increased survival in glioblastoma xenografts [115]. In pancreatic cancer, primary human PDAC CSCs (spheres) produce IFNβ, which then induces the secretion of IFN-stimulated gene 15 (ISG15) in recruited TAMs. Consequently, TAM-derived ISG15 induced CSC self-renewal and tumor-initiating properties [116]. More recently, Sainz Jr. et al. demonstrated that PDAC CSCs secrete the TGF-β superfamily members Nodal/Activin A and TGF-β1, which then induce an M2 macrophage phenotype. Coordinately, polarized TAMs secrete the antimicrobial peptide hCAP-18/LL-37, which consequently binds to its receptors (formyl peptide receptor 2 (FPR2) and P2X purinoceptor 7 receptor (P2X7R)) to enhance CSC self-renewal, invasion, and tumor-initiating properties [70]. Of note, pancreatic CSCs also overexpressed two LL-37 receptors, further indicating a role for LL-37 in pancreatic CSC maintenance. In a different study, it was shown that PDAC CSCs induce an immunosuppressive phenotype in TAMs through STAT3, ultimately leading to chemoresistance [101]. Notably, the MFGE-8 receptor, which was shown to be preferentially expressed on CSCs in colon and lung cancer cell lines, can induce M2 polarization of macrophages *in vitro* though STAT3 signaling [117]. In summary, there exists a complex relationship between CSCs and TAMs in established tumors. It appears that macrophages are not just accidental passersby that happen to secrete CSC-promoting factors, but rather, CSCs attract, reeducate, and put macrophages into their service to support primary tumor growth. While researchers are just beginning to unravel the intricacies of these processes, there is no doubt that CSC-TAM cross talk represents an important component of CSC-mediated oncogenesis.

5. Circulating Cancer Stem Cells

Distant metastases have become the leading cause of death in patients diagnosed with cancer. Metastatic spread begins with cancer cells [known as circulating tumor cells (CTCs)] detaching from the primary tumor and entering into circulation, via either blood vessels or lymphatic channels in order to colonize distant sites. These cells must acquire the ability to overcome the challenges of the hostile extratumoral conditions and adapt to different tissue environments in secondary distant organs, such as the lungs, bone marrow, or liver. It is now commonly accepted that TAMs facilitate almost every step of the metastatic cascade, from initial migration to intravasation, dissemination, extravasation, and establishment of metastasis at secondary sites [44, 51]. One

of the first definitive studies to highlight the role of TAMs in tumor metastasis was shown by Lin and colleagues in 2001. They demonstrated that CTC levels and lung metastases were significantly decreased in CSF-1-deficient mice as compared to wild type mice, supporting a role for tumor infiltrating macrophages in metastasis [118]. Additional studies targeting macrophages with clodronate liposomes, for example, have shown that elimination of macrophages significantly impacts CTC numbers and tumor metastasis [119, 120].

Once free from the tumor, CTCs can disseminate to distant organs to produce secondary metastatic lesions. Interestingly, only a minority of CTCs exhibit the capacity to successfully disseminate and proliferate in different organs, suggesting an internal hierarchy within CTCs. In fact, the existence of a small subset of CTCs with CSC properties has been shown for metastatic breast cancer [121], prostate cancer [122], small cell lung cancer [123], and PDAC [5], supporting the idea of a CSC compartment within CTCs that are distinct from CSCs of the primary tumor, enabling their escape to distant organs and subsequent growth. If this hierarchy within CTCs holds true, then TAMs likely facilitate the emergence of circulating CSCs and their intravasation and subsequent dissemination. The question remains, how do TAMs facilitate these processes in CSCs? While more studies are needed, a number of experimental systems are beginning to provide evidence that TAMs can promote an epithelial-to-mesenchymal transition (EMT) phenotype in CSCs via paracrine-secreted factors. Loss of epithelial differentiation, the acquisition of a migratory phenotype, and loss of cell adhesion are hallmarks of EMT. This process is regulated by numerous genetic modifications and a panel of well characterized transcription factors, such as SNAIL, TWIST, ZEB1, ZEB2, SLUG, BMI-1, and LOXL2 [35, 124, 125]. While numerous studies have shown that TAMs can promote an EMT phenotype in non-CSCs [68, 126–131], TAM-mediated EMT induction in CSCs was largely unappreciated until recently. In the context of pancreatic cancer, two recent studies showed that CSCs isolated from patient-derived PDAC xenografts and treated with conditioned media from M2-polarized monocyte-derived macrophages increased migration and expression of EMT genes [70, 116]. The authors identified the human cathelicidin antimicrobial peptide LL-37 and ISG15 as independent TAM-secreted mediators of these phenotypes in pancreatic CSCs. Similar TAM-mediated EMT induction has been observed in CSCs of HCC [126] and ovarian cancer [69]. STAT3 activation of target genes such as TGF-β1 and hypoxia inducible factor- (HIF-) 1α has been linked to EMT reprograming [132] and several recent studies have shown that TAM-secreted IL-6, EGF, or MFGE-8 can activate STAT3 signaling in CSCs of breast cancer [109, 133], HCC [100], or colon cancer [134]. Thus, apart from activating these pathways in CSCs to promote tumor growth as discussed above, TAM-mediated STAT3 activation may also be necessary for EMT reprogramming in CSCs. While the aforementioned studies highlight that EMT and "stemness" may go hand in hand, the implications reach beyond merely the induction of a migratory and invasive phenotype. For example, EMT transactivators have been associated with the maintenance of stem cell properties and cell survival [135], and more recently EMT induction has been shown to produce *de novo* breast CSCs [135] and to facilitate CSC maintenance in pancreatic cancer [136]. Thus, while the TAM-mediated induction of EMT in CSCs is likely necessary for the generation of migratory CSCs with invasive capacities, the implications of an EMT transcriptional signature in CSCs may be more dynamic than previously thought.

In addition to paracrine-mediated signaling, juxtacrine signaling from macrophages represents an alternate means by which TAMs can communicate with CSCs. Intravital imaging revealed that tumor cells and macrophages interact in a contact-dependent manner and comigrate *in vivo*, tumor cell migration is dependent on juxtacrine signaling, and the efficient long-distance comigration and eventual intravasation of these cells are coordinated by an EGF-CSF-1 paracrine loop [reviewed in [137]]. Along these lines, Lu et al. recently showed that TAMs physically interact with mouse breast CSCs via CD11b binding to the CSC marker CD90, leading to ephrin ligand binding to EphA4, the activation of Src and NFκB, and the subsequent secretion of various cytokines that, in turn, function to maintain the stemlike state of CSCs [113]. Taken together, these cell-cell contact-dependent interactions provide evidence of a physical CSC niche supported by TAMs; however, it is also plausible that, apart from merely interacting, CSCs and TAMs may actually fuse with one another to create a macrophage-tumor circulating cell with recombination/reprogramming of genetic material [138], analogous to that observed in stem cell fusions studies [139]. This concept, loosely known as epithelial-myeloid transition [140], was first proposed by the German pathologist Otto Aichel in 1911 to explain how a cancer cell could efficiently travel through the circulatory and lymphatic systems, while maintaining their cancer cell growth properties. Since then, the concept has slowly gained momentum [141, 142]. However, with the recent discoveries of CTCs expressing both cancer and leukocyte cell markers [143–145], the idea of "mobile hybrids" resulting from fusion events between TAMs and tumor cells is evolving as a more tangible explanation behind metastasis. Regardless of how TAMs promote CSC invasion, as stated by Qian and Pollard, macrophages "are the key that unlock the gate to allow tumor cells to escape" [44].

6. Premetastatic Niche

While many tumor cells have a predilection for metastasis, only a small percentage of CTCs (less than 0.2%) have the capacity to survive in circulation, find a suitable secondary site to support their colonization, and proliferate in their new environment [146]. In fact, apoptosis of tumor cells entering target organs represents a common early event during metastasis [147, 148], severely limiting the colonization efficiency of CTCs. Thus, while successful intravasation initiates the metastatic process, efficient survival and proliferation determine the outcome. The "seed" and "soil" theory put forth by Paget in 1889 suggested that the secondary organs themselves provide the appropriate conditions (i.e., "soil") necessary for metastatic colonization by CTCs. Our current take on Paget's theory now combines "organ selectivity" with "cell fitness,"

meaning that CTCs must also be genetically (i.e., accumulate specific mutations) or epigenetically programmed for metastasis. CSCs inherently possess the necessary "fitness" and programs for dissemination, and at the same time they bear the functional plasticity needed for transitioning between mesenchymal-like and epithelial-like states [149], the latter being necessary for CSCs to seed and resume growth at the metastatic site. In 2006, Balic et al. first linked metastasis to CSCs by demonstrating that disseminated breast cancer cells in bone marrow possessed stem cell phenotypes [150]. One year later, Hermann et al. showed that tumor metastasis in PDAC is driven by a distinct subpopulation of $CD133^+$ $CXCR4^+$ CSCs in the invasive front [5]. Today, CTCs have been shown to coexpress EMT and multiple stem markers, suggesting that CSCs are present within the CTC population [151].

In light of ever growing data supporting a role for CSCs as the "seed," CSCs are also susceptible to the harsh conditions faced during dissemination and not all cells bearing CSC markers are metastatic. Thus the "soil" counterpart of Paget's theory must also be important for CSC-mediated metastasis. Indeed, it has become evident that the formation of CSC-promoting premetastatic niches in secondary organs is not only essential but also necessary for successful CSC colonization, and current evidence suggests that resident or infiltrating immune cells, specifically macrophages, at distant sites drive the creation of premetastatic niches to facilitate successful establishment of secondary lesions. One of the earliest studies to support this hypothesis showed that not only do macrophages facilitate the growth of extravasated tumor cells, but also their elimination after initial cancer cell dissemination had been established led to a significant decrease in lung metastasis. Thus, the presence of macrophages in secondary organs is necessary for successful CTC extravasation, establishment, and growth [152].

Whether TAMs are present before the arrival of circulating CSCs or whether they are recruited following CSC extravasation remains unclear. In mouse lung or melanoma subcutaneous tumors, $CD11b^+$ myeloid cells accumulate in the lungs prior to the detection of metastatic tumor cells [153]. In studies using a genetically engineered mouse model of PDAC, infiltration of $F4/80^+CD11b^+$ macrophages in the livers of mice was observed months before tumor development and metastatic growth (M. Vallespinós and B. Sainz Jr., unpublished data). There is increasing evidence that more differentiated myeloid cells also play an important role in the development of the premetastatic niche. Specifically, van Deventer et al. observed that the recruitment of $CD11b^+Ly6C^+$ monocytes to the premetastatic lung enhances B16 cell metastasis [154], and Gil-Bernabé et al. demonstrated that $CD11b^+CD68^+F4/80^+$ recruited macrophages establish the premetastatic niche that facilitates successful breast cancer metastasis to the lungs [155]. It remains to be determined if the sum of these findings holds up in the human setting. Until then, it is interesting to speculate that primary tumor-derived secreted factors, such as soluble proteins or exosomes [156], precondition the premetastatic sites in different organs by preloading them with recruited myeloid progenitor cells. Once recruited to these sites, they can rapidly differentiate into metastasis-associated macrophages (MAMs) following the arrival of circulating CSCs, thus facilitating CSCs extravasation, survival, and subsequent proliferation via paracrine-mediated mechanism [157]. It is also important to note that, like TAMs in the primary tumor, MAMs may also facilitate CSC survival from immune cell destruction via the immunosuppressive mechanisms discussed above. Thus, the contribution of macrophages in the premetastatic and their influence in the development of metastatic lesions may be more important than their role in the primary tumor.

7. Therapeutic Strategies

Cancer has been treated with radiation therapy, chemotherapeutic drugs, and hormonal therapy for decades; however, these treatments are not tumor cell-specific and can result in severe toxicity. Tumor cells have acquired the ability to circumvent the effects of conventional therapies, leading to resistance to anticancer therapies. While there has been a recent explosion in the field of developing targeted molecular therapies that specifically block tumor cell growth and progression, a subset of cells can evade the effects of these drugs, leading to drug-resistance and/or tumor relapse. The question remains as to whether we are targeting the right population of cells.

Numerous antimacrophage strategies, including trabectedin [158], RG7155 (anti-CSF-1R) [159], and an anti-MIF (macrophage migration inhibitory factor) antibody [160], have been developed and are currently being tested in preclinical and Phase I clinical trials. However, the CSC model suggests that effective therapeutic strategies must target CSCs to not only eliminate tumor progression, but also prevent tumor recurrence after therapy. As the tumor microenvironment provides CSCs with protection from conventional therapies by promoting their "stemness" and CSCs enhance protumor properties of TAMs, disrupting CSC-TAM cross talk, or using a combined strategy to target both CSCs and TAMs, represents an exciting and promising approach for cancer therapy. A recent study demonstrated that cancer stemlike cells from chemoresistant tumors release proinflammatory cytokines that contribute to a protumor microenvironment by generating M2-like myeloid cells [161]. Mitchem and colleagues showed that targeting TAMs in PDAC reduced both CSC properties and chemoresistance [101]. These results suggest that targeting the CSC-TAM interaction is crucial for not only preventing tumor progression, but also circumventing chemoresistance.

One of the most promising antibody-mediated therapeutic strategies to date is based on inhibiting the interaction between SIRPα and CD47, a transmembrane protein expressed on many cancer cells and CSCs [162, 163], to allow for increased phagocytosis of cancer cells. Interaction of CD47 ("don't eat me" signal) with SIRPα results in the inhibition of phagocytosis by macrophages (including TAMs) through a signaling cascade mediated by phosphorylation of the immunoreceptor tyrosine-based inhibitory motif present on the cytoplasmic tail of SIRPα [164]. Numerous studies over the past few years, predominantly led by Weissman and colleagues, showed that blocking CD47 using anti-CD47

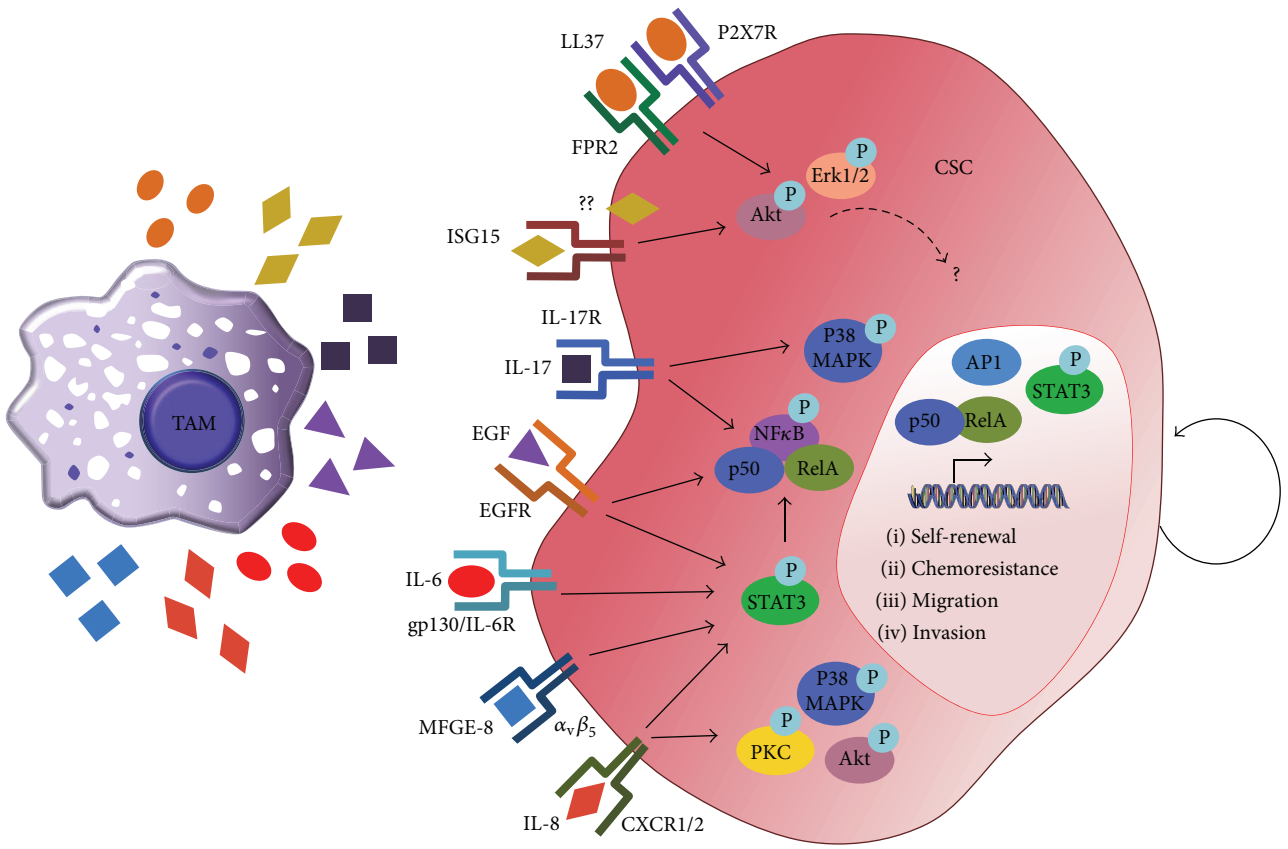

Figure 4: TAM-secreted factors regulate CSC phenotypes. TAMs have been shown to secrete LL-37, ISG15, IL-17, EGF, IL-6, MFGE-8, and IL-8 (among others), which in turn activate MAPK, STAT3/NFκB, and other yet-to-be-defined signaling pathways, leading to the activation of CSC properties, such as self-renewal, chemoresistance, migration, and invasion.

monoclonal antibodies allows for increased phagocytosis of cancer cells *in vitro* and decreased tumor burden *in vivo* [162, 163, 165, 166]. Recent work by Cioffi et al. has extended these findings to show that anti-CD47 therapy can essentially turn the tide on the relationship between CSCs and TAMs, facilitating effective phagocytosis of pancreatic CSCs, which can be further augmented with standard chemotherapeutic agents like gemcitabine or Abraxane [162]. These findings suggest that CD47 inhibition in the adjuvant setting may be an effective means for treating PDAC and potentially other cancers; however future preclinical and clinical studies will need to be performed. As we gain a better understanding of the relationship between TAM and CSCs at each stage of tumor development and progression, we will undoubtedly discover new means to interfere with the TAM-CSC cross talk.

8. Concluding Remarks

In this review, we discussed several TAM-derived factors that promote stemness and are thus potential therapeutic targets (summarized in Figure 4). The studies of the past decade have led to significant advances of our understanding of the molecular pathways regulating TAMs and CSCs; however, we are only beginning to put together the pieces that constitute the complex TAM-CSC cross talk that occurs within the host. Increasing evidence suggests that a stemlike niche composed of numerous cell types, including macrophages, is important for promoting CSC self-renewal and maintenance, and likewise, CSC-derived factors induce protumor signals in TAMs. Our current knowledge of CSCs heavily relies on tumor transplantation assays in both syngeneic and xenograft models, the latter of which does not recapitulate the complex microenvironment in which spontaneous tumor initiation occurs, nor can xenograft models accurately mimic human CSC and human TAM interactions. While many immune-compromised mice express macrophages, the macrophage response is typically elevated in these mice and it is uncertain as to whether murine macrophages communicate with human CSCs in the same way as their human counterparts. Thus, until we develop mouse models with humanized immune systems that can support the growth of human primary tumors, we will continue to rely on excellent *in vitro* systems and syngeneic mouse models to better facilitate our understanding of the relationship between TAMs and CSCs and the eventual development of novel compounds to inhibit this unconventional dependence.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Tumor-Associated Macrophages and Neutrophils in Tumor Microenvironment

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Distinct tumor microenvironment forms in each progression step of cancer and has diverse capacities to induce both adverse and beneficial consequences for tumorigenesis. It is now known that immune cells can be activated to favor tumor growth and progression, most probably influenced by the tumor microenvironment. Tumor-associated macrophages and tumor-associated neutrophils can exert protumoral functions, enhancing tumor cell invasion and metastasis, angiogenesis, and extracellular matrix remodeling, while inhibiting the antitumoral immune surveillance. Considering that neutrophils in inflammatory environments recruit macrophages and that recruited macrophages affect neutrophil functions, there may be various degrees of interaction between tumor-associated macrophages and tumor-associated neutrophils. Platelets also play an important role in the recruitment and regulation of monocytic and granulocytic cells in the tumor tissues, suggesting that platelet function may be essential for generation of tumor-associated macrophages and tumor-associated neutrophils. In this review, we will explore the biology of tumor-associated macrophages and tumor-associated neutrophils and their possible interactions in the tumor microenvironment. Special attention will be given to the recruitment and activation of these tumor-associated cells and to the roles they play in maintenance of the tumor microenvironment and progression of tumors.

1. Introduction

Cancer-related nonresolving inflammation in the tumor microenvironment (TME) is a hallmark of cancer, and cancer cells are confronted with various types of stromal and immune cells across all stages of the disease, from early carcinogenesis to tumor progression and metastasis [1, 2]. The progression of cancer has traditionally been regarded as a multistep process with genetic and epigenetic changes targeting only cancer cells. However, studies over the past two decades have revealed that the TME is an equally important determinant of tumor behavior. The components of the TME include local stromal cells, such as resident fibroblasts and macrophages, and distant recruited cells such as endothelial cells, immune cells including myeloid and lymphoid cells, bone marrow-derived precursor cells, and circulating platelets. To note, tumor-associated myeloid cells (TAMCs) comprise five distinct myeloid populations: tumor-associated macrophages (TAMs), monocytes expressing the angiopoietin-2 receptor Tie2 (Tie2-expressing monocytes or TEMs), myeloid-derived suppressor cells (MDSCs), tumor-associated neutrophils (TANs), and tumor-associated dendritic cells (Figure 1) [3]. Of these, TAMCs result in TAMs and TANs to be discussed in this review.

2. General Characteristics of TAMs

Macrophages are the most well-characterized type of tumor-infiltrating immune cell, and it is not surprising that they play a prominent active role from early carcinogenesis to tumor progression including metastasis [4]. While macrophages involved in cancer-initiating conditions are immune activated (e.g., antitumoral), once tumors are established, the macrophages are educated to become protumoral [5]. Currently, the majority of evidence supports a tumor-promoting role of a specific subpopulation of macrophages, TAMs within the primary TME. Surprisingly, macrophages can constitute up to 50% of a tumor mass, forming a major

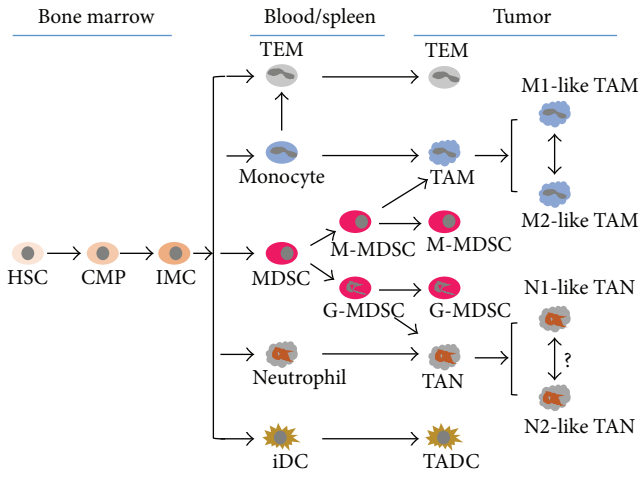

Figure 1: Differentiation of tumor-associated myeloid cells begins from hematopoietic stem cells (HSC) in the bone marrow. CMP: common myeloid progenitors, IMC: immature myeloid cells, TEM: Tie2-expressing monocyte, MDSC: myeloid-derived suppressor cell, M-MDSC: myeloid MDSC, G-MDSC: granulocytic MDSC, iDC: immature dendritic cells, TADC: tumor-associated dendritic cells, TAM: tumor-associated macrophage, and TAN: tumor-associated neutrophil [63].

component of immune cell infiltrate in the TME [4, 6, 7]. This was long considered to be an indication of antitumor immunity, considering the inherent phagocytic and cytotoxic properties of macrophages. However, high frequencies of TAMs are generally associated with poor prognosis in most human cancers [8, 9], and this is in stark contrast with the traditional notion that macrophages play host-protecting roles in inflammatory microenvironments. When exposed to signals from the TME, macrophages show a surprising degree of plasticity in functional reprogramming and adopt either pro- or anti-inflammatory phenotypes in response to environmental stimuli [10]. Importantly, another tumor-promoting structure—the TME for metastasis, consisting of macrophages, endothelial cells, and tumor cells—is recognizable in metastatic sites and has been shown to be predictive of metastatic potential in human breast cancers [11]. This observation is explained by the role of TAMs in cancer cell survival through immunosuppression, invasion, metastasis, and angiogenesis. In the transition from benign to malignant invasive cancer, the TME is flooded with cytokines and growth factors. TAMs display delayed and defective NF-κB activation in response to signals such as LPS and TNF-α and this enables TAMs to sustain "smouldering inflammation" in the TME, which is responsible for the protumor phenotypes [12].

Available information suggests that TAMs infiltrating established tumors acquire the properties of M2-like phagocytic population and phenotypes such as promotion of tumor growth and angiogenesis, remodeling of tissues, and suppression of antitumor immunity [12]. Analogously to the T helper (Th1) and Th2 dichotomy, macrophages have been classified into specific M1-like (activated) or M2-like (alternatively activated) functional status based on functional polarization by the microenvironment [13, 14]. It has been widely accepted that IFN-γ alone or with microbial LPS or cytokines such as TNF and GM-CSF induces classically activated M1 macrophages and immune complexes, IL-4, IL-6, IL-10, IL-13, IL-21, IL-33, and Notch can elicit the M2 form of macrophage activation [15, 16]. However, M1- and M2-polarized macrophages are extremes in a continuum in a wide range of functional states and truly polarized macrophages are rare [17, 18]. Instead, TAM can be described as M(IL-4), M(Ig), M(IL-10), M(GC: glucocorticoid), M(IFN-γ), M(LPS), and so forth, according to recently attempted nomenclature linked to the activation standard [19]. In turn, TAMs contribute to high IL-10 and TGF-β levels in the TME [20] and they express inflammatory cytokines (e.g., IL-1β, IL-6, IL-12, and TNF-α), albeit at low levels [21]. In response to stimuli from TEMs, TAMs can promote tumor growth through the production of activation factors for stromal and cancer cells (EGF, bFGF, VEGF, PDGF, and TGF-β) [22–25]. These findings indicate mutual interactions between TAMs and the TME for tumor progression.

Recently emerging efforts to establish a common language for describing the properties of the macrophages under investigation prefer the term "activation" rather than "polarization" for the classification of functional status of TAMs [19]. Because TAMs are not truly polarized population of macrophages, we will use the term "activation" instead of "polarization" in this review to avoid further confusions.

As macrophages in human cancer can neither be uniformly classified into classically activated M1-like or alternatively activated M2-like macrophages, they are collectively termed TAMs and the former view of TAMs as a skewed M2-like single macrophage population is an oversimplification [26]. Rather, M1- and M2-polarized macrophages are two extremes in a continuum in a wide range of functional states [17, 18, 27] and recent study with highly standardized stimulation of human macrophages showed that current M1 versus M2 polarization model can be extended to a "spectrum model" with at least nine distinct macrophage activation programs [27]. It has become clear that dynamic alterations in the phenotypes of macrophages occur during tumor initiation, progression, and metastasis and that subpopulations of TAMs are responsible for distinct tumor-promoting activities [5, 28, 29]. Notably, tumors have a diverse spectrum of disorders and the distribution and function of TAMs differ considerably in different microregions of the neoplastic tissue; recent large-scale transcriptome analyses revealed that macrophages have a mixed phenotype expressing both M1-like and M2-like markers [5, 13]. Different signals from particular locations in the TME seem to influence activation of TAMs and overall tumor prognosis [30]. For example, within cancerous tissue, TAMs can be microanatomically diverse, including the accumulation of cells with protumor properties in hypoxic areas [31] and differences in inflammatory components and pathways between tumors originating in distinct anatomical sites [31, 32]. TAMs have proangiogenic activity, and macrophage infiltration in tumors is generally associated with high vascular density [33]. M2-like TAMs, highly localized in hypoxic tumor areas, have displayed superior proangiogenic activity *in vivo*, and the numbers increased as the tumors progressed [31]. TAMs express various molecules modulating

angiogenesis, such as VEGF, bFGF, TNF-α, IL-1β, CXCL8, cyclooxygenase 2, plasminogen activator (uPA), PDGF-β, MMP7, MMP9, and MMP12 [34]. Of note, the composition of the immune microenvironment and the overall activation state of TAMs become more favorable for tumor growth during tumor progression, and the functional roles of macrophages during tumor initiation become changed during tumor progression.

Reversion of M2-like macrophages to M1-like cells and reduction of immunosuppressive effects from the M2 population have been reported when TAMs recovered an M1 phenotype following IFN-γ treatment [35, 36]. These results indicate that activation of TAMs can be reversible and suggest new possible therapeutic strategies targeting reeducation of TAMs. The identification of genetic and epigenetic mechanisms [37–39] underlying macrophage diversity in tissues and their different forms of activation may pave the way to reeducation strategies.

3. Origin and Recruitment of TAMs in Tumor Sites

It is now known that chemokines (e.g., CCL2: monocyte chemotactic protein 1), cytokines (e.g., colony-stimulating factor-1 (CSF-1)), and products of the complement cascade are major determinants of macrophage recruitment and positioning in tumors (Figure 2) [40–43]. Simply stated, peripheral blood monocytes are recruited locally and differentiate into macrophages in response to a wide spectrum of chemokines and growth factors produced by stromal and tumor cells in the TME [41]. Do TAMs differentiate only from monocytes recruited from peripheral blood? Lung alveolar and peritoneal macrophages, Kupffer cells, epidermal Langerhans cells, and brain microglia are derived from primitive yolk sac precursors and can be self-maintained locally. These are referred to as tissue-resident macrophages and the evidence that local proliferation of macrophages can contribute to the TAM pool was suggested from a Her2/Neu driven mammary carcinoma animal study [44, 45]. Though we have evidence that both tissue-resident and recruited macrophages may coexist in tumors, that TAMs in a murine mammary tumor model are phenotypically and functionally distinct from mammary tissue-resident macrophages, and also that recruited macrophages may differentiate and form the majority of TAMs, we cannot currently quantify their respective contribution to various stages of progression in many different murine and human tumors [4, 41, 46, 47]. Recently, CSF-1 whose expression was controlled by STAT1 was reported to play an important role at several levels of the monocyte-to-macrophage differentiation pathway in tumors, implying M-CSFR and GM-CSFR signaling in governing the phenotype of macrophage subsets in tumors [45, 48]. Currently, the precise origin of TAMs is thought to be either bone marrow [47] or extramedullary hematopoiesis-like spleen [49] in several studies, indicating that the dominant origin of TAMs appears to be tumor type- or stage-dependent. Overall, the understanding of both of the origin of TAMs and mechanism of their recruitment and differentiation is not completely clear.

4. General Characteristics of TANs

In inflamed tissues, neutrophils engage in sophisticated bidirectional interactions with macrophages, dendritic cells, natural killer cells, lymphocytes, and mesenchymal stem cells [50]. However, the interactions have not been significantly understood in the TME. Traditionally, the mechanism of recruitment and function of neutrophils and platelets have been studied mostly in inflammation or bleeding. Neutrophils account for about 60% of all leukocytes in the circulation and are usually the first line of defense at the site of infection or inflammation. Contrary to the well-known ability of inflammatory neutrophils to engulf bacteria, activate the immune system, and induce tissue damage in infections, it appears that TANs can function as immunosuppressive cells in the context of tumors [51]. Neutrophils may influence the phenomenon of macrophage differentiation into pro- or anti-inflammatory subtypes indicated from many studies showing that activated neutrophils, by releasing various chemokines, activate and recruit monocytes/macrophages at the site of inflammation [52]. Besides cytokines, neutrophils also secrete myeloperoxidase (MPO), also important for recruitment of monocytes/macrophages and activation of platelets [53]. These findings and some epidemiological studies indicate that the recruitment and function of neutrophils and platelets may be linked, either directly or indirectly, with those of TAMs and that they are important in cancer progression and also possibly in maintenance of the TME.

Recently, the neutrophil-to-lymphocyte ratio used in combination with elevated platelet count was found to be predictive of the future clinical course of colorectal cancer [54], and, as mentioned, products of the complement cascade are major determinants of macrophage recruitment and positioning in tumors [40–42]. Indeed, TANs have been suggested as key players in malignant transformation, tumor progression, antitumoral immunity, and angiogenesis [50]. It has been suggested that TANs from early tumors are more cytotoxic toward tumor cells and produce higher levels of TNF-α, NO, and H_2O_2 and, in established tumors, these functions are downregulated and TAN acquire a more protumorigenic phenotype [55]. Neutrophil depletion in two murine models of melanoma and fibrosarcoma reverts the increased tumor growth, angiogenesis, and metastasis observed in IFN-β-deficient mice with skewed N2 phenotypes [56], and recent review of the relationship between TAN infiltration and prognosis in human cancer demonstrates the function of TANs in murine and human tumor progression [57]. It is increasingly becoming clear and important that TANs and their myeloid precursors (peripheral neutrophils and granulocytic MDSCs [G-MDSCs]) in the spleen, bone marrow, and blood have important roles in cancer biology [58]. Neutrophils also make up a significant portion of the inflammatory cell infiltrate in many models of cancer, though they release far less cytokine when compared with other myeloid cells in the TME [59]. It was reported that, at early stages of tumor development, neutrophils are almost exclusively at the periphery of the tumor [55]. At later stages, neutrophils are also found scattered among the tumor cells.

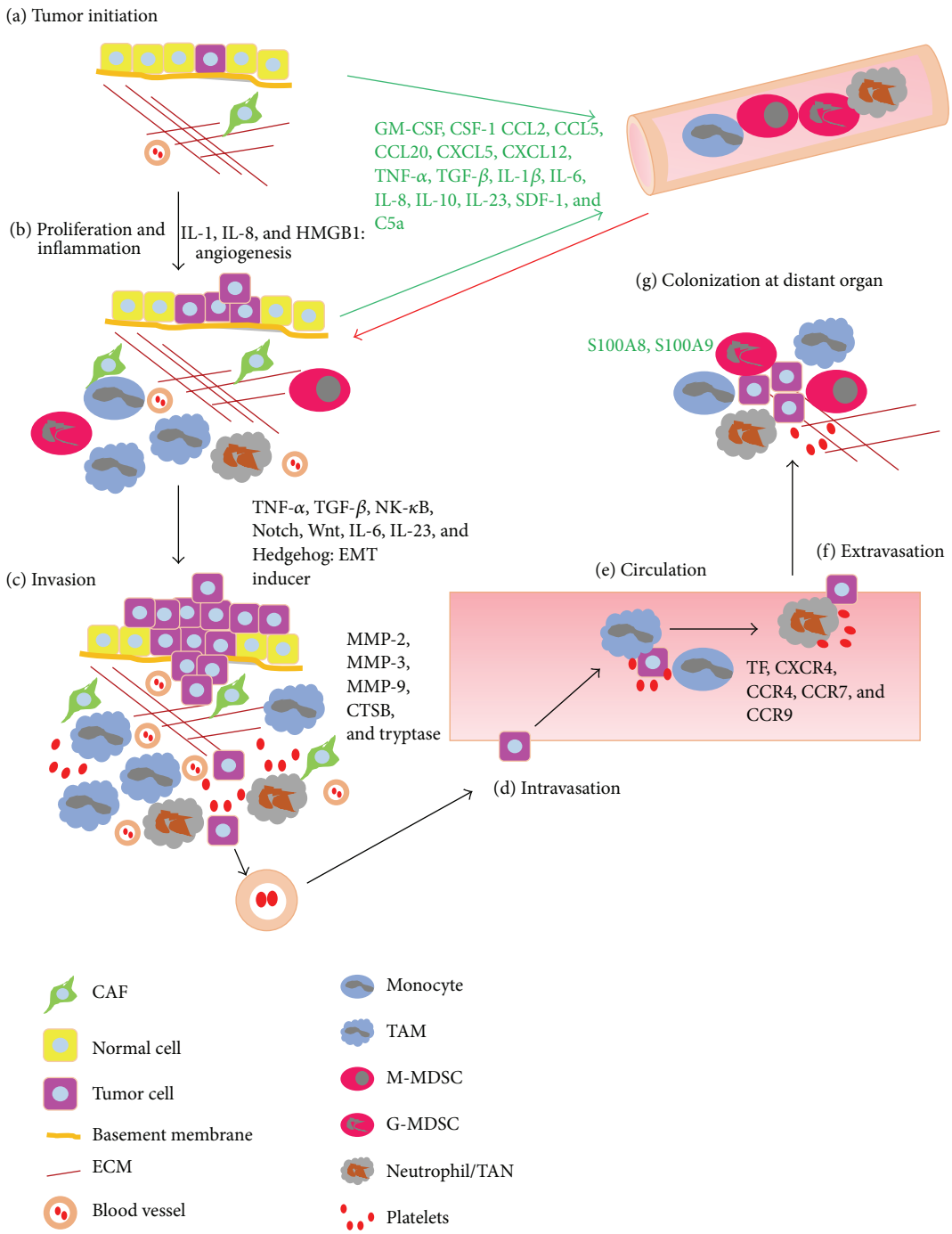

Figure 2: Recruitment pattern of myeloid cells in tumor progression and metastasis. Stages in tumor progression and metastasis including initiation, proliferation and tumor site inflammation, invasion, intravasation, circulation in blood stream, extravasation, and colonization are shown with associated myeloid cells, platelets, and cytokines. The contribution of TAM and TAN at early stage of distant colonization sites is not clear. *Green*: cytokines/chemokines in recruitment or suppression of immune cells, *black*: metastasis associated proteins, *red arrow*: movement of myeloid cells, EMT: epithelial mesenchymal transition, and CTSB: cysteine protease cathepsin B based on [105].

Studies have shown that, analogously to the M1 and M2 dichotomy, TANs develop a protumorigenic (N2) phenotype in untreated tumors, largely driven by the presence of TGF-β [58], and that blocking the effects of TGF-β or augmenting IFN-β can also alter the phenotype of TANs to a more antitumor (N1) phenotype [56]. Antitumor "N1-like" cells generated in the absence of TGF-β produced higher levels of TNF-α, MIP-1α, H_2O_2, and NO and were cytotoxic to tumor cells both *in vitro* and *in vivo* [59].

Respiratory burst and granule proteins are two main mechanisms of cell killing by neutrophils. Transcriptome analysis of naive bone marrow neutrophils (NN) from

nontumor bearing mice and G-MDSC and TAN from mice in which AB12 mesothelioma tumors were growing showed that expression levels of both proteins involved in respiratory burst and granule proteins were downregulated and that those of chemokine, cytokine, and APC genes were upregulated in TANs [58]. N2-like neutrophils may also synergistically interact with tumor-resident mesenchymal stem cells (MSCs) to prompt cancer progression [60]. TANs from established tumors produce CCL17 or CCL22, recruiting immunosuppressive regulatory T cells (Tregs) with defective cytotoxic functions into the tumor and leading to suppression of antitumoral immunity [61]. Of note, similarly to TAMs, TANs from early tumors were more cytotoxic toward tumor cells, while in established tumors TANs acquire a more protumoral phenotype, showing how the evolvement of the TME influences TAN phenotype [55]. Unlike TAMs, it is not certain whether activation of TANs is reversible, and it has been suggested that N1-like and N2-like phenotypes of neutrophils may be from different degrees of activation rather than polarization [62]. The important question whether TANs can be manipulated to undergo frank irreversible activation or possibly reversible activation states remains unresolved and should be a matter of further research.

5. Recruitment of TANs

Do we know the origin of TANs? It is known that the spleen is the site of localization of TAM and TAN precursors, from where they physically relocate to the tumor stroma, and that CXCL8 (IL-8), a chemoattractant for neutrophils, is also chiefly responsible for the recruitment of TANs (Figure 2) [49]. A recent transcriptome study showed that TANs are not "tissue-based G-MDSCs" modulated by the TME but are a different population of neutrophils from both bone marrow-derived neutrophils and G-MDSCs [58]. However, we are not sure whether the majority of TANs are actually differentiated from G-MDSCs that have been recruited to the tumor or whether they are bone marrow-/blood-derived neutrophils, converted to N2 TANs in the TME specifically by the high local concentrations of TGF-β [63]. Though the study does not clarify whether the cells were recruited from the bone marrow/blood pool of neutrophils or the splenic G-MDSC population, the two studies support the idea that TGF-β and other factors in the TME may affect the local "education" of recruited neutrophils.

6. Possible Interaction of TANs with TAMs

Do TANs then recruit TAM precursors to the tumor site or are they responsible for the M2-like activation of macrophages in the TME? It is known that activated neutrophils releasing IL-8 and TNF-α activate and recruit macrophages at the site of inflammation [64]. Neutrophils secrete MPO, and MPO binding to the MMR induces secretion of reactive oxygen intermediates, IL-8, TNF-α, and GM-CSF in chronic inflammatory environments such as rheumatoid joints [65]. M2-like macrophages express high levels of macrophage mannose receptor (MMR) and IL-10 and low levels of HLA-DR and IL-1β [66]. Though we still lack direct evidence that supports TAN and TAM interaction through MPO and the MMR, massive MPO-positive neutrophil infiltration has been found in established colorectal cancer [67] and lung cancer [68]. Also, similar influence of TGF-β on activation of macrophages and neutrophils (M2-like and N2-like, resp.) indicates a close link between TAMs and TANs in the same TME and the possibility that recruitment of macrophages by neutrophils may precede their N2-like polarization. It would be necessary to confirm whether the interaction between TANs and TAMs in the TME is similar to well-known interactions between neutrophils and macrophages in a nontumoral chronic inflammatory environment.

7. Nuclear Extracellular Trap (NET) Formation in the TME

NETs are neutrophil-derived structures composed of decompacted chromatin (DNA and citrullinated associating histones) and antimicrobial peptides, and NET-producing "NETosis" is a form of neutrophil death, distinct from apoptosis or necrosis [69]. NETs are introduced to trap and kill microorganisms and facilitate a final form of neutrophil-mediated host defense against microorganisms. They have also been found in non-microorganism-induced inflammatory environments in autoimmune diseases such as systemic lupus erythematous (SLE) and rheumatoid arthritis (RA) [70–73] and tumors [74, 75]. In autoimmune diseases such as RA, neutrophils are mostly responsible for the cytotoxic effects of immune cells and NETs appear to provide autoantigens and mediate organ damage [70, 76]. However, the function of NETs in tumor progression is still not clear, although they have been suggested to contribute to metastasis from trapping of circulating tumor cells at distant metastatic sites [74, 77] and to tumor progression at primary sites by providing a high local concentration of biologically active proteins [75, 77]. The available data indicate a lack of evidence to conclusively demonstrate whether TANs actually produce NETs and to indicate which signaling is involved in NETosis in the TME. Though we know the relationship between deposition of NETs and recruitment of MPO-rich population of neutrophils in tumors, it seems that there is not enough evidence to indicate the existence of TAN specific NETosis [74, 75, 78]. The animal studies were performed with infusion of bone marrow- or spleen-derived naive neutrophils and the localization of general neutrophils, not specifically TANs, was characterized from MPO staining in tumor. The recent identification of TAN specific signatures such as $CD62L^{lo}CD54^{hi}$ phenotype with a distinct repertoire of chemokine receptors including CCR5, CCR7, CXCR3, and CXCR4 in human lung cancer indicates that further study to validate TAN specific NETosis may be possible in animal studies [79]. Another function of NETs is to provide autoantigens. In SLE and RA patients, specific autoantigens, such as anti-dsDNA and anti-citrullinated protein antibodies and rheumatoid factor, respectively, have been detected. However, there seems to be a relative paucity of tumor-derived autoantigens identified thus far, and this suggests that a major function of tumoral NETs is more likely to trap migrating tumor cells and to provide protumoral substances rather than immunomodulating

autoantigens. Still, it is becoming clear that NETs are a very recently introduced component of the TME and that they play another protumoral role in tumor progression. Future studies will probably investigate (i) identification of the N1-like, N2-like, or general neutrophils that actually form NETs and the specific tumor progression stage to which NETs primarily contribute; (ii) whether retention of TAMs or TANs in tumors also requires formation of NETs; (iii) whether M2-like or N2-like activation requires NETs; (iv) which signals are involved in the formation of NETs.

8. Platelets as a Potential Hub for the Recruitment of Macrophages and Neutrophils

Platelets also contribute to tumor progression [80, 81]. High platelet count in blood (thrombocytosis) is associated with decreased survival in a wide range of cancers including breast, colorectal, and lung cancer [82, 83]. An increased platelet count in blood in malignancy is associated with poor patient prognosis [84, 85]. It has been suggested that platelets may protect tumor cells from immune attack in the circulation, may provide adhesive sites for tumor dissemination, may provide chemokine signals for macrophage recruitment in tumors, and may even shuttle growth factors and cytokines from one site to another [2]. By forming microthrombi, platelets may function as a "shield" to protect disseminating cancer cells in microcirculation from immune cell attack. Platelets store various chemokines and the majority (~80%) of VEGF detectable in blood and platelets induces angiogenesis *in vivo* [85].

Platelets play key roles in directing homing and retention signals for bone marrow-derived cells (BMDCs) and cancer cells and also secrete SDF-1, critical for macrophage recruitment and positioning in tumors [2]. Also, platelet-derived SDF-1 is critical for migration of CXCR4+ tumor cells, hematopoietic progenitor cells (HPCs), and endothelial progenitor cells (EPCs) [86]. This is meaningful in that BMDCs homing to the primary tumor niche may remain in an undifferentiated state in the form of HPCs, EPCs, MSCs, or GR-1+CD11b+ MDSCs or may differentiate into more specialized cell types including TAMs [2]. It is known that platelets support the recruitment of leukocytes in inflammation and vice versa and that the interaction between platelets and neutrophils can happen not only at the inflammatory site, but also in the circulation, indicating the role of platelets in metastasis [87]. Platelets can recruit themselves and neutrophils via various mechanisms, such as the formation of platelet/leukocyte complexes, secretion of serotonin, and induction of P-selectin on platelets and ICAM-1 and $\alpha v\beta 3$ on endothelial cells [87]. All of these findings indicate that platelets may play a central role in recruiting neutrophils in a chronically "persistent" inflammatory environment, that is, the TME. Tumor cells express tissue factor (TF), which is a receptor for coagulation factors VIIa and X [88, 89]. Clot formation by TF expressed by tumor cells enhances recruitment of macrophages in a lung metastasis model through various mechanisms including protease-activated receptor [90], and recruitment of granulocytic cells by the platelet-secreted CXCR2 ligands, CXCL5 and CXCL7 chemokines, upon platelet contact with tumor cells is essential mechanism for the guidance of granulocytes to form "early metastatic niches" [81, 91, 92]. Importantly, recent results indicate that complement components and platelets are key players in cancer-related inflammation and mediate recruitment of macrophages at least partially via CCL2 [40]. Summary of representative interactions between TAM, TAN, and platelets described in this review can be found in Figure 3.

All of this evidence emphasizes the role of platelets in recruitment of macrophages and neutrophils in tumor sites. Though we still lack evidence to support the role of platelets in activation of macrophages and neutrophils—and it is generally accepted that their tumor-protective role in the blood stream may be the most profound influence of platelets on tumor progression—thrombocytopenic mice show increased blood TNF-α and IL-6 and decreased TGF-β [87], possibly favoring antitumoral polarization; as stated, platelets are involved in recruitment of both macrophages and neutrophils in both primary and metastatic tumor sites. At present, there are important questions to be solved: which stages in tumor progression, including metastasis, are primarily affected by platelet functions, which of the adhesive or paracrine functions of platelets are more important for tumor progression, and which platelet factor or traditionally emphasized tissue factor is more important for the protumoral activity of the coagulation system? Further research will likely demonstrate the functional contribution of platelets in tumor progression, including the development of protumoral TAMs and TANs.

9. Clinical Implications

All the summarized data describing the protumoral role of the myeloid infiltrate of tumors in this review emphasize that TAMCs are reasonable targets for new anticancer therapeutic approaches. It is now becoming clear that host-protective properties of macrophages are suppressed in the TME and that therapeutic intervention can reverse this suppression. Recently explored strategies have focused on ablation of macrophages or reduction of recruitment of myeloid cells and repolarization of M2-like protumoral macrophages to antitumoral M1-like cells. CD40 agonist antibody [93] and TLR9 agonist (CpG-oligodeoxynucleotide) [94] have been shown to be effective in repolarizing M2-like protumoral macrophages. CCL2/CCR2 antagonist [95, 96] and CSF-1 inhibitory antibodies or Yondelis (trabectedin) [97] were effective in blocking recruitment of macrophages in tumor sites. Bisphosphonate zoledronic acid [36, 98] and clodronate [99] have been used to inhibit TAM effectors and to deplete TAMs, respectively.

Rather than depleting the entire population of neutrophils, the usual strategy is to deplete TANs or disrupt their homing ability, migration. For this purpose, the deployment of anti-CXCR2 antibodies to deplete TANs or the targeting of specific neutrophil-derived or recruiting chemokines, such as CXCL-5, Gro-α, or IL-8, was performed and reported to be successful [59]. Furthermore, targeting TGF-β or augmenting the activity of IFN-β to block its skewing of

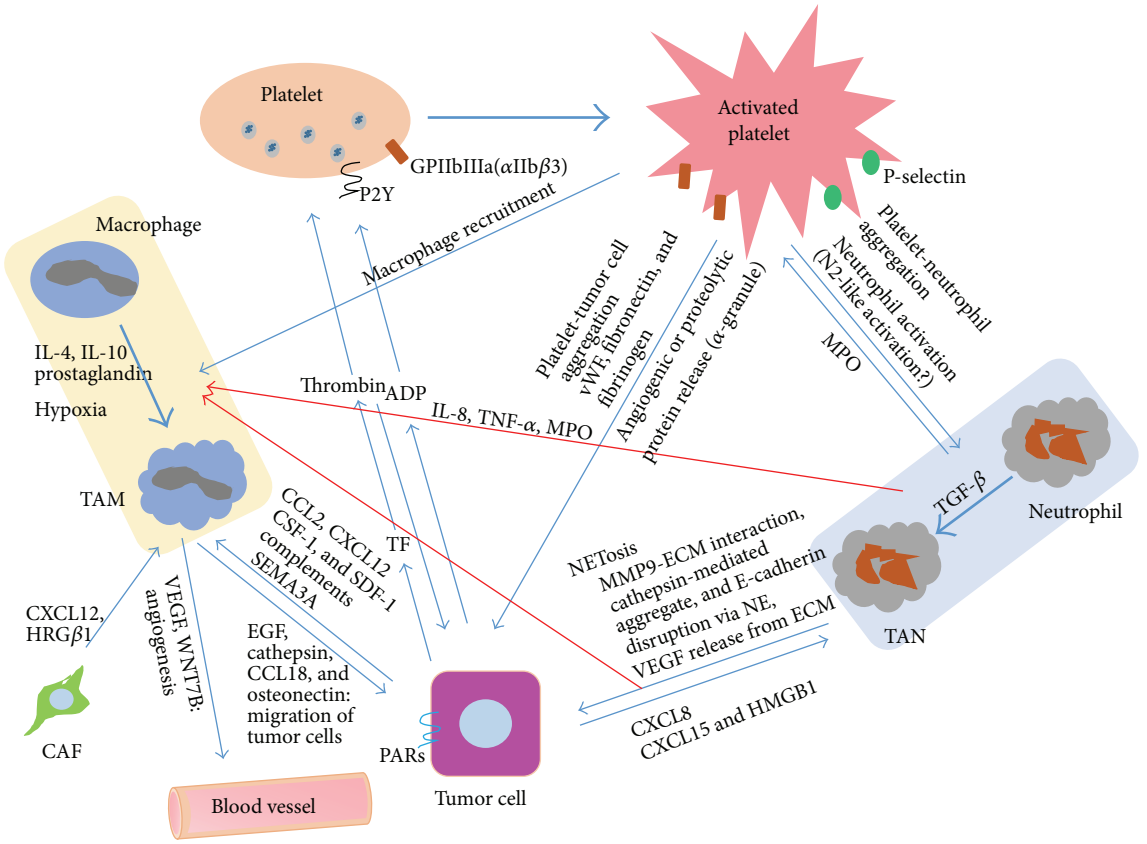

Figure 3: Summary of representative interactions between TAM, TAN, platelet, and tumor cells. The interactions between neutrophil and macrophages have not been significantly understood in the TME and the contribution of platelet in differentiation of TAM and TAN suggested in this review awaits further studies. Tumor cells, blood vessels, and CAF comprise TME. CCL2, CXCL12, CSF-1, SDF-1, complements, and SEMA3A for macrophage recruitment [30, 106]. CSF-1 prompts TAMs to produce EGF. The EGF-CSF-1 loop can be initiated by CAF derived factors, such as CXCL12 and HRGβ1 [106]. IL-4 from CD4+ T cells or tumor cells can activate macrophages to TAMs. CCL18 and osteonectin can increase migration and intravasation of tumor cells in metastasis. CXCL-8, CXCL15, and HMGB1 secreted from tumor cells can recruit TANs in metastatic sites. MPO and cytokines from neutrophil recruit platelet and macrophages. PAR and P2Y receptor are involved in thrombin and ADP mediated platelet activation, respectively. P-selectin is involved in platelet leukocyte tethering and leukocyte activation. α-granule is a storage of proteins that enhance adhesive process, angiogenesis, and extracellular matrix (ECM) degradation [81]. GPIIbIIIa mediates tumor cell and platelet interaction via vWF, fibronectin, and fibrinogen [80]. *Red arrow*: neutrophil-mediated recruitment of macrophages in tumor. *Thick arrow*: conversion of platelets, neutrophils, and macrophages to activated platelets, TAN, and TAM, respectively. GPIIbIIIa, glycoprotein IIbIIIa; vWF, Von Willebrand factor; ADP, adenosine diphosphate; PARs, proteinase-activated receptors; P2Y, P2Y receptors; TF, tissue factor; NE, neutrophil elastase; HMGB1, high mobility group protein B1; HRGβ1, heregulin β1.

TANs toward an N2 phenotype may have potential as a new therapeutic approach [56, 63]. As neutrophil-derived molecules play critical roles in a wide range of stages of tumor progression [59], targeting neutrophil-secreted enzymes or cytokines could be another effective approach [100]. Targeting TANs may indirectly affect TAM populations, considering the interaction between neutrophils and macrophages mentioned above.

Because aggressive anticoagulant therapy in cancer patients carries the risk of bleeding complications, selective inhibition of TF signaling or platelet functions should be considered for clinical settings. Currently, the benefit that direct platelet receptor antagonists may have on cancer prognosis has not been demonstrated, and the evidence to support a combined use of antiplatelet agents with current chemotherapeutic reagents is lacking [101]. The concept that tumor cells alter their gene expression profiles to acquire a genophenotype closely resembling that of platelets and express several megakaryocytic genes (adhesion receptors αIIbβ3, thrombin receptor, and PECAM/CD31 and/or platelet-type 12-LOX) to activate platelets or the coagulation cascade is referred to as "platelet mimicry" of tumor cells [102]. This well-described epiphenomenon facilitates hematogenous dissemination of tumor cells in metastasis; thus, identification of molecular targets to regulate platelet mimicry is also likely to provide new therapeutic modalities. Recently, the CXCR2 receptor for the granulocyte- and platelet-derived ligand CXCL5/7 was shown to be important for recruitment of neutrophils to early metastatic niches [92], and CXCR2 inhibitors reduce the recruitment of granulocytes in primary tumor sites as well [103, 104]. Considering that anti-CXCR2 inhibitors evaluated in the clinic for inflammatory disease are well tolerated by

most patients [103], targeting the CXCR2-CXCL5/7 axis may become effective in clinical settings.

10. Conclusions and Perspectives

There has been tremendous effort and progress in deciphering the function of myeloid cells in the TME and in tumor progression by excellent investigators in the field. Unfortunately, the wealth of mounting information regarding protumoral myeloid cells in cancer has been fragmentary and produced confusion from a disjointed view of the role of macrophages and neutrophils in the TME [19]. In addition, the absence of unique markers to differentiate each subset has obscured the nature of specific myeloid subsets in cancer. However, the contribution of TAMs and TANs to tumor progression is clear, and animal model-based or preclinical studies have shown promising results. We anticipate that the introduction of more sophisticated tumor models and techniques to differentiate different myeloid cell subsets *in vivo* will reveal fundamental information about possible spatial and temporal modulation of myeloid cells, including their interaction with platelets in each progression stage of different types of tumors.

Conflict of Interests

The authors have no conflict of interests to declare.

Acknowledgments

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Review Article

Interplay between Cellular and Molecular Inflammatory Mediators in Lung Cancer

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Inflammation is a component of the tumor microenvironment and represents the 7th hallmark of cancer. Chronic inflammation plays a critical role in tumorigenesis. Tumor infiltrating inflammatory cells mediate processes associated with progression, immune suppression, promotion of neoangiogenesis and lymphangiogenesis, remodeling of extracellular matrix, invasion and metastasis, and, lastly, the inhibition of vaccine-induced antitumor T cell response. Accumulating evidence indicates a critical role of myeloid cells in the pathophysiology of human cancers. In contrast to the well-characterized tumor-associated macrophages (TAMs), the significance of granulocytes in cancer has only recently begun to emerge with the characterization of tumor-associated neutrophils (TANs). Recent studies show the importance of CD47 in the interaction with macrophages inhibiting phagocytosis and promoting the migration of neutrophils, increasing inflammation which can lead to recurrence and progression in lung cancer. Currently, therapies are targeted towards blocking CD47 and enhancing macrophage-mediated phagocytosis. However, antibody-based therapies may have adverse effects that limit its use.

1. Non-Small Cell Lung Cancer (NSCLC)

Lung cancer remains the leading type of cancer worldwide and in Latin America [1, 2]. The disease burden is significantly high, with around 2.5 million new cases per year and 1.5 million deaths worldwide [3]. The two main histological subtypes of lung cancer are small-cell lung cancer (SCLC), which comprises 15% of cases, and non-small-cell lung cancer (NSCLC) accounting for 85% of cases [4] which include adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [5]. Among all newly diagnosed NSCLC cases, adenocarcinomas are the most frequent subgroup following by squamous cell carcinomas [6, 7]. Cigarette smoking is the major risk factor for lung cancer but around 10–20% of cases are found in never smokers; also wood-smoke is a major risk factor in countries like Mexico [8–11].

Surgery is the selected treatment for early stage NSCLC with the greatest probability of long-term survival in such patients [12]. In advanced NSCLC, conventional therapies are based on chemotherapy and radiotherapy but with low efficacy. Over the last decade, there have been advances in the study of molecular pathways underlying tumor development leading to the development of targeted therapies such as tyrosine kinase inhibitors (TKIs) and antibodies directed against the two main actionable genes in NSCLC up to now: mutations in the epidermal growth factor receptor (EGFR) gene targeted by TKIs like gefitinib [13, 14], erlotinib [9, 15, 16], and afatinib [17–19] and translocations involving the anaplastic lymphoma kinase (ALK) gene treated with the TKI crizotinib [20], alectinib [21], and ceritinib [22]. Benefits have been shown in a subset of 15–20% of patients harboring EGFR mutations which correlate with definite clinical characteristics: adenocarcinoma histology, female sex, Asian ethnicity, and nonsmokers [23–25]. Despite these improvements in therapeutic strategies, early diagnosis is very difficult; most cases are diagnosed at an advanced stage and cancer metastasis is very frequent; therefore, there is still an exceedingly low 5-year survival rate of 11–24% [26–28].

The immunotherapy approach has opened new therapeutic options in advanced NSCLC with the advent of antibodies against immune checkpoints [29, 30]. Recently, the anti-programmed death-1 (PD-1) antibodies nivolumab and pembrolizumab have been approved in the treatment of advanced metastatic NSCLC based on results from clinical trials after prior chemotherapy [31, 32]. Both antibodies block signaling through PD-1 and may restore antitumor immunity with benefits in overall survival [33, 34]. For example, nivolumab, a fully human monoclonal antibody, has recently shown greater overall survival than docetaxel [35]. Pembrolizumab has demonstrated safety and efficacy as single agent for the treatment of NSCLC [32]. These antibodies exhibit a reasonable toxicity profile but they should be administered in selected patient populations based on biomarkers such as PD-L1 expression to avoid serious immune-mediated adverse effects [36]. Although these checkpoint inhibitors have proven efficacy in patients, their mechanism of action implies side effects as the onset of autoimmune diseases and a series of endocrine disorders [37, 38]. This is the rationale for further research into other molecular and cellular factors of the immune system that could be effectively targeted to develop novel therapeutic strategies for the management of advanced NSCLC.

Recent findings indicate that inflammation plays a key role in tumor progression and survival across several cancer types [39]. Cancer related inflammation affects many aspects of malignancy including proliferation, survival, angiogenesis, and tumor metastasis [40]. Inflammatory components in the development of the neoplasm include diverse leukocytes populations, like macrophages and neutrophils, which respond immediately to inflammatory stimulus [41]. Immunoregulatory cytokines secreted in a proinflammatory environment also contribute to tumor growth and metastases and identify patient subsets in advanced NSCLC with differential prognosis [42]. Both macrophages and neutrophils are increased in patients with lung cancer; this is associated with poor clinical outcomes, suggesting that these cells might have important tumor-promoting activities [43, 44]. Tumors escape phagocytosis and immune response through overexpressing CD47 that interacts with the signal regulatory protein alpha (SIRPα) preventing engulfment [45]. Their effects are mediated through complex regulatory networks. Human cytokine profiles could define patient subgroups and represent new potential biomarkers.

2. Tumor-Associated Macrophages (TAMs)

Macrophages within the tumor microenvironment are called tumor-associated macrophages (TAMs). TAMs have a complex relationship with tumor cells; at an early stage they attack tumor cells avoiding tumor spread; however, over time they begin producing reciprocal growth factors and establish a symbiotic relationship with tumor cells [46]. Macrophages are polarized into two functionally distinct forms M1 and M2, mirroring the Th1 and Th2 nomenclature of T cells [47]. Differentiation of the M1 macrophages is induced by interferon-γ, lipopolysaccharides, tumor necrosis factor (TNF) α, and granulocyte-monocyte colony-stimulating factor. The M1 macrophages produce high levels of interleukin- (IL-) 12, IL-23, TNFα, IL-1, IL-6, CXC ligand 10 (CXCL10), inducible nitric oxide synthase (iNOS), human leukocyte antigen-(HLA-) DR, and reactive oxygen and nitrogen intermediates [47, 48]. Differentiation of the M2 macrophages is induced by IL-4, IL-10, IL-13, IL-21, activin A, immune complexes, and glucocorticoid [47]. The M2 macrophages express high levels of IL-10, IL-1 receptor antagonist, CC ligand 22 (CCL22), scavenger, mannose receptor, galactose receptor, arginase I, and CD163 antigen, reduce the expression of iNOS, and inhibit antigen presentation and T cell proliferation [47, 49].

Factors that shift TAMs towards a M2 phenotype include the location of TAMs within the tumor microenvironment, tumor stage, and type of cancer. Nevertheless, it is still not fully defined whether the diversity within the TAM population is due to the maturation of unique monocytic precursors or due to various factors within the local tumor microenvironment [50]. The M2 macrophages have been found to encourage the growth of various tumour cells *in vitro* and to increase tumor cell survival [51, 52]. M1 macrophage significantly decreased A549 cell viability and proliferation as well as invasion ability [53].

Studies suggest that in solid tumors established and progressively growing TAMs are reprogrammed to induce immune suppression *in situ* in the host through cytokines, prostanoids, and other humoral mediators [54, 55]. Tumor microenvironment can influence the functional status of macrophages *in situ* [56]. IL-1 and IL-6 expression in TAMs differs in ovarian cancer compared to peripheral blood monocytes. TAMs in the ovary produce low levels of IL1 and increase the release of IL-6, which contributes to elevated acute phase proteins and increased malignancy [55].

There is an association between the number of macrophages and prognosis in a variety of human tumors. TAM infiltration increased in carcinomas of breast, cervix, and bladder and correlates with a poor prognosis. However, in prostate, lung, and brain, increasing TAMs is associated with regression of tumors [46].

TAMs can regulate the development of new blood vessels within tumors. In hypoxic sites, they stimulate the production of enzymes and extracellular matrix molecules that regulate endothelial cell activity by stimulating factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumor necrosis factor-α (TNF-α), transforming growth factors- α and β (TGF- α, β), interferons, thrombospondin, IL-8, and epidermal growth factor (EGF) [57].

3. Tumor-Macrophage Interactions in Lung

Innate immunity in lung involves alveolar macrophages (AMs) which act as a barrier avoiding penetration of pathogens. Conversely, macrophages contribute in part to the pathogenesis of lung disease due to toxic particles ingestion, releasing lysosomal enzymes that can kill the macrophage itself, or contribute to the recruitment of new macrophages inducing chronic inflammation [58]. Clinical evidence has indicated that the activation of alveolar macrophages by SiO2 produces rapid and sustained inflammation characterized by

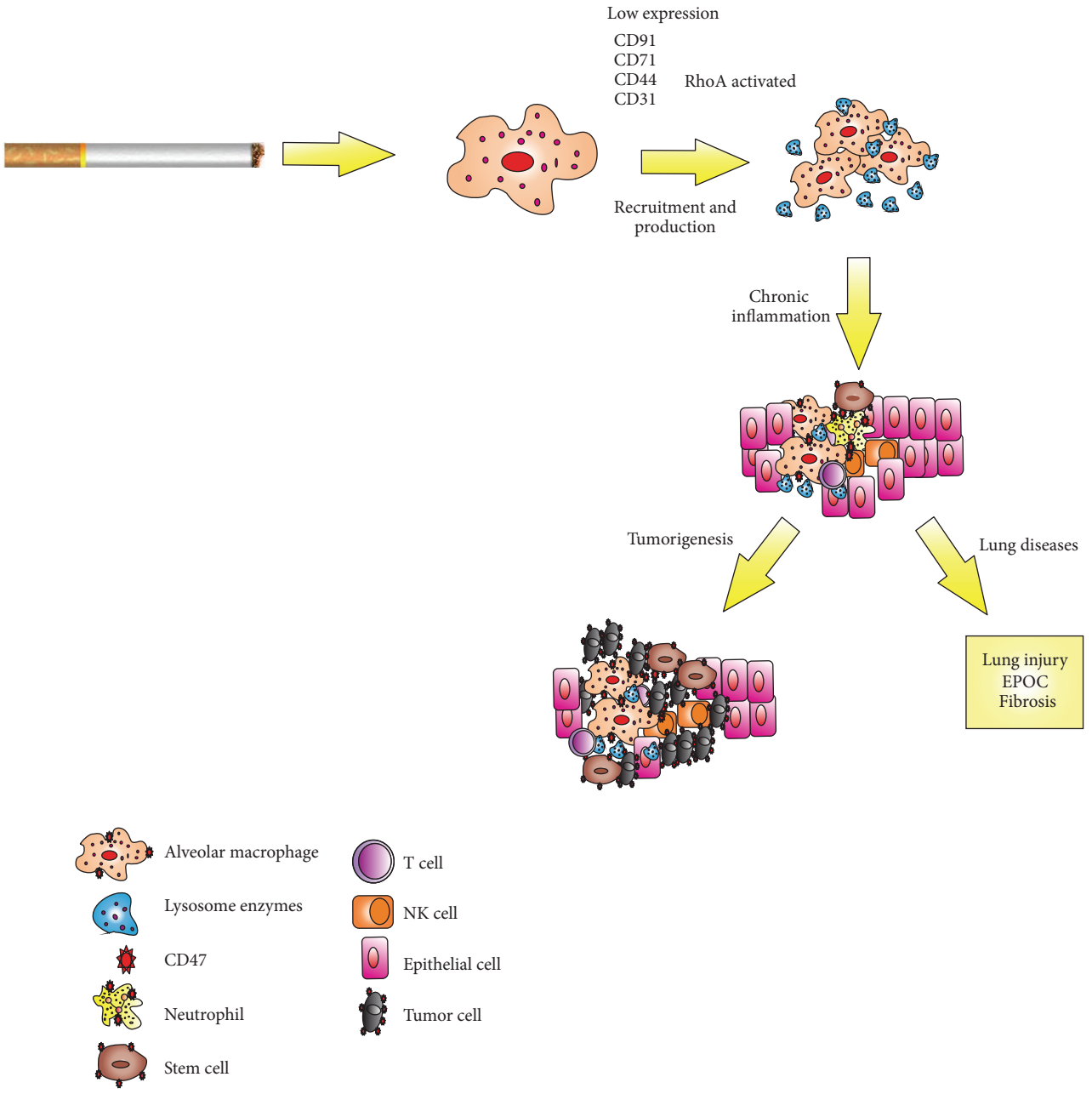

Figure 1: Smoke exposure mediated pathogenesis of pulmonary disease. The exposition to cigarette smoke by macrophages leads to release of lysosomal enzymes able to inhibit phagocytosis by macrophages. Activation of alveolar macrophages deregulates expression of adhesive molecules (CD36, CD91, and CD44) and activated RhoA inhibiting efferocytosis. The rapid and sustained inflammation may contribute to the lung injury and tumorigenesis.

the generation of monocyte chemotactic protein 1, which, in turn, induces fibrosis [59].

Exposure to cigarette smoke activates NF-E2-related factor 2 (Nrf2) in macrophages and reduces neutrophil recruitment, reduces AMs phagocytic ability and expression of several important recognition molecules, and impairs clearance of apoptotic cells through oxidant-dependent activation of RhoA [60, 61]. In current smokers, the exposure to cigarette smoke affects several important recognition molecules on AMs and downregulates CD31, CD91, CD44, and CD71 on these cells [60]. AMs with defective phagocytosis lead to chronic inflammation and significantly increase the likelihood of developing chronic obstructive pulmonary disease, lung injury, and cancer [62] (Figure 1).

The infiltration of alveolar macrophages promotes the death of tumor cells in those sites of primary tumor growth and/or metastasis in lung [63]. The antitumor activity of alveolar macrophages from lung cancer patients decreases with increased metastasis, tumor size, and development of pleural invasion [64]. The onset of malignant disease triggers

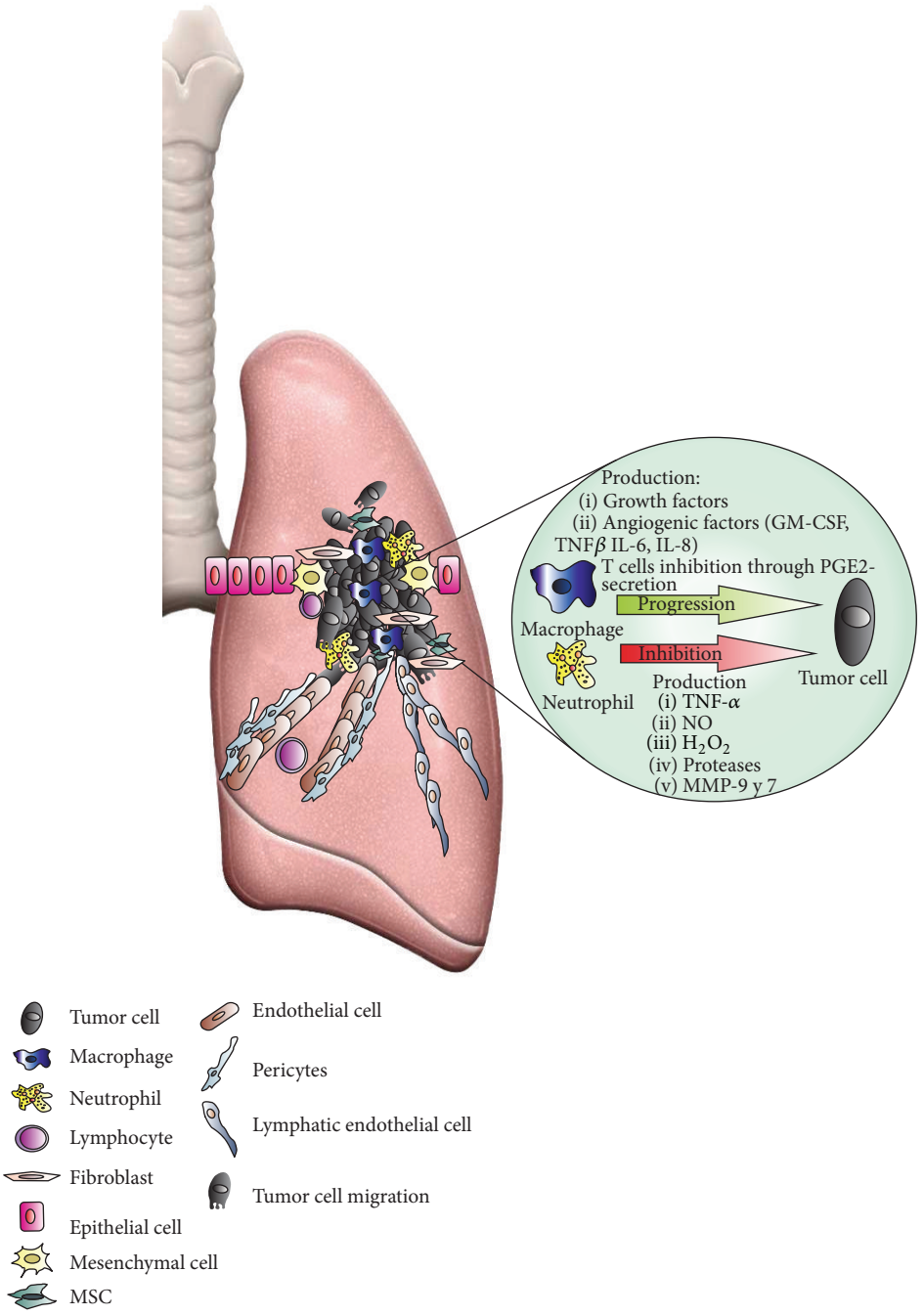

Figure 2: Inflammation: a component of the tumor microenvironment. During malignant transformation until progression disease, the recruitment of immune cells and secretion of soluble factors play an important role in tumor genesis. Tumor killing is promoted for proinflammatory microenvironment where polarized M1 macrophages and N1 neutrophils are recruited. The production of soluble factors, such as TNF-α, NO, H_2O_2, proteases, and metalloproteinase by immune cells, inhibits tumor growth. However, the generation of an anti-inflammatory environment and the alternative activation of M2 macrophages and N2 neutrophils promote tumor growth. Also, growth factors and angiogenic factors (GM-CSF, TNF-β, IL6, and IL8) contribute to tumor proliferation and the inhibition of immune response through prostaglandins.

the immune response recruiting TAMs into the tumor site. High numbers of intratumor TAMs have been linked with invasion, angiogenesis, hypoxia, and early occurrence of metastasis in different tumor types including lung cancer [48, 50] (Figure 2).

In patients with NSCLC, the M1 macrophage phenotype has been associated with the expression of IL-1, IL-12, tumor necrosis factor-α (TNF-α), and iNOS and also has been correlated with extended survival time [65]. In a study, M1 TAMs were identified using CD68 and iNOS markers in

tumor compared to nontumor tissue in NSCLC patients. Results indicate that iNOS expression is lower in tissues from patients with adenocarcinoma and squamous cell carcinoma compared to nontumor tissues but surprisingly this was not the case in large cell lung carcinomas [66]. The classically activated M1 macrophages produce effector molecules such as reactive oxygen intermediates, reactive nitrogen intermediates, and TNFα, to limit tumor growth. Overall there is an association of M1 TAMs with better lung cancer prognosis.

At the other end are the alternatively activated M2 macrophages which have been correlated with tumor initiation, progression, metastases, by secretion of matrix-degrading enzymes, angiogenic factors, and immunosuppressive cytokines chemokines, inhibiting inflammation [65, 67, 68]. M2 macrophages polarized by cigarette smoke lead to proliferation, migration, and invasion of alveolar basal epithelial cells, and exposition to these cigarette smoke-induced M2 macrophages also significantly increased the cell population in G2/M phase causing proliferation in lung cancer cells [69].

Patients with combination gene signature of M1/M2 macrophages exhibited high median overall survival [53]. In NSCLC, the concentration of macrophages M2 was 70% in comparison with 30% M1. Density of macrophages M1 in the tumor islets, stroma, or islets and stroma was positively associated with patient's survival time [66]. Also, M1 in islet is a predictive response value to survival [66].

4. Tumor-Associated Neutrophils (TANs)

Neutrophils are also polarized into N1/N2 subgroups, N1 being proinflammatory, while N2 is anti-inflammatory. N1 and N2 represent a dichotomy in neutrophil subpopulations present in patients and animal models with cancer where they play distinctive roles in the pathogenesis of disease [70]. TANs have a complex interaction with T cells in the tumor microenvironment [71]. They displayed an activated phenotype that included chemokine receptors as CCR5, CCR7, CXCR3, and CXCR4. Also, TANs produced proinflammatory factors MCP-1, IL-8, MIP-1α, and IL-6, as well as the anti-inflammatory IL-1R antagonist [72]. Also, TANs exhibit high activated phenotype compared with peripheral neutrophils. In cancer patients, TANs could drive antitumoral immunity through regulating cytotoxic T lymphocytes. In early stages of lung cancer disease, TANs increased T cell IFN-γ production and activation and increase T cell proliferation [72]. The blockage of TGF-β is able to polarize N2 TANs to N1 TANs in murine models of mesothelioma and lung cancer [73].

Resolution of inflammation involves cessation of neutrophils recruitment and initiation of apoptosis and clearance [74]. If apoptotic neutrophils within the tissues are not removed in an efficient and timely manner, they will become necrotic and release cytotoxic granule proteins that may perpetuate host tissue damage. Thus, neutrophils apoptosis and clearance is a critical limiting factor for the successful resolution of inflammation [75]. In colon adenocarcinoma cell line, massive infiltration of neutrophils showed regression of tumor [76].

So far, the possible mechanisms by which neutrophils are increased in NSCLC patients have not been described; despite this, these cells are dysfunctional [77]; increased levels of IL-8 could explain this accumulation; however, the mechanisms by which this occurs are not known [42].

5. CD47 and Immune Evasion

Chronic inflammation confers higher risk of developing cancer. Neutrophils are recruited to tumor sites through transendothelial migration involving the CD47:SIRPα recognition (signal regulatory protein alpha) creating an inflammatory environment [78]. Malignant cells escape phagocytosis displaying high levels of CD47 on their surface which binds to SIRPα in macrophages and dendritic cells. After binding to SIRPα, CD47 induces a dephosphorylation cascade preventing phagocytosis through impaired synaptic myosin accumulation [79]. In this way, CD47 can regulate the function of cells in the monocyte/macrophage lineage [80–82].

CD47 is a ubiquitous cell-surface molecule from the immunoglobulin (Ig) superfamily that interacts with SIRPα, thrombospondins, and integrins [83]. CD47 was first isolated in association with integrin in neutrophil granulocytes and was later shown to regulate integrin function [84, 85]. It plays a role in cellular processes like proliferation, apoptosis, adhesion, and migration [86] and in immunological processes such as inflammatory response, immune response, and tumor immunity [87, 88]. This receptor is recognized as a marker of "self" [89] highly expressed by circulating hematopoietic stem cells, red blood cells, macrophages, macrophages neutrophils, and many cancer types [90]. CD47 has also been identified as a tumor marker, and its dysregulation contributes to cancer progression and evasion of antitumor immunity [91–94].

CD47 is expressed ubiquitously whereas its counter-receptor SIRPα is more abundant in myeloid-lineage cells such as macrophages, neutrophils, and dendritic cells [95]. Several processes are regulated through the CD47:SIRPα signaling system of macrophages, including phagocytosis mature red blood cells (RBCs) in the spleen, phagocytosis of senescent cells and apoptotic bodies, rejection of transplants of hematopoietic stem cells (HSCs), and immunosurveillance thereby preserving tissue integrity and function [96–99]. Remarkably, there are many factors positively regulating phagocytosis while SIRPα-CD47 is the only negative regulator preventing self-phagocytosis [88].

CD47 is critical for transepithelial and transendothelial migration of neutrophils or polymorphonuclear leukocytes (PMN) facilitating diapedesis through endothelial cells while targeted CD47 deletion decreases neutrophil extravasation [100, 101]. The SIRPα-CD47 interaction initially recruits PMNs to tumor sites or sites of injury but later negatively regulates these cells to end the inflammatory response. However, in a postacute stage of inflammation, neutrophils experience cleavage of the cytoplasmic signaling domains of SIRPα, correlating with increased recruitment and neutrophil-associated damage. Truncated SIRPα acts like a decoy, able to bind CD47 but not signaling intracellularly

therefore maintaining the inflammatory microenvironment and being a caveat for CD47 targeted therapies [102–105]. Additionally, SIRPα binding to CD47 *in vitro* downregulates CD18 as marker of neutrophil activation thus playing a role in the inflammatory activation state of PMNs [106, 107].

The dual role of CD47 in promoting inflammation through neutrophil migration and recognition of self through blocking phagocytosis in macrophages plays a role in the development of cancer and later in tumor immune evasion. Loss of CD47 induces phagocytosis by macrophages *in vitro* and blocks tumor development and metastasis *in vivo* [108]. This receptor is strongly overexpressed in several cancer types including both hematological and solid tumors [80, 91, 109, 110]. A high CD47 expression has been a poor prognostic factor for patients with these diseases [80, 111, 112]. CD47 is also highly expressed in tumor initiating cells (TICs) or cancer stem cells (CSC) where it is a marker of more aggressive tumor cells, with higher metastatic potential, and less sensitive to engulfment by macrophages, thereby escaping from immune surveillance while increasing cell proliferation through activation of the PI3K/Akt pathway [92, 113–116]. Therefore, CD47 becomes an attractive target for therapeutic approaches with both antitumor and anti-inflammatory properties and anti-CD47 antibodies are being tested with positive results in preclinical and clinical settings [80, 111, 112, 117].

In lung cancer and in several types of cancers including breast, bladder, colon, pancreatic, and hematological cancers, blocking CD47 in tumor cells leads to increased phagocytosis by macrophages and later activation of T cells [94]. The CD47:SIRPα interaction is involved in the pathogenesis of lung cancer and other cancer types when tumors release cytokines promoting tumor growth and stimulating the conversion of macrophages from M1 to M2 phenotype [118]. Systemic administration of nanoparticles with anti-CD47 siRNA showed efficient inhibition of lung metastasis to about 30% of controls [94]. In patients with lung metastasis, the number of circulating tumor cells (CTC) with the phenotype EPCAM(+)CD44(+)CD47(+)MET(+) were associated with poor overall survival and increased metastasis and CD47 was a marker associated with the fraction of metastasis-initiating cells within the pool of CTCs [119].

Antisense suppression of CD47 in squamous lung tumors prior to irradiation showed benefit obtaining a 71% tumor size reduction. This protection could possibly be exerted through thrombospondin-1 signaling to recover from radiation stress, revealing a strategy to protect normal tissues from radiation damage using anti-CD47 antibodies which could be useful in the application of combined radiation with targeted therapies in lung cancer [120].

There is a close relationship between macrophage, neutrophil infiltration, and upregulation or CD47 with poor prognosis and lack response to treatment. Nowadays, therapies are developed to block the interaction of tumor cells with macrophages through CD47, thereby offering an opportunity to turn TAMs against NSCLC cells by allowing the phagocytic behavior of resident macrophages. Also, anti-CD47 could regulate the recruitment of neutrophils into tumor and diminish the chronic inflammation Figure 3.

6. Therapeutic Approaches: TAMs and TANs

Preclinical studies showed that peptide to M2-like TAM improves survival of tumor bearing mouse [121]. Inhibition of CSF-1 receptor, which is essential for macrophage differentiation significantly increased survival and suppressed established tumors, accompanied by decreased M2-like TAM [122]. Treatment with metformin is able to reduce the metastases *in vivo*, through blocked matrix metalloproteinase-9 and expression of MMP-2, maintaining the components of the extracellular matrix, avoiding the separation of tumor cells, inhibiting the growth and metastasis of tumors [123]. Also, metformin prevented M2-polarization of macrophages regulated AMPKα1 and, besides, inhibited IL-1 induced release of the proinflammatory cytokines IL-6 and IL-8 in macrophages [124, 125]. Combination of metformin with TKI inhibitor reduces pulmonary fibrosis trough decreased TGF-beta [126].

Glycodelin (gene name PAEP) is a proliferation suppressor and apoptosis inducer of T cells, monocytes, B cells, NK, and regulated pulmonary immune response in asthmatic inflammation. However, atypical expression is observed in squamous cell carcinomas and adenocarcinomas of NSCLC [127]. *In vitro*, silencing by siRNA-transfection of PAEP in two NSCLC cell lines resulted in significant upregulation of immune system modulatory factors such as PDL1, CXCL5, CXCL16, MICA/B, and CD83 as well as proliferation stimulators EDN1 and HBEGF [127]. This kind of therapy provides a mechanism to overcome tumor immunosurveillance.

As mentioned above, currently the only FDA-approved immunotherapies for the treatment of NSCLC are nivolumab and pembrolizumab. These antibodies inhibit checkpoint molecules such as CTL-4 and PDL-1, improving the survival and response to treatment [128]. CTLA-4 is thought to regulate T cell proliferation early in an immune response, primarily in lymph nodes, whereas PD-1 is upregulated in current smokers and suppresses T cells [129]. These antibodies switch on immune system cells mediated by T cells, increasing their ability to recognize and destroy cancer cells [128, 130]. Monoclonal antibodies specific for tumor cell antigens, coupled with appropriate cytokines, may provide rational basis for designing trials to employ the neutrophil cytotoxic potential as adjuvant therapy in cancer patients [131].

7. Conclusion

Chronic inflammation seems to play a major role in the onset and development of cancer. Understanding the interaction between the cellular and molecular factors that mediate inflammation in NSCLC, including the rather unexplored components of innate immunity such as macrophages and neutrophils, can elucidate novel targets affecting key oncogenic pathways in this malignancy and allow preventing cancer cell proliferation, angiogenesis, and metastasis. Inhibiting CD47 as promoter of neutrophil extravasation and migration may reduce inflammation thereby preventing cancer, and blocking the antiphagocytic signal of CD47 on the surface of tumor cells can overcome immune suppression, harnessing

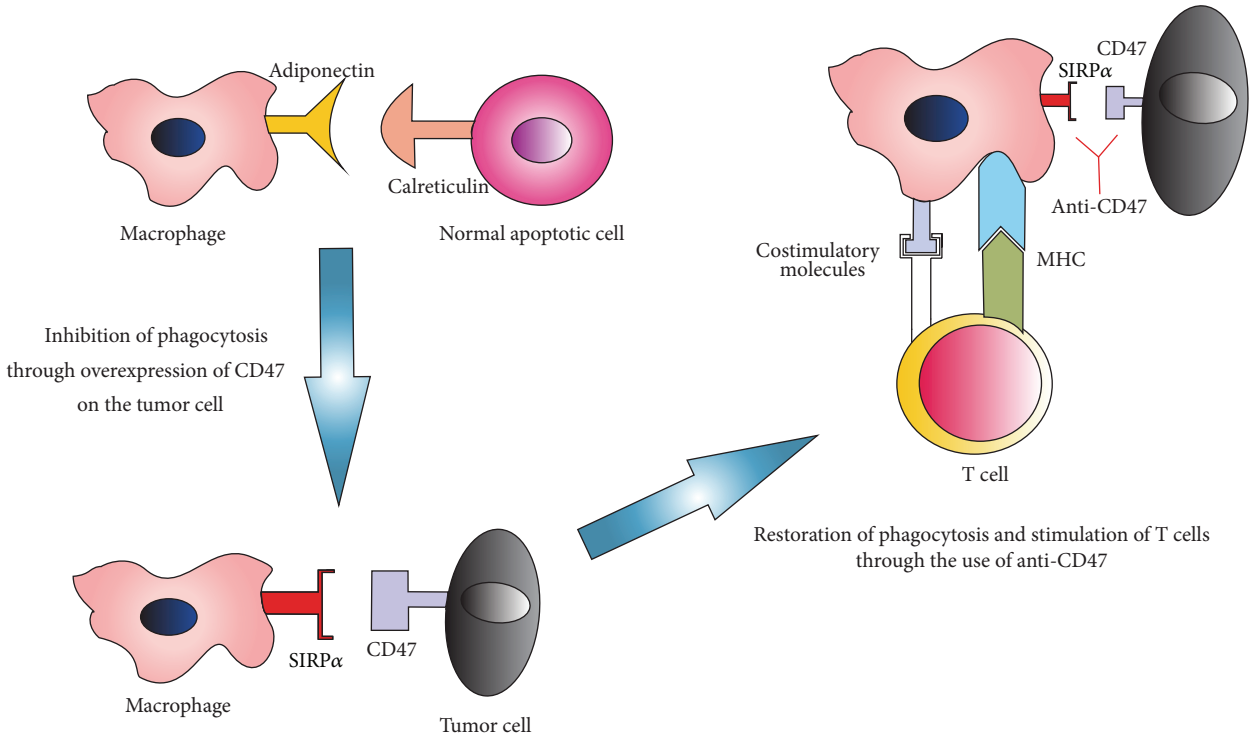

Figure 3: Target CD47. Macrophages maintain tissue homeostasis through phagocytosis of apoptotic cells. Interaction with other cell types can increase or inhibit their activity. Adiponectin via calreticulin leads to the uptake of early apoptotic cells by macrophages. However, as an immune evasion mechanism, tumor cells can deregulate the expression of CD47 and thereby inhibit phagocytic activity. Currently, the administration of anti-CD47 antibody activates phagocytosis by blocking interaction SIRP-α/CD47 and inhibits the migration of neutrophils to the tumor site inhibiting their growth.

the immune system to target malignant cells more effectively. On the other hand, the potential side effects should be addressed by careful selection of patient populations based on biomarkers such as tumor CD47 overexpression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Macrophages: Regulators of the Inflammatory Microenvironment during Mammary Gland Development and Breast Cancer

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Macrophages are critical mediators of inflammation and important regulators of developmental processes. As a key phagocytic cell type, macrophages evolved as part of the innate immune system to engulf and process cell debris and pathogens. Macrophages produce factors that act directly on their microenvironment and also bridge innate immune responses to the adaptive immune system. Resident macrophages are important for acting as sensors for tissue damage and maintaining tissue homeostasis. It is now well-established that macrophages are an integral component of the breast tumor microenvironment, where they contribute to tumor growth and progression, likely through many of the mechanisms that are utilized during normal wound healing responses. Because macrophages contribute to normal mammary gland development and breast cancer growth and progression, this review will discuss both resident mammary gland macrophages and tumor-associated macrophages with an emphasis on describing how macrophages interact with their surrounding environment during normal development and in the context of cancer.

1. Introduction to Macrophages

As a cell of the innate immune system, macrophages play critical roles in both host defense against pathogens and proper tissue development. During embryonic development, a population of macrophages derived from yolk sac hematopoiesis can be found throughout the organism and are thought to contribute to the populations of tissue-resident macrophages in the adult. This process occurs prior to the induction of hematopoiesis in the bone marrow, strongly suggesting a unique origin and function for these embryonic macrophages [1, 2]. Additionally, embryonically derived, tissue-resident macrophages have been found in a diverse array of organs and tissues, including the mammary gland, and the maintenance of these populations does not require monocyte precursors [3]. Postnatally, however, the multistep differentiation program that leads to mature macrophages begins in the bone marrow with hematopoietic stem cells (HSCs) [4]. These c-kit$^+$/Sca-1$^+$/lineage (Lin)$^-$ HSCs give rise to two distinct multipotent progenitor populations: the c-kit$^+$/Sca-1$^+$/Lin$^-$/IL-7Rα^+ common lymphoid progenitor (CLP), which differentiate into B cells, T cells, NK cells, and a subset of dendritic cells (DCs), and the c-kit$^+$/Sca-1$^-$/Lin$^-$/IL-7Rα^- common myeloid progenitor (CMP), which can populate the erythrocyte, megakaryocyte, myeloid-derived DC, granulocyte, and monocyte compartments [4, 5]. More specific precursors of the monocyte/macrophage lineage have been identified, including the c-kit$^+$/Lin$^-$/CX3CR1$^+$ monocyte-macrophage DC progenitors (MDP) that give rise to both monocytes and dendritic cells [6]. Recent work has also identified a CD135$^-$/Ly6C$^+$ committed progenitor derived from the MDP that is restricted to the monocyte-macrophage lineage [7]. Mature CD11b$^+$/CD115$^+$ monocytes can then enter the circulation

in order to be distributed around the body. Circulating monocytes are a heterogeneous population themselves, consisting of so-called patrolling monocytes and inflammatory monocytes [8]. Patrolling monocytes are responsible for crawling along the luminal side of the endothelium to monitor for danger-associated molecular patterns (DAMPs) and, upon encountering such a signal, rapidly entering the tissue and beginning to recruit additional effector cells in order to start a productive immune response [9]. A major function of inflammatory monocytes is to respond to sites of inflammation and tissue damage. Monocytes are recruited to these sites by following a variety of chemokine gradients, the most well-characterized of which is chemokine (C-C motif) ligand 2 (CCL2) [10, 11]. Upon arriving in the vasculature near the site of inflammation, monocytes begin a process of rolling adhesion in which selectin molecules on the surface of the endothelial cells bind to selectin ligands on the monocytes [12, 13]. These interactions then allow tight binding to occur between vascular cell adhesion molecule 1 (VCAM1) on the endothelium and integrin molecules on the monocytes [14–16]. Finally, the monocytes are arrested and can exit the circulation and enter the inflamed tissue, a process known as diapedesis [12]. Once in the tissue, monocytes can be further differentiated to macrophages in the presence of colony stimulating factor-1 (CSF-1) to carry out effector functions involved in pathogen clearance, wound healing, and developmental regulation [17, 18].

Macrophages are a cell type with exquisite plasticity and are able to carry out a diverse array of functions. In order to accomplish this, macrophages respond to signals from cytokines, chemokines, growth factors, and pathogen-derived factors in the microenvironment. In the early stages of an infection, macrophages are activated by interferons produced by infected cells and by bacterial-derived compounds such as flagella, lipopolysaccharide (LPS), and unmethylated CpG motifs [19–21]. These signals typically induce a proinflammatory response in macrophages to limit pathogen spread and recruit additional innate and adaptive immune cells to the site of infection. After the infection has been controlled and the pathogen cleared, macrophages are instrumental in the resolution of inflammation to prevent further tissue damage. Cytokines such as interleukin-4 (IL-4) and IL-13 can promote an anti-inflammatory response in macrophages to block additional activation of immune cells in the tissue and promote tissue remodeling and collagen deposition [22, 23].

2. Resident Macrophages in the Mammary Gland

2.1. Mammary Gland Development. In addition to their roles in pathogen clearance and wound healing, macrophages can also respond to cytokines present in the tissue microenvironment during development, where complex and reciprocal interactions take place between epithelial and stromal cells. One particular site where such interactions take place is in the developing mammary gland. Beginning early in embryogenesis, patterning of the mammary glands occurs with the specification of the sites of the developing glands [24–26]. As development continues, epithelial cells invaginate into the surrounding mesenchyme and form the mammary bud. Just prior to birth, the cells begin to proliferate and allow the bud to invade into the adjacent fat pad. Once this has occurred, the mammary epithelial cells (MECs) begin a process of ductal morphogenesis to generate a rudimentary ductal tree [27, 28]. A prominent structure in pubertal mammary gland development is the terminal end bud (TEB), the site of actively proliferating epithelial cells. These organized structures are found at the distal end of the mammary ducts and contain cap cells and body cells, which give rise to cells of the myoepithelial and luminal lineages, respectively [29, 30]. As the cells proliferate, the TEBs advance through the fat pad until they reach the edge, at which time they regress to form the terminal ducts. At this point, side branching occurs to create secondary and tertiary ducts from the main ducts to fill the entire fat pad laterally. The mammary gland undergoes large-scale expansions and regressions during repeated estrous cycles, with new epithelial buds sprouting from the ducts and subsequently disappearing as estrogen and progesterone levels rise and fall [31, 32]. During pregnancy, however, these hormone-induced changes stop being cyclical and the gland enters a state of preparation for lactation. Alveolar buds form in response to prolactin and develop into mature alveoli to produce milk [33, 34]. After weaning, the mammary gland must return to its resting, prepregnancy state through a tightly regulated process of programmed cell death called involution [35]. At this time, the mammary gland begins to expand and regress again during estrous cycles and is ready to expand again in response to another pregnancy.

During postnatal development, numerous cytokines and hormones regulate further growth of the mammary gland. Previous work has shown that cytokines IL-4 and IL-13 are critical for promoting the differentiation and maturation of luminal epithelial cells [36]. Additionally, the requirement of estrogen receptor (ER) and progesterone receptor (PR) signaling in pubertal development has been demonstrated through elegant tissue recombination studies. While embryonic development is unaffected, mammary glands of ERα-null mice fail to elongate through the fat pad during pubertal development and lack defined TEBs [37]. Despite this lack of outgrowth, ERα-null epithelium is still responsive to progesterone and form alveoli during pregnancy. The requirement of ER signaling is limited to the epithelial cells, as transplantation of wild-type MECs into an ERα-null fat pad results in normal ductal morphogenesis [37]. Additional studies have shown a differing role for PR signaling, with transplantation of PR-null MECs into wild-type fat pad resulting in the formation of a normal ductal tree [38]. As expected, however, PR-null MECs fail to respond to progesterone during pregnancy and do not form alveolar structures. Intriguingly, transplantation of wild-type MECs into a PR-null fat results in a modest defect in ductal outgrowth, suggesting a role for PR signaling in stromal cells regulating MEC proliferation in a paracrine manner [38]. Notably, ER and PR signaling promote MEC proliferation in a paracrine manner, with previous reports demonstrating that proliferating cells are not contained within the ER^+ or PR^+

compartments [39–41]. Hormone signaling is a tightly regulated process, with any deviations above or below the optimal levels resulting in similar defects. Exposure to exogenous estrogen treatment results in decreased ductal elongation, similar to results seen in ERα-null MEC transplants; however, estrogen treatment also leads to increased lateral branching [42]. Thus, keeping hormone levels and signaling within a specified range is of critical importance for maintaining mammary gland integrity.

2.2. Macrophages in the Developing Mammary Gland. As a cell type that serves to act as a first line of defense against foreign substances and pathogens, it is only logical to have macrophages dispersed throughout the body. But in addition to their role as immunological surveyors, macrophages also play critical roles in regulating mammary gland development. Previous studies have indicated that macrophages are found in close association with MECs at many well-characterized stages of mammary gland development [43]. Immunostaining of mammary glands for the macrophage marker F4/80 indicates the presence of macrophages surrounding the body cells of the TEB [43, 44]. These macrophages are poised to phagocytose cellular debris from MECs undergoing apoptosis while generating the hollow lumen of the mammary ducts [45]. At maturity, macrophages can be found lining the mammary ducts where they promote epithelial cell proliferation and differentiation through production of growth factors, chemokines, and inflammatory mediators. During lactation, $F4/80^+$ macrophages have been observed in close proximity to the alveoli and are a major cellular component of milk [43, 44, 46]. Once lactation is completed and weaning occurs, the mammary gland undergoes involution to return to its prepregnant state, involving large amounts of apoptosis and extracellular matrix (ECM) remodeling. Again, macrophages are major contributors to this process, phagocytosing apoptotic cellular debris and producing matrix remodeling factors to facilitate the transition back to the fully involuted state [47, 48].

Numerous studies have been undertaken using genetic and biochemical approaches to deplete macrophages during mammary gland development. Mice homozygous for a null mutation in CSF-1, the critical factor required for macrophage differentiation, show significant impairment in ductal elongation during mammary gland development [49]. This defect can be rescued through the use of a tetracycline-inducible transgene to reexpress CSF-1. Architecturally, organization of collagen I into long fibers around the neck of the TEBs is impaired in CSF-1-deficient mice while total collagen I deposition is unaffected, implicating a specific role for macrophages in regulating collagen organization but not collagen biosynthesis [50]. The contributions of macrophages to estrous-cycle induced changes were described elegantly using the CD11b-DTR inducible mouse model of macrophage depletion. Macrophages are found at different frequencies in the mammary gland during the estrous cycle, reaching a maximum during diestrus. Depletion of macrophages resulted in a nearly 50% reduction in alveolar bud formation in response to progesterone treatment and an overall decrease in MEC proliferation [51]. Additional work using sublethal irradiation has demonstrated that cells of the hematopoietic lineage are required for the formation of TEBs during pubertal development and that macrophages modulate their immunostimulatory profile over the course of the estrous cycle [45, 52].

While these studies clearly demonstrate that a role for macrophages is regulating mammary gland development, the mechanism by which this occurs remains unclear. One possible mechanism is that macrophages in the microenvironment respond to the same cytokines and growth factors required for epithelial cell development and respond in a unique way. IL-4 and IL-13 have been implicated in mammary epithelial cell differentiation and are found at measureable amounts in the developing mammary gland [36]. When exposed to these cytokines, macrophages respond by producing a host of anti-inflammatory factors and tissue remodeling agents known to be needed during mammary gland development. Studies of macrophages in infection models have illustrated that tissue-resident macrophages are more predisposed to an anti-inflammatory response compared to monocyte-derived macrophages recruited from the circulation [53, 54]. Transforming growth factor-beta (TGF-β) and members of the matrix metalloproteinases (MMP) family are produced by macrophages at high levels in response to IL-4/IL-13 stimulation *in vitro* [19, 55]. In the setting of the mammary gland *in vivo*, MMPs are required to degrade and remodel the ECM to allow further ductal elongation to occur through the fat pad, while TGF-β plays a suppressive role to limit the extent of ductal branching [56–59]. Thus, it is possible that IL-4 and IL-13 play dual roles in the microenvironment: promoting MEC differentiation and stimulating tissue-resident macrophage function. While ductal elongation is driven primarily by ovarian-produced estrogen, studies in breast cancer have shown that macrophages themselves are capable of producing estrogen locally through the expression of the estrogen synthesizing enzyme aromatase [60]. There is a relative lack of knowledge to date regarding the role of macrophage-produced estrogen, but it is tempting to speculate that macrophages associated with the TEBs or lining the mammary ducts could regulate development and proliferation directly by creating pools of locally concentrated estrogen. Further studies are warranted to determine if macrophages express aromatase *in vivo* and how the resulting rise in estrogen levels in the mammary gland affects development. In addition, the increased estrogen and proliferative signals in the mammary gland may also help establish a protumorigenic environment, in which the MECs are primed for the tumor initiation when exposed to an oncogenic insult. Understanding how changes that take place in the mammary gland during development can affect tumor initiation at a later point in life is critical in developing preventative strategies through life-style changes and therapeutic intervention.

2.3. Effects of Inflammation on Resident Macrophages. Recent evidence has supported the long-postulated idea that chronic inflammation enhances the risk of developing cancer [61–64].

Furthermore, diseases with systemic inflammatory components are major risk factors for certain types of cancer, including breast cancer [61, 65]. In patients with Crohn's disease, increased expression of the proinflammatory cytokine tumor necrosis factor-alpha (TNFα) recruits inflammatory macrophages and leads to the production of additional proinflammatory factors, initiating a feed-forward loop which leads to tissue damage and predisposition to oncogenic initiation [66]. One of the most common diseases associated with cancer risk is obesity, with 34.9% of adults in the United States being classified as obese [67]. Patients with obesity often have elevated serum levels of proinflammatory molecules, such as IL-6, which induce a systemic chronic inflammatory state [68]. In the mammary gland microenvironment specifically, obesity is directly linked with increased IL-6 signaling and increased macrophage recruitment compared to normal-weight mammoplasty specimens [69]. In a resting state, the amount of proinflammatory and anti-inflammatory signals are maintained in a state of equilibrium (Figure 1). However, in pathologic settings such as obesity, inflammatory homeostasis is lost and the balance is tipped in favor of proinflammatory factors. In these cases, the increased abundance of proinflammatory factors relative to anti-inflammatory factors affects cells in the microenvironment. Once macrophages are exposed to proinflammatory factors they upregulate the production of additional proinflammatory factors, creating a feed-forward loop that further upsets inflammatory homeostasis. It is interesting to speculate why obese patients with increased levels of IL-6 have a predisposition to developing ER^+ breast cancers specifically [70]. Studies focused on endometrial carcinoma have revealed a paracrine signaling axis whereby cancer cells produce IL-6 to stimulate stromal cells to upregulate aromatase and produce estrogen, thus inducing a cycle of increased cancer cell proliferation and IL-6 production [71]. It remains to be seen if a similar axis exists in breast cancer, but with their role in regulating mammary gland development, it is not difficult to hypothesize that macrophages may upregulate aromatase expression in response to IL-6 in the context of obesity, thus providing a mechanistic explanation of the propensity for obese women to develop ER^+ breast tumors.

In addition to pathologic inflammatory conditions, acute inflammatory responses in the context of normal tissue processes can have profound impacts on the microenvironment. In the 5-year period following childbirth women are susceptible to developing postpartum breast cancer with a particularly poor prognosis [72]. Elegant xenograft studies in mice have revealed that the microenvironment of the involuting mammary gland significantly enhances tumor growth compared to nulliparous mammary glands [73]. Most recently, an overall profile was created to determine the relative abundance of immune cells during the process of involution compared to nulliparous and lactating glands. While modest changes were observed in DC recruitment at all time points of involution, a near 10-fold increase in macrophage recruitment is observed during the first week of involution and remains elevated at 4 weeks after weaning [47]. This increased macrophage recruitment was accompanied by increased $CD4^+$ and $CD8^+$ T cell recruitment and an increased presence of $FOXP3^+$ regulatory T cells. Mechanistically, the microenvironment of the involuting mammary gland induces macrophages to take on an immunosuppressive profile by producing IL-10 and suppressing T cell activation [47]. This acute disruption of inflammatory homeostasis results in the formation of a protumorigenic niche through direct suppression of adaptive immunity. A better understanding of the critical balance between proinflammatory and anti-inflammatory factors is clearly needed in order to develop new therapeutic regimens for the treatment and even prevention of breast cancer.

3. Macrophages in the Tumor Microenvironment

In addition to their contributions to normal mammary gland development, macrophages are well-established constituents of the breast tumor microenvironment. Increased macrophage density in pretreatment biopsies of breast cancer patients correlates with reduced recurrence-free and overall survival [74–76]. Therefore, efforts have focused on understanding the mechanisms through which macrophages contribute to breast cancer growth and progression and these topics have been reviewed extensively [19, 55, 77–79]. Myeloid cells in the tumor microenvironment, in particular macrophages, have been shown to contribute to tumor growth and progression in a variety of ways. Tumor-associated macrophages (TAMs) secrete soluble factors, such as vascular endothelial growth factor (VEGF), which induce angiogenesis and partially relieve the hypoxic stress within fast-growing tumors [80]. In addition to promoting angiogenesis, TAMs support tumor cell survival, migration, and invasion through the secretion of growth factors such as EGF and FGFs and chemokines such as CXCL1/2 [81–84]. Of note, TAMs not only secrete factors but also facilitate the release of protumorigenic factors from the ECM, a topic discussed later in this review. In recent studies using intravital imaging techniques, Lohela et al. demonstrated that prolonged depletion of myeloid-derived cells in a model of breast cancer resulted in delayed tumor growth, decreased angiogenesis, and fewer lung metastases [85]. Furthermore, production of growth factors and ECM remodeling by TAMs have been implicated in promoting breast cancer resistance to chemotherapeutic agents such as paclitaxel, doxorubicin, and etoposide (reviewed in [86]). Finally, numerous studies have provided evidence of TAMs interacting with cells of the adaptive immune system, mainly $CD4^+$ and $CD8^+$ T lymphocytes, and both directly and indirectly suppressing their antitumor effects [87–89].

Understanding macrophage functions in the context of normal tissue development can provide insights into the functions of macrophages during tumor growth and progression. Specifically, there are parallels between the mechanisms of macrophage recruitment and macrophage-mediated alterations in ECM in both the normal mammary gland and the tumor microenvironment. Furthermore, it is becoming clear that the balance between proinflammatory and anti-inflammatory factors is key to the regulation of

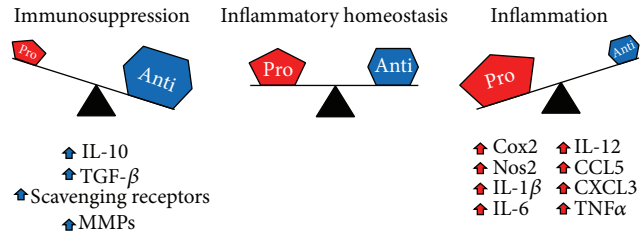

Figure 1: Tissue-resident macrophages are important for maintaining a state of inflammatory homeostasis. Under normal conditions, pro- and anti-inflammatory signals are maintained in a balanced state referred to as inflammatory homeostasis (center). During the early stages of infection or tissue damage, increased production of proinflammatory factors can tip the balance towards an overall inflammatory state (right). During late stages of infection and wound healing, the production of anti-inflammatory factors is significantly increased, leading to an immunosuppressive state (left). A failure to return to inflammatory homeostasis leads to chronic inflammation or immunosuppression and can lead to the development of numerous pathologies, including cancer.

macrophage function within the tumor microenvironment. Therefore, further discussion will focus on macrophage recruitment, polarization, and regulation of ECM within the tumor microenvironment.

3.1. Recruitment of Macrophages to the Tumor Microenvironment. As mentioned above, CCL2 and CSF-1 are important for both recruitment and differentiation of macrophages in the normal mammary gland. Likewise, these factors have been implicated in recruitment of macrophages to both primary and metastatic tumor sites. Using genetic approaches, seminal studies demonstrated that CSF-1 is critical for macrophage recruitment and differentiation in tumor microenvironment of MMTV-PyMT mice [90]. These studies demonstrated that reduced macrophage infiltration significantly reduced the ability of the tumor cells to metastasize to the lung. Tumor cell-derived CSF-1 has also been linked to the proliferation of a protumor subset of $CD11b^{lo}F4/80^{hi}$ macrophages in the MMTV-Neu transgenic model of mammary tumor growth [91]. In these studies, administration of the CSF-1R inhibitor GW2580 to tumor bearing mice drastically reduced the numbers of $CD11b^{lo}F4/80^{hi}$ macrophages in S phase. These, and other recent studies, suggest that in addition to recruitment of monocytes from the bloodstream, certain TAM populations are able to proliferate within the tumor microenvironment [91–93]. Taken together, these studies indicate that therapies aimed at targeting the accumulation and/or proliferation of TAMs may improve clinical outcomes for breast cancer patients, and as a result CSF-1R inhibitors and blocking antibodies have entered clinical trials for various cancer types, including breast cancer. In a recent report, Ries et al. described a significant depletion of $CD68^+/CD168^+$ macrophages in a small cohort of breast cancer patients and among those receiving the highest protocol dose, analysis revealed a switch of lymphocyte infiltrates from $CD4^+$ T cells before treatment to $CD8^+$ T cells after treatment [94]. This study provides proof-of-principal that blockade of the CSF-1/CSF-1R pathway results in fewer macrophages recruited to human breast tumors, and this change in myeloid recruitment affects the overall composition of the tumor microenvironment.

Another key chemokine that has been implicated in macrophage recruitment to the tumor microenvironment is CCL2/MCP-1. Numerous studies have found that tumor cell-derived CCL2 promotes macrophage recruitment both *in vitro* and *in vivo* [95–97]. In recent studies, both CCL2 and CCL5/RANTES were found to correlate with increased macrophage recruitment in human patient samples, and specifically in ER^+ samples [98]. Using estrogen-supplemented oophorectomized mice bearing MMTV-PyMT mammary tumors, further studies demonstrated that inhibition of either CCL2 or CCL5 using blocking antibodies resulted in reduced macrophage infiltration and reduced tumor growth [98]. In addition to promoting recruitment of macrophages to the primary tumor site, CCL2 has also been implicated in indirectly promoting the seeding and growth of tumor cells in the metastatic site. Specifically, CCL2 was found to recruit a distinct population of macrophages termed metastasis-associated macrophages, defined as $CD11b^+Ly6C^{high}$, to the lung metastatic site [10]. Once localized to this site, CCR2 activation stimulates macrophages to secrete an additional chemokine, CCL3, which contributes to tumor cell-macrophage interactions and retention in the metastatic site through activation of CCR1 [99]. Taken together, these studies suggest that blocking macrophage recruitment through inhibition of chemokine signaling may effectively reduce macrophage contributions during tumor growth and progression. However, some challenges have been associated with targeting chemokines including the induction of compensatory mechanisms in response to chemokine inhibition. In a recent study evaluating CCL2 blockade, Bonapace et al. found that while blocking CCL2 reduced lung metastasis, which was maintained upon continuous CCL2 inhibition, cessation of CCL2 neutralization led to increased metastasis and accelerated death [100]. Assessment of combinatorial therapies, which included targeting additional cytokines, such as IL-6, that were increased in the lungs upon treatment cessation, alleviated the increase in metastasis. Thus, these studies suggest that targeting chemokines, such as CCL2, as a therapeutic strategy should be approached with caution and could possibly require combination-based approaches for success.

In addition to CSF-1 and CCL2, other chemokines have also been linked to macrophage recruitment in the primary tumor site. Using an inducible model of mammary tumorigenesis, we identified CX3CL1 as a mediator of macrophage recruitment to early stage mammary hyperplasias [101]. More recent studies have linked CX3CL1 expression with poor outcome in breast cancer patients [102], although whether high CX3CL1 is linked to macrophage recruitment in human breast cancer samples remains to be determined. Boyle et al. recently reported that CCL20-CCR6 axis is important for regulating macrophage recruitment into mammary tumors of MMTV-PyMT mice [103]. In these studies, growth of mammary tumors in CCR6-knockout mice led to reduced mammary tumor initiation and growth. Further analysis of these tumors revealed a reduction in immune cell infiltration along with changes in macrophage polarization as shown by reduced expression of IL-4R and CD206. Importantly, reconstitution of TAMs into CCR6-knockout mice bearing orthotopically transplanted MMTV-PyMT tumors restored tumor growth demonstrating the importance of this chemokine axis for mammary tumor growth. In addition to general recruitment to the tumor microenvironment, a subpopulation of macrophages is also known to accumulate in hypoxic regions within tumors. Recruitment of macrophages into hypoxic regions is mediated through soluble factors such as VEGF, endothelin-2, and angiopoietin-2 [104, 105]. Semaphorins, such as Sema3A, were recently linked to recruitment of macrophages to hypoxic regions via a neuropilin-1-dependent mechanism [106]. Additional recent studies have also found that hypoxic cancer cells produce chemoattractants that promote macrophage recruitment, including oncostatin M and eotaxin, which also act to polarize macrophages to a protumor phenotype and are required for tumor progression [107]. Taken together, these studies demonstrate that macrophage recruitment into the tumor microenvironment can be driven by many different factors, highlighting the complexity of the mechanisms driving macrophage infiltration.

Although less extensively studied compared with tumor cell-derived chemokines, stromal cells, including carcinoma associated fibroblasts (CAFs), mesenchymal stem cells (MSCs), and endothelial cells, also produce chemokines that can potentially recruit macrophages into the microenvironment. Stimulation of CAFs and MSCs with tumor cell-derived conditioned media leads to upregulation of various chemokines, including CCL2, CXCL8, and CCL5 [108]. Furthermore, Yoshimura et al. demonstrated that stromal cell-derived CCL2 contributes to macrophage recruitment to 4T1 tumors and that loss of stromal cell CCL2 leads to decreased lung metastasis [109]. Recent genetic studies have demonstrated a critical role for BMP signaling in the regulation of chemokines from fibroblasts. Specifically, loss of BMPR2 from fibroblasts led to increased metastasis of MMTV-PyMT tumors corresponding with increased chemokine expression and increased infiltration of myeloid cells [110].

In addition to chemoattractants derived from tumor and stromal cells, there is evidence that tumor-associated ECM may also contribute to macrophage recruitment. For example, collagen fragments are known to be chemotactic for inflammatory cells [111]. Furthermore, it has been proposed that proteolysis of collagen I promotes macrophage recruitment into the involuting mammary gland, which is characterized as a tumor-promoting environment [112]. Another ECM component linked to macrophage recruitment is hyaluronan, which is a glycosaminoglycan consisting of repeating disaccharide subunits of glucuronic acid and N-acetylglucosamine. Macrophages are often associated with a hyaluronan-containing matrix within the tumor environment, and studies have suggested that hyaluronan can act directly on macrophages to regulate their migration [113]. Specifically, hyaluronan has been shown to promote macrophage chemotaxis using *in vitro* chemotaxis assays [114]. Consistent with these findings, *in vivo* studies have demonstrated that reduction of hyaluronan in the mammary tumor stroma correlates with decreased macrophage infiltration [115]. Taken together, the numerous studies focusing on macrophage recruitment demonstrate that macrophage infiltration into the tumor microenvironment can potentially be mediated by a variety of factors (Figure 2). Further studies are warranted to understand the relative contributions of tumor cell versus stromal cell derived chemokines and ECM components to macrophage recruitment during tumor growth and progression.

3.2. Macrophage Polarization within the Tumor Microenvironment. Once recruited to the tumor microenvironment, macrophages respond to the plethora of stimuli within the microenvironment and differentiate into various effector subsets. Numerous studies have focused on defining macrophage subsets within the tumor microenvironment. Currently, the most widely accepted classification of macrophage polarization is based on descriptions of classical (M1) versus alternative (M2) polarization, which were developed as a result of initial studies investigating macrophage responses to helper T cells 1 (Th1) and helper T cell 2 (Th2) derived molecules [116]. Classically activated macrophages develop in response to interferon-gamma (IFNγ) and pathogen-derived toll-like receptor ligands [19, 117]. This response is characterized by the production of cytotoxic factors such as reactive oxygen species and nitric oxide, increased rates of phagocytosis, and enhanced antigen presentation on the cell surface. Alternatively activated macrophages, on the other hand, develop as part of the wound healing program and as such are thought to antagonize inflammation. M2 macrophages are induced by the Th2 cytokines IL-4 and IL-13, as well as in response to IL-10, immunoglobulins, and glucocorticoids [55, 118]. These cells, in turn, secrete factors that promote angiogenesis, upregulate expression of scavenging receptors, and produce enzymes to remodel the surrounding extracellular matrix. As interest and work in the field of macrophage biology has expanded, the nomenclature describing the activation status of macrophages has become complex and often confusing. In an attempt to streamline the methods used to generate and describe the cells used by the different research groups, Murray et al. published a comprehensive set of recommendations which will undoubtedly simplify future analysis and comparison of macrophage subsets [119].

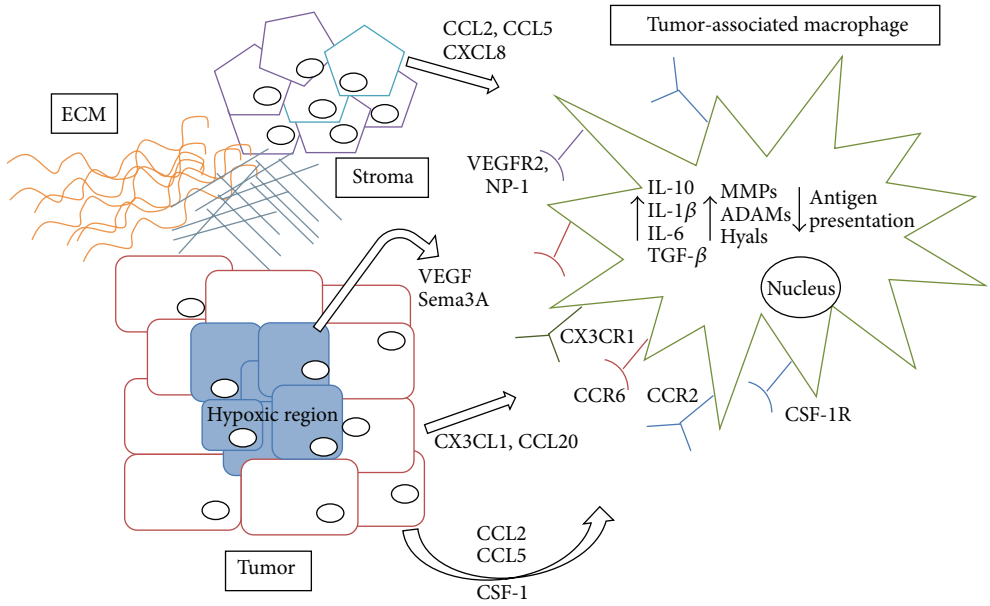

Figure 2: Complex interactions in the tumor microenvironment. Breast cancer cells located in the tumor periphery (red rectangles) secrete cytokines and chemokines, which recruit monocytes from the circulation and differentiate them into tumor-associated macrophages (TAMs). Tumor cells located in the inner, hypoxic region (blue rectangles) develop a more specialized array of molecules to recruit macrophages poised to help the hypoxic cells survive and proliferate. The stromal cells of the tumor, along with components of the extracellular matrix (such as collagen I and hyaluronan), additionally contribute to the recruitment and retention of TAMs. Once educated by the tumor microenvironment, TAMs upregulate pathways associated with both M1- and M2-activated macrophages and actively support the survival, proliferation, and metastasis of breast cancer cells.

Based on their functions within the tumor microenvironment, TAMs have been generally characterized as M2-like [55]. Several studies have demonstrated that TAMs express higher levels of scavenging receptors, angiogenic factors, and proteases, similar to M2 macrophages. Furthermore, TAM polarization to the M2-like phenotype in the MMTV-PyMT model has been attributed to IL-4-producing Th2 cells within the tumor microenvironment [89]. However, there is evidence that macrophages exhibit different phenotypes during different stages of tumor initiation and progression. During early stages of transformation, recently recruited macrophages are exposed to a wide variety of proinflammatory signals derived from the epithelial cells and the surrounding stroma and often express M1-related factors that have protumorigenic properties, such as IL-1β and IL-6 [120, 121]. As a component of the proinflammatory response, production of reactive oxygen and nitrogen species could also potentially enhance the rate of epithelial cell mutation and thus accelerate tumorigenesis [122]. In established tumors, macrophages exhibit alternatively activated functions including the production of immunosuppressive factors, such as IL-10 and TGF-β, which are capable of actively suppressing the antitumor immune response [79, 88, 89]. These macrophages also produce growth factors and remodel the matrix, supporting tumor cell growth and enhancing invasion. Therefore, TAM phenotypes are now thought to include a combination of markers typically assigned to the M1 and M2 phenotypes. Thus, as efforts are being made to "repolarize" macrophages within the tumor microenvironment towards the M1/classically activated phenotype, care must be taken to ensure that the potentially protumorigenic functions of these macrophages are suppressed.

Recent sophisticated analyses utilizing genomewide studies and RNA-sequencing have revealed that macrophage phenotypes *in vivo* are far more heterogeneous and complex than initially expected. Xue et al. performed a detailed transcriptome analysis of primary human monocytes stimulated with 28 different signals, the results of which suggest a "spectrum" model where 9 different macrophage activation programs were identified in response to different combinations of stimuli [123]. Analysis of the enriched gene sets in human macrophages derived from smokers and COPD patients revealed activation programs within these primary macrophages that were significantly different from the hypothesized phenotypes. In smokers' samples, a complex network of stimuli including glucocorticoids, free fatty acids, and IL-4 were detected, while in COPD patient samples the previously published IL-4/IL-13 associated gene signatures were not reproduced and instead a profound loss of inflammatory genes was reported [123]. These results demonstrate the complexity of activating signals responsible for the phenotypes of macrophages in human pathologies, and they suggest that a simple bipolar M1/M2 paradigm may not be sufficient to describe macrophages associated with disease states. Based on the observation that the microenvironment of lung disease is capable of producing a spectrum of macrophage activation states, it seems likely that this heterogeneity would also be observed in the tumor microenvironment. Indeed, while performing gene-expression profiling on TAMs and mammary tissue macrophages from

tumor bearing MMTV-PyMT mice, Franklin et al. observed few canonical M2 markers to be upregulated in the TAM population [92]. Instead, they reported TAM differentiation to be dependent on signaling of the transcription factor *Rbpj*, a key regulator of canonical Notch signaling.

In addition, recent evidence suggests that individual tumors may contain several different subsets of macrophages and those might differ in their functions. Movahedi et al. reported the presence of two distinct TAM populations in mammary TS/A tumors, distinguishable most easily by the level of MHCII expression on their surface [124]. $MHCII^{lo}$ macrophages were shown to reside mainly in hypoxic tumor regions and expressed markers associated with M2 polarization. The $MHCII^{hi}$ subset, however, expressed M1-signature genes such as *Cox2, Nos2,* and *IL-12*. These cells were shown to secrete proinflammatory cytokines and chemokines such as IL-6, CCL5, and CXCL3, which could in turn serve to further recruit additional proinflammatory cells to the tumor margins. However, both macrophage subsets were shown to be poor antigen presenting cells and were able to suppress T cell proliferation, indicating that both subsets might be capable of contributing to protumor immunosuppression. Interestingly, Ruffell et al. observed a similar localization of $MHCII^{lo}$ and $MHCII^{hi}$ TAMs in mammary tumors derived from MMTV-PyMT mice; however, the ability of TAMs to suppress $CD8^+$ T cell proliferation was limited to the $MHCII^{lo}$ subset of cells [88]. These findings indicate that some TAM properties are most likely universal (recruitment, localization), whereas other properties (specific interactions with other infiltrating cells) might be dependent on the tumor model under investigation. In a recent study examining macrophage localization within human breast tumors, high $CD68^+$ macrophage staining within gaps of ductal tumor structures correlated with reduced lymph node metastasis [125]. Taken together, these data suggest that TAMs represent a macrophage population that is distinct from both M1 and M2 macrophages as they are canonically described in the setting of infection, but there is most likely a spectrum of TAMs whose phenotype and function depend on tumor type and location within the tumor.

3.3. Macrophage Regulation of ECM within the Tumor Microenvironment. One of the identified mechanisms through which macrophages may regulate ductal elongation during mammary gland development is through organization of ECM, such as collagen [50]. While some functions of TAMs in the tumor microenvironment, including promotion of tumor cell migration and invasion, angiogenesis, and suppression of adaptive immune responses, have been extensively examined, the contributions of macrophages to the modulation of ECM remain relatively understudied. Macrophages actively contribute to the changes in ECM through the production of ECM components and through the release of factors that cleave ECM. Consequently, ECM components and their fragments can act directly on macrophages to promote their recruitment, retention, and function. One of the mechanisms through which alternatively activated macrophages contribute to resolution of inflammation is through producing and remodeling the ECM. Therefore, it is not surprising that alternatively activated macrophages produce ECM. Macrophages have been found to produce fibronectin [126] and collagen [127], including high levels of type VI collagen, which is increased in alternatively activated macrophages and promotes monocyte adhesion [128]. While studies focusing on the contributions of macrophages to ECM deposition in the context of breast cancer are limited, it is worth noting that collagen VI is found at the invasive edge of breast tumors, where macrophages are known to localize, and promotes epithelial-mesenchymal transition of cancer cells [128]. Thus, studies aimed at determining whether macrophages at the leading edge contribute to these high levels of collagen VI are warranted.

In addition to producing ECM, macrophages express high levels of proteases that can contribute to the cleavage and remodeling of ECM. Gene profiling of TAMs demonstrates increased expression of proteases including MMPs, ADAMs, and cathepsins [81]. Macrophage-derived proteases can contribute to protumor alterations in the stroma in a number of ways including facilitating ECM breakdown for subsequent invasion and migration, liberation of tumor-promoting factors from the ECM, and generation of bioactive ECM fragments. For example, macrophage-derived MMPs have been linked to the release of angiogenic factors, such as VEGF and FGFs, in the tumor microenvironment [81]. In addition, studies demonstrated that alternatively activated macrophages are directly involved in collagen turnover, specifically through uptake and degradation of collagen by CX3CR1-positive cells involving the mannose receptor [129]. Uptake of collagen requires MMP activity, potentially linking macrophage regulation of collagen to both cleavage and uptake.

Macrophages may also regulate hyaluronan in the tumor microenvironment. Hyaluronan is generated as a high molecular weight glycosaminoglycan that can be broken down into fragments that are characterized as inflammatory and protumorigenic [113]. Hyaluronan cleavage occurs through enzymatic degradation by hyaluronidases (Hyals) or by mechanisms involving reactive oxygen and nitrogen species [130]. Macrophages have been found to express hyaluronidases [131] and can thus potentially contribute to the breakdown of hyaluronan during inflammation and tumor progression. In turn, it has been suggested that hyaluronan can direct macrophage function. Exposure of macrophages to hyaluronan, either purified or tumor-derived, leads to increased expression of various inflammatory mediators including IL-1β [132] and IL-10 [133]. In recent studies, tumor-derived microvesicles were found to induce IL-10 expression in macrophages using a hyaluronan-dependent mechanism through the PI3K/Akt/mTOR pathway [134]. Together, these studies suggest a link between hyaluronan and regulation of macrophage function, possibly through enhancing immunosuppressive function. Together, these observations suggest that macrophages are likely to be important regulators of ECM production and remodeling in the tumor microenvironment, and further studies are warranted to define the specific functional consequences of these actions.

4. Summary

In conclusion, it is clear that inflammation is a complex process that has evolved to resolve damage to the body caused by pathogens or disease. In the normal mammary gland, tissue-resident macrophages play a vital role in the regulation of development and maintenance of tissue homeostasis. Pro- and anti-inflammatory factors produced in the microenvironment act not only on epithelial cells, but also on macrophages and lead to the further disruption of inflammatory homeostasis and the creation of a protumorigenic niche that is primed for oncogenic initiation. Tumor cells acquire the capacity to harness the functions of inflammatory cells, such as macrophages, to aid in their growth and progression. Experimental studies have demonstrated that macrophages interact with cancer cells and their phenotype and function evolve as the tumor itself evolves. However, recent studies demonstrating the complexity of macrophage polarization and the impact of macrophage localization within the tumor microenvironment suggest that the contributions of macrophages to breast cancer growth and progression are likely to be quite complex. Therefore, it will be critical to obtain a better understanding of the mechanisms that drive macrophage recruitment, polarization, and function within the tumor microenvironment at different stages of breast cancer formation and progression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Nicholas J. Brady and Pavlina Chuntova contributed equally.

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Research Article

Blood Genome-Wide Transcriptional Profiles of HER2 Negative Breast Cancers Patients

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Tumors act systemically to sustain cancer progression, affecting the physiological processes in the host and triggering responses in the blood circulating cells. In this study, we explored blood transcriptional patterns of patients with two subtypes of HER2 negative breast cancers, with different prognosis and therapeutic outcome. Peripheral blood samples from seven healthy female donors and 29 women with breast cancer including 14 triple-negative breast cancers and 15 hormone-dependent breast cancers were evaluated by microarray. We also evaluated the stroma in primary tumors. Transcriptional analysis revealed distinct molecular signatures in the blood of HER2− breast cancer patients according to ER/PR status. Our data showed the implication of immune signaling in both breast cancer subtypes with an enrichment of these processes in the blood of TNBC patients. We observed a significant alteration of "chemokine signaling," "IL-8 signaling," and "communication between innate and adaptive immune cells" pathways in the blood of TNBC patients correlated with an increased inflammation and necrosis in their primary tumors. Overall, our data indicate that the presence of triple-negative breast cancer is associated with an enrichment of altered systemic immune-related pathways, suggesting that immunotherapy could possibly be synergistic to the chemotherapy, to improve the clinical outcome of these patients.

1. Introduction

Breast cancer, the most diagnosed malignancy in women [1], is a highly heterogeneous disease presenting a broad range of molecular, biological, and clinical characteristics. Despite the advances in molecular classification of breast cancer [2–5], identifying of clinically relevant subgroups is still based on the status of estrogen and progesterone receptor (ER and PR) and human epidermal growth factor receptor 2 (HER2) along with clinicopathological variables. Currently, breast cancer is categorized into three main therapeutic groups: ER-positive (ER+), HER2-positive (HER2+), and triple-negative breast cancer (TNBC/ER−PR−HER2−). ER+ tumors account for about 70% of breast cancer that respond well to endocrine therapy and have a good prognosis and survival (5-year survival rate of 85%) [6]. Among ER+ tumors, HER2 negativity is associated with a better prognosis when compared with HER2+ tumors. Overall, overexpression of HER2, identified in about 20% of breast cancer, is associated with a more aggressive phenotype but, however, survival of these patients has been dramatically improved by the development of drugs targeting this receptor (trastuzumab, lapatinib,

and pertuzumab) [7]. Unlike the ER+ or HER2+ breast cancers, triple-negative tumors lack a validated targeted therapy, with conventional chemotherapy remaining the standard of care. As a result, TNBC subtype tends to have a poor clinical outcome and an increased risk of recurrence and distant metastasis. Therefore, there is a major concern regarding the identification of new therapeutic targets for this subtype and developing an effective targeted therapy for these patients.

Gene expression profiling of peripheral blood cells arises as a valuable tool to evaluate gene signatures related to solid tumors. The reason to use blood cells as "sensors" to characterize tissue tumors is based on the fact that blood circulating cells monitor the body's physiological status and modify their expression pattern in response to pathological changes. Previous studies on peripheral blood revealed specific signatures related to lymphomas and leukemia as well as inflammatory and autoimmune diseases [8–10]. Gene expression signatures in peripheral blood of breast cancer patients were associated with early detection of tumors [11, 12], predicting metastasis [13, 14], or treatment response to therapy [15]. However, the tumor-blood communication involves a large spectrum of signaling molecules and deciphering their role still represents a great challenge.

In line with this view, the overall aim of this study was to evaluate the mRNA-peripheral blood profile of two HER2− breast cancer subtypes, including hormone-dependent breast cancer (ER+PR+HER2−) and triple-negative breast cancer (TNBC/ER−PR−HER2−), known to have the best and the worst prognosis, respectively.

2. Materials and Methods

2.1. Blood Sample Collection and Processing. Twenty-nine female breast cancer patients were recruited for this study between August 2010 and September 2012 at The Oncology Institute "Prof. Dr. Ion Chiricuta," Cluj-Napoca (IOCN), Romania. The study was approved by the ethical committees of the University of Medicine and Pharmacy "Iuliu Hatieganu," Cluj-Napoca, Romania, and the IOCN, the coordinators of this study. All patients provided informed consent in accordance with the Declaration of Helsinki. The patients were included in the study if they met the following criteria: (a) were recently diagnosed with invasive breast cancer, (b) had negative HER2 status (HER2−) in the primary tumors, (c) did not present metastasis or secondary malignancies, and (d) were not treated prior to or during the collection of biological samples. The status of ER, PR and HER2 was assessed by immunohistochemistry and staging was done according to AJCC criteria by a certified pathologist (Table 1). Additionally, a group of 7 healthy women was considered as control (CTR).

From each subject, 4 mL of peripheral blood was collected in EDTA anticoagulant tubes. At the time of collection, none of the participants had fever or any acute diseases, followed anticoagulant therapy, or received chemotherapy, radiotherapy, or immunotherapy. Blood samples were collected between 8 a.m. and 12 p.m. for all subjects. They were immediately stored on ice and processed following a standardized protocol. Briefly, after plasma removal and RBC lysis, total RNA from nucleated blood cells was extracted using TriReagent (Ambion/Thermo Fisher, Waltham, MA, USA) and purified with the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The quality of purified RNA was assessed with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and a Nanodrop-ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to quantify the extracted RNAs.

RNA Integrity Number (RIN) was used to define the quality of RNAs. All extracted RNAs had RIN between 8 and 10 and were considered for further analysis. The RNAs were stored at −80°C until further processing for microarray.

2.2. Microarray Experiment. One hundred nanograms of total RNA was used for the synthesis of one-color microarray probes using the Agilent Low Input Quick Amp Labeling Kit according to Agilent's protocols. Before hybridization, microarray probes (cRNA-Cy3) were purified with the RNeasy Mini Kit (Qiagen, Hilden, Germany). All microarray probes with a minimum concentration of 1.65 μg and specific activities of 6 pmol/μL Cy3 were considered for hybridization. The cRNAs-Cy3 probes were hybridized for 17 hours at 65°C on human gene expression 4 × 44 k v2 microarray slides (G4845A) following the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). Slides were scanned with an Agilent G2565CA microarray scanner, and image processing was done using Feature Extraction Software v.11.0.

2.3. Microarray Data Analysis. The datasets containing array signal intensities were imported and analyzed in Gene Spring GX v.11.5. Quantile normalization was used to correct for interarrays global differences. Nonuniform, outlier, and saturated spots were filtered, and only sequences with acceptable flags in minimum 90% samples were retained for analysis. Differences in gene expression between the three studied groups (ER−PR−HER2−, ER+PR+HER2−, and CTR) were tested by using one-way ANOVA followed by Tukey's post hoc test. When two groups were considered (all breast cancer samples, named as BC, versus CTR) differences in gene expression were assessed by unpaired t-test. For all of the comparisons, p values were adjusted for multiple testing by the Benjamini-Hochberg FDR method. Genes were considered to be differentially expressed when their expression exceeded 1.5-fold between groups and the adjusted p value was less than 0.05. Differentially expressed gene profiles were further used to compute a supervised cluster based on the Euclidean distances and Ward algorithm.

2.4. Ingenuity Pathways Analysis (IPA). The four lists of differentially expressed genes between groups, containing Agilent probe set IDs and fold changes, were uploaded into IPA (Ingenuity Systems, http://www.ingenuity.com/) and queried against a background specific model (Agilent Whole Human Genome Microarray 4 × 44 k v2). IPA Core Analysis function was used to examine which biological processes and pathways were affected by gene expression changes observed

Table 1: Baseline clinical and histological characteristics of the HER2− patients.

Number	Age	Clinical stage	TNM staging	Nottingham score	Menopause age	ER/PR status
1	58	II B	T2N1M0	II	#	ER−/PR−
2	53	III A	T2N2M0	II	50	ER−/PR−
3	40	III B	T4bN2M0	II	39	ER−/PR−
4	45	III A	T3N1M0	III	#	ER−/PR−
5*	48	II B (R)/I A (L)	T2N1M0(R)/T1N0M0(L)	III	32	ER−/PR−
6	49	II B	T2N1M0	III	#	ER−/PR−
7	50	III B	T4bN1M0	II	#	ER−/PR−
8*	55	III B (R)/I B (L)	T4bN2M0(R)/T1N0M0 (L)	III	51	ER−/PR−
9	56	II B	T2N1M0	III	N/A	ER−/PR−
10	60	II A	T1N1MO	III	45	ER−/PR−
11	35	II B	T2N1M0	III	#	ER−/PR−
12	53	III A	T2N2M0	III	50	ER−/PR−
13	40	II B	T2N1M0	III	#	ER−/PR−
14	74	III B	T4bN2M0	III	48	ER−/PR−
15	50	II B	T2N1M0	II	50	ER+/PR+
16	45	II B	T2N1M0	I	45	ER+/PR+
17	52	III A	T2N2M0	III	N/A	ER+/PR+
18	54	III A	T2N2M0	II	#	ER+/PR+
19	65	III B	T4bN2M0	II	47	ER+/PR+
20	50	I	T1cN0M0	III	45	ER+/PR+
21	62	III B	T4bN2M0	II	54	ER+/PR+
22	52	III B	T4bN2M0	III	50	ER+/PR+
23	62	III A	T3N1M0	II	52	ER+/PR+
24	68	II B	T3N0M0	I	50	ER+/PR+
25	49	III A	T3N1M0	I	#	ER+/PR+
26	43	III A	T3N1M0	I	44	ER+/PR+
27	63	III B	T4aN0M0	II	40	ER+/PR+
28	52	II B	T2N1M0	II	52	ER+/PR+
29	48	II A	T2N0M0	II	#	ER+/PR+

*Patients with bilateral breast cancer: R, right breast tumor; L, left breast tumor.
#: the patient has been diagnosed before reaching menopause; N/A: missing data.

in our datasets and also to identify upstream regulators (UR) and their targets that could control these processes. The significance of the association between each dataset and functional categories or canonical pathways stored in Ingenuity Knowledge Base was tested by Fisher's exact test. The threshold of significance was set at 0.05. In order to predict significant URs, an overlap p value and an activation z-score were computed for each potential UR. The overlap p value was estimated by Fisher's exact test, indicating whether there is a significant overlap between the genes in our dataset and the genes known to be modulated by an UR. An overlap p value less than 0.01 was considered significant. Activation z-score was assigned based on the consistency between the expected effects (activation or inhibition) of an UR on each target gene and the observed changes in gene expression. Thus, UR was predicted to be in an "activated" state if z-score > 2; otherwise UR was predicted to be in an "inhibited" state (z-score < -2).

2.5. Quantitative Real-Time PCR (qRT-PCR). Quantitative real-time PCR analysis was used to validate the microarray results. One hundred nanograms of total RNA for every sample was reverse-transcribed to cDNA using First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany). Two and a half microliters of 1 : 10 (v/v) diluted cDNA was amplified with 1 μM of specific primers and 0.2 μM of fluorescence probes in a final volume of 10 μL using LightCycler Taqman Master Kit (Roche Applied Science, Penzberg, Germany). Roche Applied Science software was used to design the structure of primers and specific Universal Probe Library (UPL) probe for every gene as follows: PTGS2 (NM_000963.2): F-cttcacgcatcagtttttcaag, R-tcaccgtaaatatgatttaagtccac, UPL (#23); IL-8 (NM_000584.3): F-gagcactccataaggcacaaa, R-atggttccttccggtggt, UPL (#72); TREM1 (NM_018643.3): F-tctggactgtatcagtgtgtgatct, R-ccaggggtccctgaaaaa, UPL (#75); AREG (NM_001657.2): F-tgatcctcacagctgttgct, R-tccattctcttgtcgaagtttct, UPL (#73); RNA18S5 rRNA (NR_003286.2): F-gcaattattccccatgaacg, R-gggacttaatcaacgcacgc, UPL (#48); RPLP0 (NM-001002.3): F-gatgcccagggaagacag, R-tctgctcccacaatgaaacat, UPL (#85). Thermal cycling conditions were performed in a ViiA7 system (Applied Biosystems) and included a denaturation step

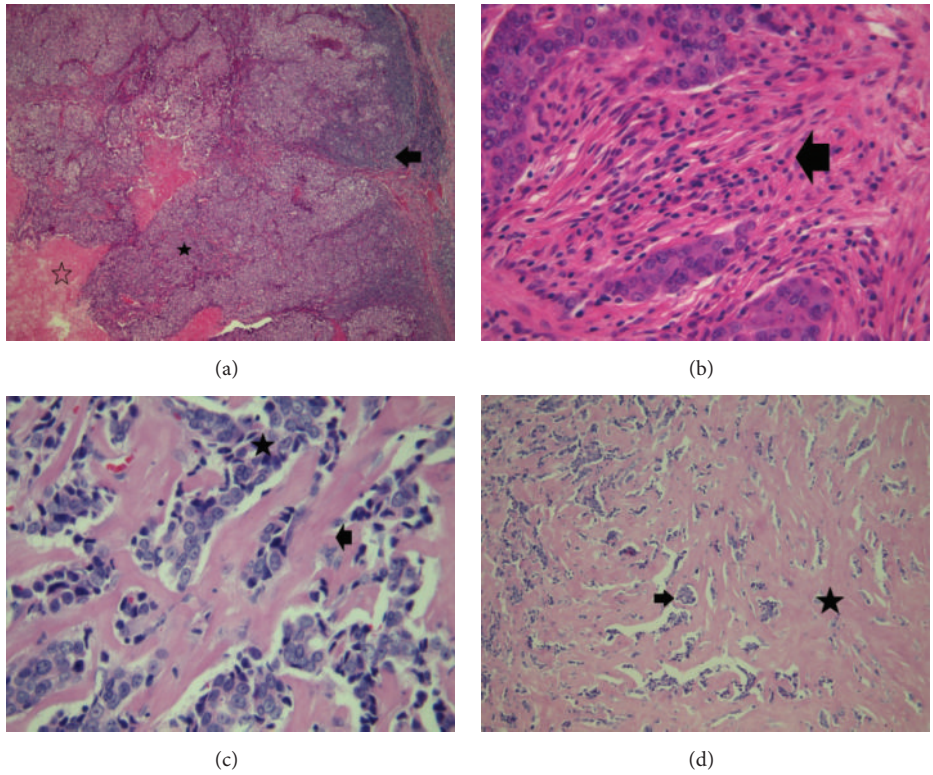

Figure 1: Stroma evaluation for ER−PR−HER2− (TNBC) and ER+PR+HER2− breast cancer subtypes: (a) TNBC mammary carcinoma (40x): black star, viable tumor, hollow star, necrosis, and black arrow, heavy inflammation; (b) TNBC mammary carcinoma (400x): black arrow, desmoplastic stroma with moderate inflammation; (c) ER+PR+HER2− mammary carcinoma (400x): black arrow, fibrohyaline stroma, and black star, viable tumor; (d) TNBC mammary carcinoma (40x): black arrow, viable tumor, and black star, desmoplastic stroma.

at 95°C for 15 seconds followed by 40 cycles of amplifications consisting of an annealing step at 60°C for 20 seconds and extension at 72°C for 1 second. RPLP0 and RNA18S5 housekeeping genes were used to normalize the genes of interest, and their relative expression was calculated using ΔΔCt relative quantification method.

2.6. Stroma Evaluation. Hematoxylin and Eosin- (H&E-) stained slides on 5 μm tissue sections were used for stroma evaluation. Stroma was classified as desmoplastic or fibrohyaline taking into account the density of the stromal cells, stromal edema, and collagen density in intercellular space. Necrosis was evaluated in terms of presence or absence, while two kinds of inflammatory grade including no-weak and medium-intense reaction have been established.

2.7. Statistical Analysis. Statistical analysis was performed using SPSS 16.0 software. Association between clinicopathological characteristics was assessed by Fischer's exact test for categorical variables and Mann-Whitney test for quantitative variables. Normality of qRT-PCR data was tested using Shapiro-Wilk test. According to data distribution, the differences in gene expression between studied groups, evaluated by qRT-PCR, were assessed by parametric tests. When two groups were considered, the comparison was made using Student's t-test, while for three groups we used one-way ANOVA followed by Tukey's post hoc test. A p value lower than 0.05 was considered significant.

3. Results

3.1. Association between Clinicopathological Parameters of the Patients and Breast Cancer Subtype. All patients included in the study were diagnosed with HER2 negative invasive ductal carcinomas. ER+PR+ and ER−PR− subtypes were approximately equally distributed among HER2− breast cancer cases (51.7% and 48.3%, resp.). ER+PR+HER2− and ER−PR−HER2− groups were comparable in age, and more than 60% of the patients had reached menopause at the date of the diagnosis. All of the TNBC cases had positive lymph nodes, and about 71% of them were Nottingham grade III. None of the patients had detectable metastasis at diagnosis. The majority of ER+PR+HER2− samples presented fibrohyaline stroma with no or weak inflammation, while in the case of ER−PR−HER2− subtype 9 out of 14 samples presented desmoplastic stroma with medium or intense inflammation (Figure 1 and Table 2).

Associations between the clinicopathological parameters and ER, PR status are presented in Table 2. TNBC subtype was significantly associated with high Nottingham grade, desmoplastic stroma, inflammation, and necrosis. No association was found for lymph nodes, tumor size, clinical stage, or menopausal status.

Table 2: Association between ER, PR status and clinicopathological parameters.

Characteristics	Number of patients (%)	ER+PR+HER2−	ER−PR−HER2−	p value
Study population	**29 (100%)**	**15 (51.7%)**	**14 (48.3%)**	
Median age (years)		52	51.5	0.41
Menopausal status				
Pre	9 (31%)	11	7	0.24
Post	18 (62.1%)	3	6	
N/A	2 (6.9%)	1	1	
Clinical stage[§]				
I-II	13 (44.8%)	6	7	0.71
III	16 (55.2%)	9	7	
Tumor size[§]				
T1-T2	16 (55.2%)	7	9	0.46
T3-T4	13 (44.8%)	8	5	
Lymph nodes[§]				
N0	4 (13.8%)	4	0	—
N1	15 (51.7%)	6	9	
N2	10 (34.5%)	5	5	
Nottingham grading				
I-II	16 (55.2%)	12	4	**0.009**
III	13 (44.8%)	3	10	
Stroma				
Fibrohyaline	17 (58.6%)	12	5	**0.025**
Desmoplastic	12 (41.4%)	3	9	
Inflammation				
No-weak	18 (62.1%)	13	5	**0.008**
Medium-intense	11 (37.9%)	2	9	
Necrosis				
Absent	21 (72.4%)	15	6	**0.0007**
Present	8 (27.6%)	0	8	

[§]Two patients with bilateral cancer. The higher value for clinical stage, tumor size, and nodes was considered.

3.2. Gene Expression Profiling of Whole Blood. We have generated genome-wide transcriptional profiles of blood samples from 14 patients with triple-negative breast cancer (TNBC/ER−PR−HER2−), 15 patients with hormone-dependent breast cancer (ER+PR+HER2−), and seven healthy donors (CTR). Microarray-based gene expression analysis revealed different molecular blood signatures according to the ER/PR status. We found 371 genes with at least 1.5-fold differential changes in TNBC compared to CTR samples. Of these genes, 177 were overexpressed, and 194 were underexpressed. Following the same criteria of selection, we identified 579 genes differentially expressed in ER+PR+HER2− compared to CTR (314 upregulated and 265 downregulated genes) and 172 genes with altered expression in TNBC versus ER+PR+HER2− samples (79 upregulated and 93 downregulated genes).

Intersecting the results for all three comparisons yielded a 108-specific signature for hormone-dependent subtype (sequences with differential expression in ER+PR+HER2− compared to CTR and TNBC but not in TNBC versus CTR) and 34-specific genes signature for TNBC subtype (sequences with differential expression in TNBC compared to CTR and ER+PR+HER2− but not in ER+PR+HER2− versus CTR) (Figure 2). The full lists of specific genes for ER−PR−HER2− and ER+PR+HER2− subtypes are presented in Additional file 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/3239167.

In order to have a global image of the transformations that occur in the blood cells of the patients with breast cancer pathology, we further considered all 29 blood samples from breast cancer patients as a whole group (BC). A disease signature with differential expression of 290 genes with greater than 1.5-fold expression changes (155 upregulated and 135 downregulated genes) was identified by microarray analysis in BC compared to CTR. The supervised hierarchical clustering of these profiles revealed two distinct clusters corresponding to CTR and BC groups. Although different molecular profiles were observed in the ER−PR−HER2− and ER+PR+HER2− subtypes, the pattern for these subgroups in the BC cluster was mixed and did not cluster according to the ER, PR status (Figure 3).

3.3. Assessment of Deregulated Pathways and Biological Processes. In order to identify the molecular pathways and biological processes affected by the transcriptional changes

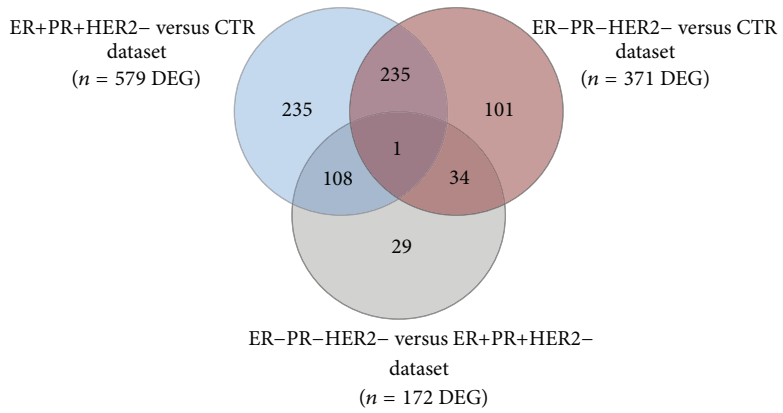

Figure 2: Venn diagram of genes/sequences with differential expression exceeding 1.5-fold in the compared groups. The overlap areas show unique signatures for ER+PR+HER2− (108) and ER−PR−HER2− (TNBC) (34) subtypes.

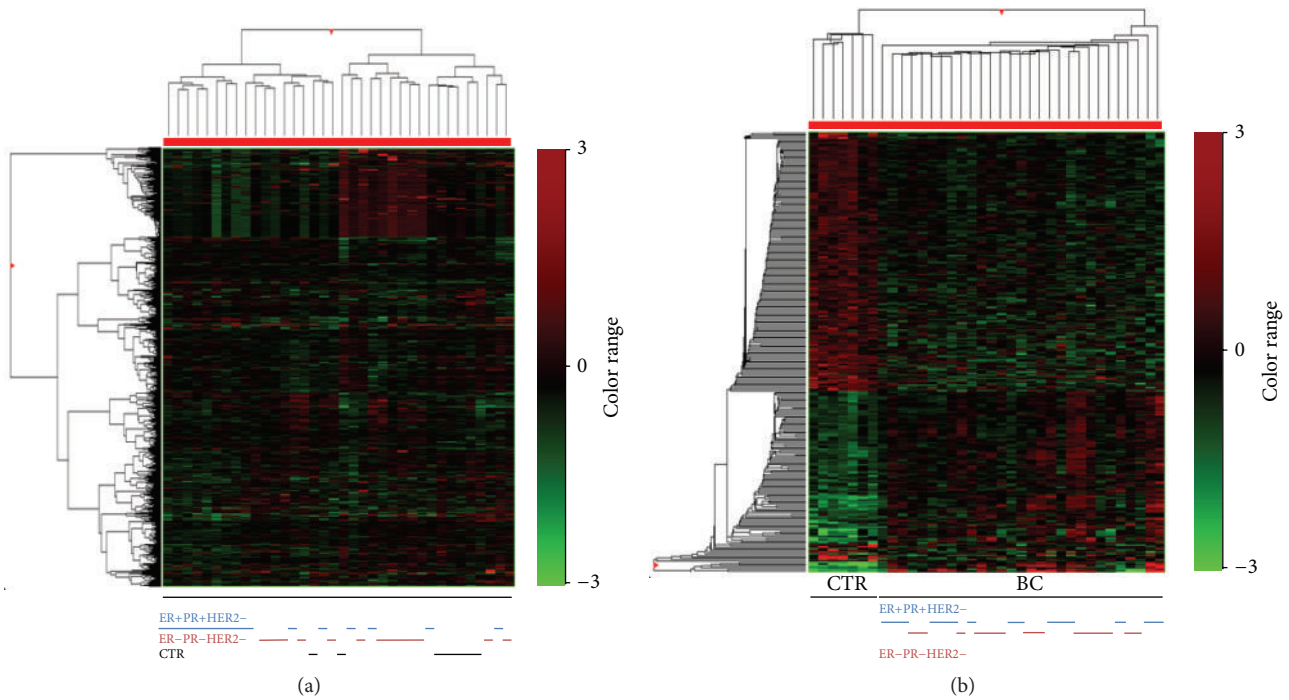

Figure 3: Unsupervised (a) and supervised (b) hierarchical clustering of blood samples from 29 BC and 7 CTR. The hierarchical clusters were computed using Euclidean distances and Ward method. The color indicates the level of mRNA expression: red, higher level of expression; green, lower level of expression; black, no expression change. All samples are represented by columns and genes by rows.

observed in the peripheral blood of patients with breast cancer, we analyzed our data using IPA software. We ran Core Analysis for BC versus CTR, ER−PR−HER2− versus CTR, ER+PR+HER2− versus CTR, and ER−PR−HER2− versus ER+PR+HER2− datasets. IPA analysis revealed 43 significant canonical pathways ($p < 0.05$) across the four datasets (Figure 4). The color code in the heat map of the canonical pathways is related to p value obtained by Fischer's exact test; the darkest color was assigned for the dataset in which the canonical pathway is the most significant. We identified 15 significant canonical pathways in the peripheral blood of ER+PR+HER2− patients, respectively, and 18 significant pathways in TNBC patients when compared to control group. Specific canonical pathways such as "communication between innate and adaptive immune cells," "differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F," and "differential regulation of cytokine production in intestinal epithelial cells by IL-17A and IL-17F" were activated just in TNBC when compared to control group, while "chemokine signaling," ephrin B signaling, and PTEN signaling were found only in TNBC when compared to ER+PR−HER2− subgroup.

In order to better understand the differences observed in the peripheral blood cells of patients with breast cancer

BC versus CTR	ER+PR+HER2− versus CTR	TNBC versus CTR	TNBC versus ER+PR+HER2−	
$4.9E-06$	$2.2E-05$	$3.5E-06$	ns	Granulocyte adhesion and diapedesis
$5.1E-05$	$1.7E-04$	$3.6E-05$	ns	Agranulocyte adhesion and diapedesis
$3.4E-03$	$1.9E-02$	$5.2E-03$	N/A	IL-17A signaling in gastric cells
$5.8E-03$	$2.1E-02$	$5.1E-03$	ns	Glucocorticoid receptor signaling
$1.2E-02$	$2.3E-02$	$1.4E-02$	N/A	L-Dopachrome biosynthesis
$1.8E-02$	ns	$2.4E-02$	N/A	Differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F
$2.9E-02$	ns	$3.8E-02$	N/A	Differential regulation of cytokine production in intestinal epithelial cells by IL-17A and IL-17F
$2.4E-02$	ns	$4.2E-02$	ns	Aryl hydrocarbon receptor signaling
$1.2E-02$	ns	ns	N/A	IL-17 signaling
$1.6E-02$	ns	ns	ns	Adipogenesis pathway
$1.7E-02$	ns	ns	N/A	D-myo-Inositol (1, 4, 5, 6)-tetrakisphosphate biosynthesis
$1.7E-02$	ns	ns	N/A	D-myo-Inositol (3, 4, 5, 6)-tetrakisphosphate biosynthesis
$2.7E-02$	ns	ns	N/A	D-myo-Inositol-5-phosphate metabolism
$2.8E-02$	ns	ns	N/A	3-Phosphoinositide degradation
$3.6E-02$	ns	ns	N/A	3-Phosphoinositide biosynthesis
$4.0E-02$	ns	ns	N/A	ERK5 signaling
$4.2E-02$	ns	ns	ns	Role of tissue factor in cancer
$4.8E-02$	ns	ns	N/A	Spermine and spermidine degradation I
$4.8E-02$	ns	ns	N/A	NAD biosynthesis III
$4.8E-02$	ns	ns	N/A	Eumelanin biosynthesis
$1.1E-02$	$2.9E-03$	$1.4E-02$	N/A	Role of IL-17A in psoriasis
ns	$2.5E-02$	ns	N/A	Cell cycle: G2/M DNA damage checkpoint regulation
ns	$1.3E-02$	ns	N/A	HIPPO signaling
ns	$2.0E-02$	ns	ns	RhoA signaling
ns	$3.6E-02$	ns	ns	Role of JAK2 in hormone-like cytokine signaling
ns	$4.9E-02$	ns	ns	TGF-β signaling
ns	$2.3E-02$	$4.8E-02$	ns	IGF-1 signaling
ns	$2.6E-02$	$4.8E-02$	ns	ERK/MAPK signaling
ns	ns	$1.0E-02$	N/A	Hematopoiesis from pluripotent stem cells
ns	ns	$2.8E-02$	N/A	PI3K/AKT signaling
ns	ns	$3.4E-03$	N/A	Communication between innate and adaptive immune cells
ns	ns	$3.8E-02$	N/A	Polyamine regulation in colon cancer
ns	ns	$3.9E-02$	ns	PPAR signaling
ns	ns	$4.1E-02$	N/A	Role of IL-17A in arthritis
ns	ns	$4.7E-02$	ns	IL-8 signaling
N/A	$5.3E-03$	N/A	$2.6E-03$	Extrinsic prothrombin activation pathway
N/A	$2.3E-02$	N/A	$4.8E-03$	Sorbitol degradation I
ns	ns	ns	$5.3E-03$	Ephrin B signaling
ns	ns	ns	$9.6E-03$	Ephrin receptor signaling
N/A	ns	ns	$1.9E-02$	PTEN signaling
ns	ns	ns	$3.0E-02$	Molecular mechanisms of cancer
N/A	N/A	ns	$3.1E-02$	Glioma invasiveness signaling
ns	ns	ns	$4.2E-02$	Chemokine signaling

Figure 4: Heat map of the significant canonical pathways in the four datasets of differentially expressed genes: BC versus CTR, ER+PR+HER− versus CTR, TNBC (ER−PR−HER−) versus CTR, and TNBC versus ER+PR+HER− subgroup. The significance of the association between canonical pathways and each dataset was assessed in IPA by Fischer's exact test ($p < 0.05$). The darkest color was assigned to the smallest p value for a canonical pathway among all datasets, while uncolored boxes indicate the nonsignificant canonical pathways (ns) or their absence (N/A).

we further focused on the implications of immune cells in tumor development. We identified the statistically significant biofunctions induced by innate and adaptive immune cells in breast cancer patients (Additional file 1). By evaluating the genes involved in movement, adhesion, migration, and infiltration of immune cells, we identified several upregulated proinflammatory modulators including CXCL1, CXCL2, CXCR2, CXCR4, CCL3, CCL4, CCL3L1/CCL3L3, EGR1, EGR2, EGR3, IL-8, PTSG2, PLAU, OSM, and TREM1. The assessment of key modulators, based on IPA Upstream Regulator Analysis, has highlighted two of the mentioned proinflammatory factors, PTGS2/COX-2 (z-score = 2.322 and overlap p value = $6.41E-09$) and TREM1 (z-score = 2.685 and overlap p value = $8.11E-08$), as upstream regulators when comparing all breast cancer samples with healthy donors (Table 3). The relationship between upstream regulators and their targets is presented in Figure 5. AREG/AREGB and F7 were predicted to be upstream regulators in hormone-dependent subtype, whereas only AREG/AREGB was identified as an upstream regulator in the dataset (Table 3).

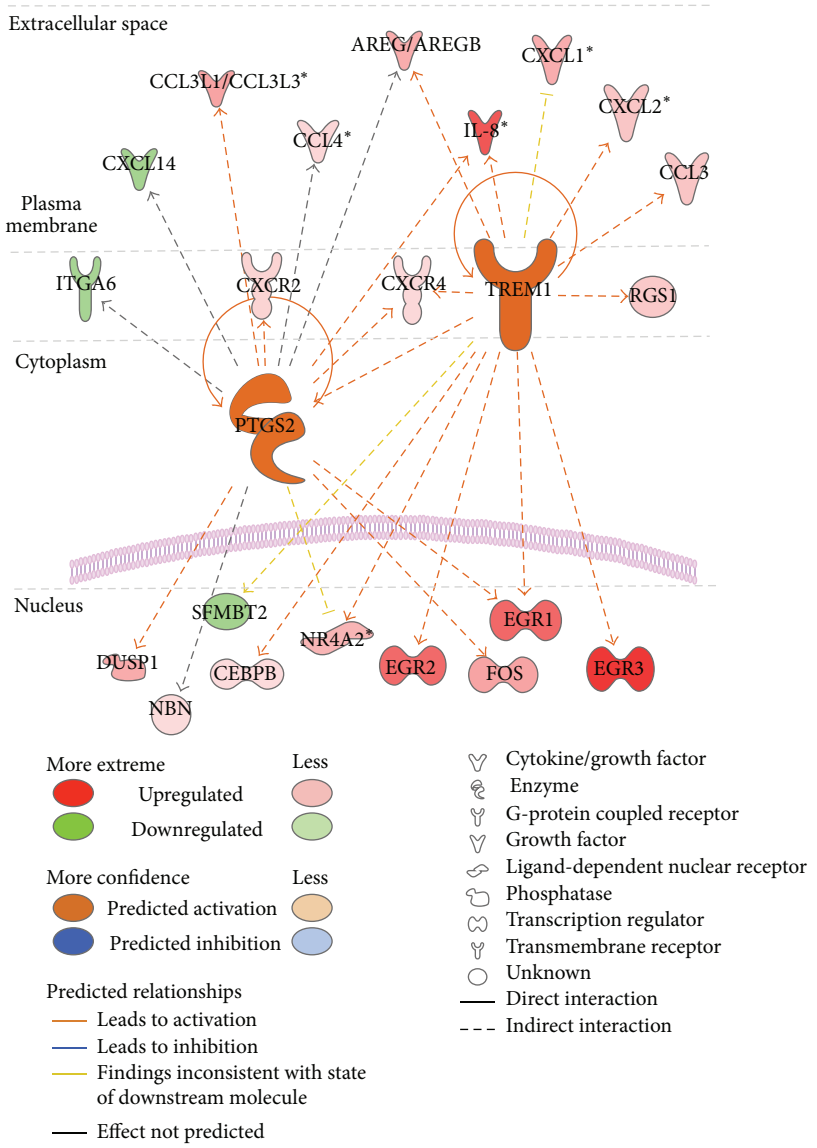

Figure 5: The network of TREM1 and PTGS2 (COX-2) upstream regulators and their target molecules, evaluated in the peripheral blood cells of breast cancer patients compared with healthy donors (Ingenuity Pathway Analysis).

3.4. qRT-PCR Data Validation. The common upstream regulators (PTGS2 and TREM1) and two of their common targets (IL-8 and AREG) were evaluated by qRT-PCR (Table 4). We found full concordance between qRT-PCR data and microarray results in terms of magnitude and the direction of expression changes. For PTGS2, differences in expression assessed by qRT-PCR were higher than those obtained by microarray in all comparison, while for TREM1, IL-8, and AREG the expression was comparable between microarray and qRT-PCR data.

4. Discussion

The interaction between tumor and host is not limited to communication with its local microenvironment but also affects distant anatomic sites and systemic immune response. Consequently, these interactions could be reflected by a tumor-related blood gene expression signature. In this study, we explored transcriptional profiles in the peripheral blood cells of HER2 negative breast cancer patients according to ER/PR status to evaluate whether there are transcriptional differences and to gain a better understanding of the changes triggered into the blood circulating cells.

Our results highlighted the implication of tumor-related inflammation as well as the immune response in all blood samples from breast cancer patients, with an enrichment of these processes in the TNBC subtype. Tumor microenvironment (TME), a springboard for tumor growth, represents a complex cooperation between tumor, stroma, and blood cells [16, 17]. In the new conceptual rationale, Hanahan and Coussens [18] described the TME including tumor-promoting inflammation as a new hallmark of cancer,

Table 3: Upstream regulators predicted by IPA in the peripheral blood cells of breast cancer patients compared with healthy donors.

Upstream regulator	Fold change	Molecule type	Predicted activation state	z-score	p value of overlap	Target molecules in dataset
BC versus CTR						
PTGS2	5.764	Enzyme	Activated	2.322	$6.41E-09$	AREG/AREGB, CCL3L1/CCL3L3, CCL4, CXCL14, CXCR2, CXCR4, DUSP1, EGR1, FOS, IL-8, ITGA6, NBN, NR4A2, PTGS2
TREM1	2.051	Transmembrane receptor	Activated	2.685	$8.11E-08$	AREG/AREGB, CCL3, CEBPB, CXCL1, CXCL2, CXCR4, EGR1, EGR2, EGR3, IL-8, NR4A2, PTGS2, RGS1, SFMBT2
ER+PR+HER− versus CTR						
F7	−2.303	Peptidase	Activated	2.736	$1.03E-07$	CXCL2, EGR1, FOS, IER2, IL-8, JAG1, KLF5, ZFP36
AREG	4.422	Growth factor	Activated	2.395	$2.45E-06$	AREG/AREGB, CXCR4, EGR1, FOS, NPPC, PTGS2
ER−PR−HER2− versus CTR						
AREG	2.803	Growth factor	Activated	2.407	$1.12E-06$	AREG/AREGB, CXCR4, EGR1, FOS, PLAU, PTGS2

Table 4: Relative expression of PTSG2, TREM1, IL-8, and AGREG assessed by qRT-PCR in BC, ER−PR−HER2−, and ER+PR+HER2− samples. Statistical significance was assessed using one-way ANOVA followed by Tukey's post hoc test.

	BC versus CTR		ER−PR−HER2− versus CTR		ER+PR+HER2− versus CTR	
	FR	p value	FR	p value	FR	p value
PTGS2	11.36	<0.0001	11.21	<0.0001	11.50	<0.0001
TREM 1	1.88	0.005	1.93	0.013	1.83	0.043
IL-8	6.99	<0.0001	5.98	<0.0001	7.94	<0.0001
AREG	4.16	<0.0001	4.07	<0.0001	4.26	<0.0001

with immune cells being recognized to facilitate the cancer progression. Inflammatory cells and inflammatory mediators including chemokines, cytokines, and prostaglandins were identified in the TME of most tumors, including breast cancers [19, 20].

Increasing evidence suggests that, besides tumor cells, cancer-associated fibroblasts (CAFs), tumor associated macrophages (TAMs), and tumor-associated neutrophils (TANs) are involved in the production of proinflammatory chemokines and cytokines. Cancer-associated fibroblasts (CAFs), an activated population of stromal fibroblasts with a role in tumor development, represent the most important stromal cell type of mediators of tumor-promoting inflammation [21]. Previous studies have shown that CAFs production of CXCL1, CXCL2, PTGS2/COX-2, and IL-6 in breast cancer can modulate the functions of immune cells in TME [22, 23]. Our data showed overexpression of proinflammatory factors including CXCL1, CXCL2, CXCR4, CCL3, CCL4, IL-8, and PTGS2/COX-2 in the peripheral blood of patients with breast cancer both when considering the subtypes individually and when considering all breast cancer samples as a group. Furthermore, we found that PTGS2/COX-2's targets were highly enriched in BC versus CTR samples (overlap p value = $6.41E-09$) (Table 3). Recent studies indicated that tumor expression of COX-2 can lead to epithelial to mesenchymal transition in breast tumor cells [24], and the pharmacological inhibition of COX-2 reduces breast tumor development [25, 26]. Although PTGS2/COX-2 was identified as a possible predictive marker of micrometastasis of breast cancer in the bone marrow [27], currently there are no studies to show its overexpression in the blood of patients with breast cancer. In preneoplastic lesions, proinflammatory cytokines and chemokines secreted by CAFs are involved in complex regulatory signals promoting macrophage recruitment, angiogenesis, and tumor growth [28].

On the other hand, circulating monocytes represent the source of TAMs that are selectively attracted in TME by tumor-derived attractants such as chemokines and cytokines [29]. Substantial evidence suggests that TAMs accumulate preferentially in hypoxic regions of tumors, leading to overexpression of COX-2, VEGF, IL-8, CXCL12, or CXCR4 [30]. COX-2 overexpression in turn leads to increased production of proangiogenic factors such as VEGF, IL-8, and CXCL1 with a role in vascular channel formation [31, 32]. Our data showed a significant activation of "IL-8 signaling" pathway

in the blood cells of TNBC patients correlated with increased inflammation and necrosis in primary tumors.

It is known that IL-8 released by tumor cells represents a chemoattractant for neutrophils to TME [33]. Additionally, in a similar manner to that in wounds, neutrophils secrete cytokines including IL-6, IL-8, TNF-α, and GM-CSF, enhancing angiogenesis and tumor progression. Most of TNBC are highly proliferative tumors and have poor developed vascular networks that generate susceptibility to hypoxia and implicit necrosis. Tumor necrosis has been associated with a poor outcome in breast carcinoma [34] and usually generates cytokine-like IL-1 and HMGB1 with a role of promoting inflammatory response and neoangiogenesis [35]. Our data highlight a high cellular density of inflammatory and fibroblastic cells in stroma of TNBC subtypes. In a recent study, Pierobon et al. [36] demonstrated that, in hypoxic conditions, TREM1, a transmembrane receptor expressed in myeloid cells, is involved in the inflammatory responses mediated by neutrophils and monocytes. TREM1 is considered to amplify inflammation by triggering the secretion of some important inflammatory factors including TNF-α, IL-6, CXCL8, CCL4, and CCL5 [37]. In our study, TREM1 was revealed as an upstream regulator in the peripheral blood of BC patients (z-score = 2.685; overlap p value = $8.11E-09$). Our data indicates that TREM1 activates important proinflammatory factors including IL-8, CXCL1, CXCL2, CXCL3, AREG, and transcription regulators such as EGR1, EGR2, and EGR3, suggesting enhancing in differentiation and mitogenesis. Balzarolo et al. [38] showed that EGR1, when activated, acts as a brake on TNF-related apoptosis-inducing ligand (TRAIL) expression in NK cells. The role of the soluble protein TREM1 as possible prognostic marker to detect lung metastasis was previously reported [39]; nevertheless no study has investigated the role of TREM1 in the peripheral blood of breast cancer patients.

Cross talk between innate and adaptive immune systems has already been demonstrated to have profound effects on cancer development, including breast carcinogenesis [40, 41]. The immune system balance was also confirmed by our study. Our data revealed that "differential regulation of cytokine production in macrophages and T helper cells by IL-17A and IL-17F" canonical pathway was activated just in the blood of TNBC patients but not in ER+PR+HER2− patients when compared to CTR group (Table 3). The role of IL-17A and IL-17F was previously related to neutrophils recruitment during inflammation. These molecules activate fibroblasts from both innate and epithelial cells from TME to produce proinflammatory cytokines and chemokines [42]. Furthermore, an enrichment of "chemokine signaling" was observed in the blood of patients with TNBC subtype compared to hormone-dependent subtype.

Our results highlighted activation of processes such as mobilization, migration, infiltration, and accumulation of leucocytes in the blood of the patients with breast cancer (Additional file 1). The set of cytokines, chemokines, and biomediators identified as upregulated in peripheral blood cells of breast cancer patients have been shown to stimulate innate peripheral blood immune cells such as phagocytes and granulocytes to migrate and infiltrate to TME. It is known that leucocytes represent crucial regulators of cancer development by altering local homeostasis and declining immune balance between antitumor responses and oncogenic pathways [43]. Our data suggest a more extensive immune response in patients with TNBC compared to that with ER+PR+HER2− subtype by a significant accumulation, trafficking/migration, and adhesion of phagocytes and granulocytes. Processes such as migration of inflammatory leucocytes were also revealed as more significantly upregulated in TNBC versus ER+PR+HER2− samples. Furthermore, human breast carcinoma that contains infiltrates of innate-immune cell types such as macrophages was associated with increased angiogenesis and unfavorable clinical prognosis [44]. An increased process related to the accumulation of macrophages ($p = 0.0059$) (Additional file 1) and more pathways involved in modulation of angiogenesis (Figure 3) were observed in TNBC versus hormone-dependent subtype.

We noticed more functions related to proliferation, differentiation, and migration of peripheral blood lymphocyte in BC patients, with more intense T cells activities, especially in the TNBC subtype (Additional file 1). It is widely known that T cells are the most potent cells of the immune system. In TNBC subtype, we observed a shifting of immune responses toward accumulation, transmigration, and conversion of naive T lymphocyte, balancing innate, and adaptive immunity but accompanied by inhibition of B lymphocytes maturation.

The interactions between TME and cancer cells represent intrinsic features of breast cancer subtypes. In a recent study, Camp et al. [45] identified specific microenvironment features when comparing basal-like with luminal breast cancer subtypes. They observed that basal-like cells respond to stromal interactions by increasing migration, including important proinflammatory mediators such as IL-6, IL-8, CXCL1, and oncostatin M (OSM). Our data also indicate the presence of these molecules in the peripheral blood cells of patients with breast cancer regardless of subtype. Furthermore, validation of our results on larger cohorts could contribute to a better understanding of the role of immune system in HER2− breast cancer subtypes, allowing new immunotherapeutic approaches.

In conclusion, our data show distinct molecular signatures in the blood of HER2 negative breast cancer patients according to ER/PR status. We noticed a significant enrichment of altered systemic immune-related pathways in the blood of TNBC patients correlated with an increased inflammation and necrosis in primary tumors suggesting that immunotherapy could possibly be synergistic to the chemotherapy to improve the clinical outcome of these patients.

Conflict of Interests

None of the authors has any competing interests.

Authors' Contribution

Ovidiu Balacescu and Loredana Balacescu contributed equally to this work.

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Review Article

Distinct Functions of Neutrophil in Cancer and Its Regulation

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Neutrophils are the most abundant of all white blood cells in the human circulation and are usually associated with inflammation and with fighting infections. In recent years the role immune cells play in cancer has been a matter of increasing interest. In this context the function of neutrophils is controversial as neutrophils were shown to possess both tumor promoting and tumor limiting properties. Here we provide an up-to-date review of the pro- and antitumor properties neutrophils possess as well as the environmental cues that regulate these distinct functions.

1. Introduction

Neutrophils are the most abundant of all white blood cells and play a key role in host protection against microbial infections and in inflammation. Chronic inflammation has been associated with increased susceptibility for cancer. Hepatitis B [1] and inflammatory bowel disease [2] are common examples for this correlation, leading to hepatocellular carcinoma and colorectal cancer, respectively. Neutrophils, as a key component in inflammation, may play a crucial role in inflammation driven tumorigenesis. This was well exemplified when neutrophils were shown to directly promote carcinogenesis in a mouse model of colitis [3]. Indeed, neutrophils at the primary tumor site were shown to provide a wide range of different tumor promoting functions. Neutrophils were shown to support angiogenesis via secretion of proangiogenic factors as well as the proteolytic activation of proangiogenic factors. Neutrophils were also implicated in promoting tumor growth through the proteolytic release of EGF, TGFβ, and PDGF from the extracellular matrix (ECM). Neutrophils express high levels of metalloproteinases which can also modify the ECM to allow tumor cell dissemination thereby promoting tumor spread. Furthermore, neutrophils were shown to recruit other tumor promoting cells to the tumor bed. Finally, immature neutrophils, also termed G-MDSC (granulocytic myeloid derived suppressor cells), were implicated in the establishment of an immunosuppressive tumor microenvironment thereby limiting antitumor immunity. On the other hand, neutrophils were shown to have antitumor properties including the capacity to kill tumor cells either through direct cytotoxicity or via antibody dependent cell cytotoxicity (ADCC) [4]. Similar conflicting reports were made as to the role neutrophils play in the premetastatic niche. Neutrophils accumulate in large numbers in premetastatic organs [5–7]. The fact that bone marrow derived cells were implicated in priming of the premetastatic niche prompted the hypothesis that neutrophils may be the cells that mediate this process. Indeed, neutrophils were shown to have a positive effect on tumor cell seeding in the premetastatic site [6]. In contrast, we and others have shown that neutrophils actively limit metastatic seeding by killing tumor cells [5, 7].

Interestingly, while neutrophils play a role in modulating tumor cell seeding in the metastatic site, it seems like they do not affect the growth rate of the metastatic nodules [5, 7]. This suggested that neutrophil antitumor functions are not always manifested inside the tumor and may depend on

the chemokine landscape in the tumor microenvironment. This notion was further supported by findings showing that upon entering the tumor microenvironment neutrophils acquire a different set of traits. This was referred to as "polarization" of neutrophils toward a tumor promoting or an antitumor phenotype which is mediated via cytokines available in the tumor microenvironment (i.e., TGFβ and IFNs, resp.). Furthermore, recent studies suggested that neutrophils are not a homogeneous population of cells and may consist of both pro- and antitumor subpopulations [8]. Together, the observations made thus far suggest that the mere accumulation of neutrophils in the tumor site may not necessarily be indicative of their contribution or of their prognostic value. Along these lines, the ongoing efforts to correlate neutrophil counts, or the ratio between neutrophils and other immune cells, with patient prognosis and ultimate outcome are conflicting and show that neutrophil abundance may correlate with a better prognosis in some studies and with a worse prognosis in others [9].

2. Molecular Mechanisms of Neutrophil Polarization in the Tumor Microenvironment

Neutrophils were shown to have diverse functions in the tumor microenvironment including both promoting and inhibiting tumor growth. As neutrophils are quick to respond to environmental cues, the most plausible explanation for the different neutrophil phenotypes was that neutrophil function is dictated by the local chemokine milieu. Advances in our understanding of how neutrophil function is regulated in cancer have led to the realization that neutrophils may be directed towards a specific phenotype, be it tumor promoting or tumor limiting, upon entering the tumor. Here we will discuss how interferons and TGFβ polarize neutrophils in the tumor microenvironment.

2.1. Interferons. Type I interferons (IFNs) were first characterized in the process of viral interference. However, since then IFNs were found to be involved in a wide range of biological processes. In the context of cancer, IFNs show strong antitumor function as they inhibit tumor cell proliferation and promote apoptosis [10]. However, IFNs were also found to play a key role in mounting an antitumor immune response through the activation of T-cells, NK cells, and macrophages [11]. In recent years it has become apparent that IFNs also affect neutrophil function and promote antitumor processes mediated by neutrophils. Jablonska et al. have shown that IFN-β is critical for suppressing the expression of proangiogenic factors, such as VEGF and MMP9, in tumor infiltrating neutrophils leading to enhanced tumor vascularization and growth in IFN-β deficient animals [12]. Furthermore, IFN-β was found to play a significant role in regulating the recruitment of neutrophils and their longevity in the primary tumor [13, 14]. Finally, type I IFN activity was found to inhibit neutrophil-mediated formation of "fertile" premetastatic niche [15].

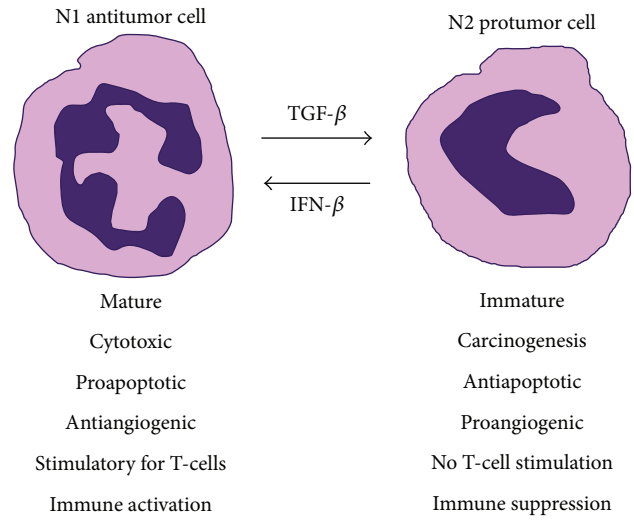

Figure 1: Neutrophil function in cancer is dictated by environmental cues. Neutrophils may be divided into N1 antitumor and N2 protumor cells. TGFβ is a potent driver of the transition from N1 to N2 phenotype whereas IFN-β is a potent driver of the transition in the opposite direction. This exemplifies the notion that neutrophil function in cancer is determined by the chemokine milieu in the microenvironment.

2.2. TGFβ. TGFβ is a multipotent molecule known to have diverse effects in cancer. One of the most explored functions of TGFβ in cancer is its role in generating an immunosuppressive tumor microenvironment. A groundbreaking study by Fridlender and colleagues [16] demonstrated that TGFβ plays a critical role in suppression of antitumor neutrophil cytotoxicity. In this study, the authors showed that blocking TGFβ signaling leads to a change in the cellular composition of the tumor and allows the influx of large numbers of neutrophils. More importantly, they showed that tumor-associated neutrophils (TANs) recruited in the absence of TGFβ signaling have an antitumor N1 phenotype. The authors concluded that TGFβ in the tumor microenvironment is involved in polarizing TAN towards N2 protumor phenotype. This concept was supported by other studies showing that TGFβ can directly block antitumor neutrophil cytotoxicity [5] and that TGFβ receptor deficient myeloid cells, including neutrophils, maintain an antitumor phenotype and limit tumor growth [17].

The conflicting effects of TGFβ and IFNs on neutrophil function in the context of cancer are an example of how neutrophils respond to cues in the microenvironment (Figure 1). While understanding the mechanisms that regulate neutrophil function is clearly important from a therapeutic point of view, the realization that neutrophils may play conflicting roles, depending on their context, is an important notion.

3. Antitumor N1 Phenotype

Antitumor N1 neutrophils act to limit tumor growth and metastatic progression. This is accomplished via distinct mechanisms including direct and antibody dependent cytotoxicity as well as through the activation of other cell types including T-cells and dendritic cells.

3.1. Direct Cytotoxicity. Direct cytotoxicity of neutrophils towards tumor cells is not a novel concept and was first observed in the early 1970s [18]. Neutrophils are highly motile phagocytic cells whose primary function is antimicrobial protection of the host. Accordingly, neutrophils generate a variety of antimicrobial molecules. However, most of these molecules are harmless to eukaryotic cells. Still, the reactive molecules generate by the NADPH oxidase complex, superoxides, H_2O_2, and HOCl. Indeed, these molecules were found to be directly involved in antitumor neutrophil cytotoxicity [19–21]. Several studies have shown that physical contact is required for neutrophil cytotoxicity. However, stimulating cultured neutrophils with a potent agonist, such as PMA, leads to the generation and secretion of very high levels of H_2O_2 alleviating the need for physical contact [5].

3.2. ADCC. Antibody dependent cell-mediated cytotoxicity (ADCC) is another mechanism for neutrophil antitumor cytotoxicity. Tumor cell-specific antibodies may be successfully used as an anticancer therapy. Antibody labeled cells are susceptible to destruction by immune cells expressing Fc receptors (FcR). Neutrophils express several FcRs that can mediate ADCC including FcγRI (CD64), FcγRIIa (CD32), FcγRIIIa (CD16a), and FcγRIIIb (CD16b) [22–24]. Indeed, neutrophils were shown to take part in ADCC in several types of cancer including glioma, squamous cell, and ovarian carcinoma. Neutrophils were also shown to contribute to the antitumor ADCC in Non-Hodgkin's Lymphoma [25], in breast cancer using [26], and in B-cell lymphoma [27].

3.3. Stimulation of T-Cells and DCs. Neutrophils, on top of having a role in killing tumor cells directly, can also stimulate adaptive antitumor immune responses. This was well exemplified by experiments showing that neutrophils are required for proper antitumor $CD8^+$ T-cell immune response [16, 28–30]. Stimulation of adaptive antitumor immunity by neutrophils has two arms, the recruitment of other immune cells and their antigen presenting abilities.

(a) Recruitment of Immune Cells. Neutrophils secrete several cytokines including TNFα, Cathepsin G, and neutrophil elastase which have a direct effect on T-cells and promote their proliferation and cytokine production. Neutrophils, under these conditions, act to recruit and activate T-cells and enhance the overall adaptive immune antitumor response. Specifically, TAN were shown to stimulate T-cell proliferation and IFNγ secretion in early stage lung cancer patients [31].

(b) Neutrophil Extracellular Traps (NETs). Production of extracellular traps by neutrophils is an interesting feature in neutrophil biology. These NETs are composed of chromatin fibers decorated with histones and other proteins and are considered as an additional tool in neutrophils' arsenal of antimicrobial properties. However, Tillack and colleagues showed that NETs may also be utilized to prime T-cells [32]. This was also linked to a possible role of NETs in immunoediting in cancer and the propagation of antitumor immune responses [33].

(c) Antigen Presentation. For a long time antigen presentation was thought to be exclusively mediated by macrophages and more so by dendritic cells (DCs). However, in 2007 Beauvillain and colleagues demonstrated that neutrophils can efficiently process and present antigens to directly stimulate T-cell immune responses [34]. While this does not directly link neutrophil presentation of antigens to antitumor cytotoxicity, Fridlender and colleagues showed in 2009 that N1 TANs require T-cells for their antitumor activity in the primary tumor [16], an observation that may be explained by neutrophils' ability to present tumor antigens to stimulate T-cells.

4. Protumor N2 Phenotype

Neutrophils have been traditionally considered as guards of the host immune system. However, in the context of tumor, the function of these cells is frequently modified to act against the host and promote tumor growth and metastasis formation. A possible reason for this could be tumor-secreted factors that elicit wound-repair responses by neutrophils that in turn inadvertently stimulate tumor progression [35]. Moreover, wound-infiltrating neutrophils are rapidly diverted from a wound to preneoplastic cells and such interactions lead to increased proliferation of the preneoplastic cells. Prostaglandin E2 (PGE2) seems to be the factor responsible for this process [36]. These results have shown that repeated wounding with subsequent inflammation leads to a greater incidence of local melanoma formation. Along these lines, several studies have shown that infiltration of tumors by neutrophils is associated with poor clinical outcome. Tumor-associated neutrophils (TANs) have been shown to promote tumor growth and progression via a variety of mechanisms, including extracellular matrix remodeling, promotion of tumor cell invasion and metastasis, angiogenesis, lymphangiogenesis, and immune suppression [12, 13, 15].

4.1. Protumor Cytokines. One of the mechanisms responsible for neutrophil-mediated tumor angiogenesis, growth, and metastasis is the secretion of protumor cytokines by these cells [37]. Depending on the cytokine milieu, neutrophils are able to secrete multiple growth factors such as EGF, TGFβ, PDGF, HGF, VEGF, and oncostatin M [12, 38–41].

Evidence suggests that EGF and its receptor EGFR are involved in the pathogenesis and progression of different carcinoma types [42]. Amplification of the EGFR gene and mutations of the EGFR tyrosine kinase domain have been recently demonstrated to occur in carcinoma patients. EGFR causes neoangiogenesis but also increased proliferation, decreased apoptosis, and enhanced tumor cell motility [43] since its receptor (EGFR; HER1; erbB1) is highly expressed on

variety of human tumors including non-small cell lung cancer (NSCLC) and breast, head and neck, gastric, colorectal, esophageal, prostate, bladder, renal, pancreatic, and ovarian cancers [42, 44].

TGFβ is frequently upregulated in human cancers [45] and has been linked to the regulation of tumor initiation, progression, and metastasis [46]. Tumor-secreted TGFβ is usually sequestered to the extracellular matrix as an inactive complex and becomes activated through enzymes such as neutrophil-derived elastase and MMP9 [46]. Furthermore, reactive oxygen free radicals produced by activated neutrophils can activate latent TGFβ [47]. Thus, activated neutrophils, through production of elastase, MMP9, and ROS, may contribute to TGFβ-mediated immunosuppression [9]. Furthermore, TGFβ has been shown to be a potent chemoattractant for neutrophils facilitating their recruitment to sites of inflammation [48, 49] and to promote their protumor N2 phenotype, as mentioned above [16].

Another important neutrophil-derived growth factor is platelet-derived growth factor (PDGF). Interestingly, this growth factor was shown to be chemotactic for monocytes and neutrophils [50]. It was recently established that PDGF stimulation cooperates with genetic changes caused by retroviral insertions in induction of fully malignant tumor phenotype [51]. Moreover, the autocrine PDGF signaling seems to play a role in the growth and metastasis of epithelial cancers.

VEGF is a very potent proangiogenic factor but also serves as a potent chemoattractant for neutrophils. It has been implicated as the key endothelial cell-specific factor required for pathological angiogenesis, including tumor neovascularization. Inhibition of the VEGF signaling blocks angiogenesis in growing tumors, leading to regression of tumor growth [52]. The function of vascular endothelial growth factor (VEGF) in cancer is not limited to angiogenesis and vascular permeability [53]. VEGF-mediated signaling occurs in tumor cells, and this signaling contributes to key aspects of tumorigenesis, including the function of cancer stem cells and tumor initiation [54]. Autocrine VEGF signaling can promote the growth, survival, migration, and invasion of cancer cells [55–57].

Oncostatin M is another pleiotropic cytokine that is secreted by neutrophils [58]. It has been shown to exert proinflammatory effects by inducing adhesion and chemotaxis of neutrophils and chemokine production by endothelial cells [59]. Although oncostatin M was originally identified as an inhibitor of tumor cell growth *in vitro* [60, 61], it is increasingly apparent that this cytokine plays a role in breast cancer cell detachment [62] and angiogenesis [41].

In addition to growth factors, neutrophils are able to secrete other cytokines that influence tumor development and spreading. For instance, neutrophil delivered TNFα, IL-6, and IL-17 were shown to promote tumor growth by modifying the function of stromal cells surrounding the tumor [63, 64]. TNFα produced by tumor cells or inflammatory cells in the tumor microenvironment can promote tumor cell survival through the induction of NFκB-dependent antiapoptotic molecules [65]. TNFα was also shown to promote angiogenesis [66] and induce the expression of VEGF and HIF-1α in tumor cells [67]. IL-6 promotes angiogenesis and the expression of VEGF [68] through JAK2/STAT3 signaling [64] and the tumor promoting effects of IL-17 are in part mediated through upregulation of IL-6 [63, 64].

4.2. Angiogenesis and Modulation of the ECM. Angiogenesis is one of the hallmarks of the development of malignant neoplasias. Primary tumors of a certain size require the growth of new blood vessels in order to be supplied with nutrients and oxygen. Accordingly, at a size of 1-2 mm^3, tumors alter their angiogenic phenotype and support continuous proliferation of endothelial cells. This "angiogenic switch" is activated by disturbed balance between endogenous pro- and antiangiogenic factors. It leads to the uncontrolled growth of blood vessels, mainly via stimulation of VEGF. Importantly, experimental *in vivo* models of angiogenesis have demonstrated that neutrophils affect neovascularization in the tissues [69]. Accordingly, Gr-1-mediated neutrophil depletion was found to significantly reduce tumor angiogenesis [70, 71]. Notably, in patients with myxofibrosarcoma, elevated numbers of neutrophils were observed in high-grade malignant tumors and this correlated positively with increased intratumoral microvessel density [72]. The mechanism by which tumor-associated neutrophils modulate tumor angiogenesis has not been fully elucidated. Activated neutrophils can release a variety of proteases that can degrade and remodel the ECM, a process that is crucial for angiogenesis. These cells have recently been shown to express high amounts of VEGF and MMP9 that is known to be responsible for initiation of the angiogenic switch and to support vessel growth in tumors [12]. MMP9 has been shown to have the most profound effects in mediating tumor angiogenesis [73]. Proteolysis of the ECM by this MMP releases such potent angiogenic factors such as vascular endothelial growth factor (VEGF) and FGF2 that are usually sequestered in an inactivated form to the ECM [74, 75]. MMP9 is also involved in the regulation of leukocytosis, for example, by potentiating proangiogenic and neutrophil attracting IL-8 expression [76] and by the release of hematopoietic progenitor cells from the bone marrow [77]. Huang et al. could show that MMP9-deficient mice display significantly reduced tumor microvessel density, compared with wild-type mice [78]. Neutrophil-derived MMP9 has also been shown to contribute to tumor angiogenesis and progression of squamous cell carcinoma [74]. Finally, Bv8, a potent proangiogenic factor, was shown to be upregulated in neutrophils in the context of cancer and to directly contribute to tumor angiogenesis and progression [79, 80].

4.3. Tumor Cell Dissemination. Metastasis is a highly complex process requiring tumor cell detachment from the primary tumor and migration to secondary target organs through the lymphatic or blood circulatory systems [81]. Neutrophils can exhibit both pro- and antimetastatic properties under certain conditions [82–85]. In prometastatic state neutrophils secrete soluble factors, including proteases and cytokines, that activate endothelium and parenchymal cells, leading to improvement of adhesion of circulating tumor cells in distant sites [74, 83, 86] and enhanced metastasis formation. Moreover, contact-dependent mechanisms, whereby

neutrophils act as a bridge, tethering circulating tumor cells (CTCs) to target organ endothelium, have been described [87]. Such interaction is mediated by the binding of $\beta 2$ integrins on neutrophils to ICAM-1 on tumor cells and was described for lung and liver metastasis model [84, 88]. In studies by Spicer et al. neutrophils promote cancer cell adhesion within liver sinusoids and their depletion before cancer cell inoculation resulted in decreased number of metastases in an intrasplenic model of liver metastasis [84]. Another interesting study showed that neutrophils can support lung metastasis development through physical interaction and anchoring of circulating tumor cells to endothelium [89]. It is not clear if this process supports tumor cell extravasation into target organ or neutrophils hold melanoma cells in the capillaries until they grow into a secondary tumor [89].

In addition to the mechanisms proposed thus far, novel aspects of neutrophil biology recently got attention as possible mechanism that contributes to cancer progression and metastasis. Recent studies suggest that NETs are able to trap tumor cells and depending on neutrophil activation such sequestered tumor cells can be destroyed by ROS that results in inhibition of metastasis formation [82] or be kept in place thus supporting early adhesion of tumor cells to distant organ sites and metastatic processes [90].

In the recent work of Wu et al. an inhibitory role of endogenous type I IFNs on neutrophil-mediated metastasis formation could be shown. The lack of endogenous type I IFNs drives neutrophils to prometastatic phenotype at least in two ways, supporting neutrophil migration and the formation of the premetastatic niche in the lung and inhibiting neutrophil cytotoxicity against tumor cells in circulation.

4.4. Formation of the Premetastatic Niche. Tumor induced changes in the microenvironment of distal organs make tissues more receptive to colonization of migrating tumor cells [91, 92]. Consequently, bone marrow derived cells, including neutrophils, are mobilized and accumulate in the future site of metastasis [93] where they participate in the formation of supportive metastatic microenvironment termed "premetastatic niche" [94–96]. These cells are recruited by Bv8, MMP9, S100A8, and S100A9 [6, 97] and this process seems to be strongly dependent on granulocyte colony-stimulating factor (G-CSF) [6].

Recent studies have shown that neutrophils make up the main cell population involved in formation of premetastatic niche [82]. This process seems to be enhanced by the absence of type I interferons that results in upregulation of CXCR2 expression on neutrophils from these mice. Moreover, prometastatic molecules like S100A8, S100A9, Bv8, and MMP9 are upregulated in lungs of Ifnar1$^{-/-}$ mice. Both S100A8 and S100A9 are known to influence tumor cell proliferation, survival, and migration [97, 98] but also to stimulate migration and proliferation of neutrophils. Bv8, next to induction of tumor cell extravasation [6], increases neutrophil accumulation within premetastatic tissue. MMP9 is responsible for formation of leaky vasculature in the premetastatic lung [99] and support of tumor cells survival in this organ [100].

4.5. Recruitment of Other Cells and Immune Evasion. The immune regulatory functions of neutrophils are recently getting growing attention. Interactions between neutrophils and other immune cells obviously are regulating many inflammatory processes, including tumorigenesis. There is evidence that activated neutrophils can interact with T-cells in dichotomous ways. Several studies have shown that neutrophils can present antigens and provide accessory signals for T-cell activation [101–103]. Other studies have suggested that neutrophils can suppress antigen-nonspecific T-cell proliferation [104, 105]. The suppressive function of granulocytic cells in cancer patients has generally been attributed to a circulating low-density granulocytic myeloid derived suppressor cell (G-MDSC) population [60–62]. However, there is some uncertainty about whether G-MDSCs do exist in humans. In mice this heterogeneous group of cells consists mainly of immature neutrophils and monocytes.

Neutrophil-mediated T-cell suppression requires arginase 1 or ROS [105–107]. In humans with metastatic cancer disease, arginase 1-mediated suppression of lymphocytes was reported [108, 109]. Lately, mature blood neutrophil subset was shown to suppress T-cell activation in cancer [8] and during severe inflammation [104]. This suppression requires release of H_2O_2 into the immunological synapse in a Mac-1 (CD11b/CD18) dependent manner.

Very recent studies show that neutrophils cooperate with $\gamma\delta$ T-cells in promotion of breast cancer metastasis [110]. Neutrophil depletion in the highly aggressive metastatic breast cancer mouse model KEP results in significant reduction of both spontaneous pulmonary and lymph node metastasis [110]. Moreover, combined depletion of both neutrophils and CD8$^+$ cells results in inhibition of metastasis formation, implicating cooperation of these cells during this process.

5. Recruitment of Neutrophils into Tumor and Premetastatic Sites

Neutrophils make up substantial population of cells infiltrating tumors and premetastatic niche, in mice and human [12, 15, 111]. Many cell subtypes, including tumor cells, produce chemokines that attract neutrophils, for example, CXCL1 or CXCL2.

5.1. Factors That Mediate Neutrophil Recruitment. The migration of neutrophils into solid tumors depends on chemotactic factors. There are several chemotactic factors that may stimulate the migration of neutrophils, but the most potent are members of the CXCL chemokine family. Human CXCL8 (IL-8) is one of the best studied neutrophil chemoattractants with respect to tumor biology and is overexpressed in different human carcinomas and tumor cell lines including breast, colon, cervical, lung, brain, prostate, ovarian, and renal cell carcinomas, acute myelogenous and B-cell lymphocytic leukemia, melanoma, and Hodgkin's disease [112]. Importantly, both stromal and tumor cells can produce CXCL8. Other human chemokines such as CCL3 (MIP-1α) and CXCL6 (huGCP-2) or murine chemokines CXCL1, CXCL2, and CXCL5 are potent chemoattractants and activators for neutrophils [12] and are produced by many tumors [113–116].

Recent study on hepatocellular carcinoma indicated importance of CXCL16 and its receptor CXCR6 in neutrophil recruitment and tumor progression, due to its ability to stimulate tumor cells to release CXCL8. Another recent study shows that human metastatic melanoma cells entrapped in the lungs secrete IL-8 to attract neutrophils, which promotes tumor cell tethering to the vascular endothelium. Prolonged cell retention in the lungs facilitated transendothelial migration and metastasis development [89]. Experiments have shown that inhibition of neutrophil migration, for example, by blocking of chemokine receptor CXCR2 or CXCR2$^{-/-}$ in mice, leads to reduced tumor angiogenesis and growth in B16F10 melanoma [14, 117] and Lewis lung carcinoma model [107]. Inhibited myeloid cell infiltration due to the loss of CXCR2 was also shown to be responsible for significantly suppressed chronic colonic inflammation and colitis-associated tumorigenesis [118].

A number of additional mediators might serve as chemoattractants for neutrophil recruitment to the tumor tissue. It has been shown that bioactive lipids, such as sphingosine-1-phosphate (S1P), could promote neutrophil activation and chemotaxis [119, 120]. Similarly, the hypoxia-inducible factor 1-α and its downstream products like CXCL12, VEGF, or MMP9 are involved in recruitment and retention of neutrophils in angiogenic environments [12, 121]. VEGF, in addition to its proangiogenic role during tumor growth, is also capable of inducing neutrophil adhesion to postcapillary venules followed by homing to tissues of its high expression, for example, tumor or premetastatic niche [122, 123].

Recent studies suggest that the myeloid-related proteins (MRPs) are also involved in neutrophil migration. The MRPs S100A8 and S100A9 are strongly expressed by tumors and in the premetastatic niche and act as strong chemoattractants for neutrophils into these sites [82, 97, 124]. However, the exact mechanism of MRPs mediated neutrophil mobilization is not clear and still needs to be investigated.

5.2. Survival of Neutrophils in Tumor Microenvironment. Due to their proinflammatory functions and potential toxicity against host tissue, the neutrophil life span is strictly regulated [125]. In the absence of inflammatory stimuli, neutrophils are removed from circulation shortly after their mobilization from the bone marrow, mainly by apoptosis. Importantly, several proinflammatory cytokines have been shown to influence the longevity of neutrophils [126]. Recent observations of Andzinski et al. [13] show that the life span of tumor-associated neutrophils is remarkably prolonged in tumor-bearing IFN-β deficient (Ifnb1$^{-/-}$) mice, compared to wild-type controls. This is apparently due to the fact that IFN-β is able to influence both the extrinsic and the intrinsic apoptosis pathways of neutrophilic granulocytes. Lower expression of Fas, reactive oxygen species, active Caspases 3 and 9, as well as a change in expression pattern of proapoptotic and antiapoptotic members of the Bcl-2 family and the major apoptosome constituent Apaf-1, is observed under such conditions. The death receptor Fas on neutrophils has been shown to be involved in spontaneous extrinsic cell death signaling [127]. Fas has been shown to play a role in type I IFN-induced apoptosis in several types of neoplasias such as melanoma, multiple myeloma, and chronic myeloid leukemia cells [128, 129].

ROS production by neutrophils might also play an important role in regulation of life span of neutrophils. For example, a delayed spontaneous apoptosis was shown in patients deficient for NADPH oxidase [130, 131]. It has also been shown that hypoxia or pharmacological inhibition of NADPH oxidase and hydrogen peroxide scavengers decreases the rate of neutrophil apoptosis [132]. Recent data indicate that spontaneous production of ROS is diminished in the absence of endogenous IFN-β, potentially contributing to the delayed apoptosis of tumor infiltrating neutrophils in Ifnb1$^{-/-}$ mice [13]. G-CSF is one of the major survival factors of neutrophilic granulocytes and has been reported to reduce Bax expression and redistribution [133] and restore its phosphorylation status thus leading to its inactivation. This mechanism is responsible for G-CSF-mediated repression of Caspase activation [134]. Regulation of G-CSF expression is responsible for altered neutrophils survival in tumors.

6. Concluding Remarks

Neutrophil function in cancer has long been a matter of debate as these cells were shown to possess a range of tumor promoting as well as tumor limiting properties. We propose that these conflicting observations stem from the fact that neutrophils are not a homogeneous population of cells. Neutrophil heterogeneity stems from two facts that are not mutually exclusive and have to do with the changes in the chemokine milieu in the context of cancer: The first is the fact that neutrophils are highly responsive to cues in their microenvironment and may adopt a protumor phenotype in certain conditions and an antitumor phenotype in others. The second is the fact that there are distinct neutrophil subsets which differ in their contribution in the context of cancer. Together, these observations support the notion that neutrophil function in cancer may be dictated in a context dependent fashion (Figure 1). These observations also identify potential elements which may be therapeutically targeted to enhance antitumor neutrophil activity while limiting their protumor properties.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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