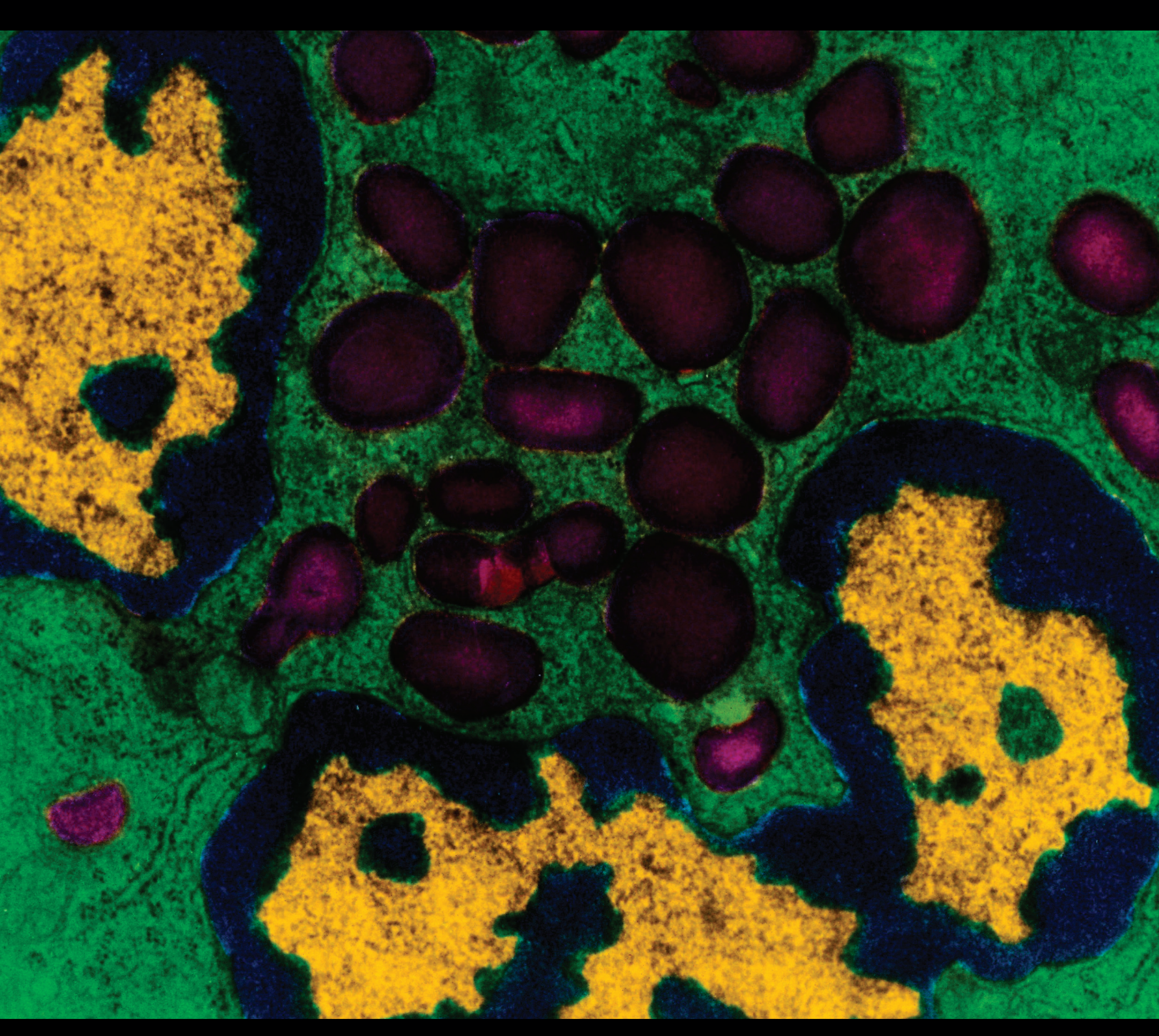
Mediators of Inflammation

Inflammation as a Target in Cancer Therapy

Lead Guest Editor: Sonia Leon-Cabrera
Guest Editors: Kaylee Schwertfeger and Luis I. Terrazas



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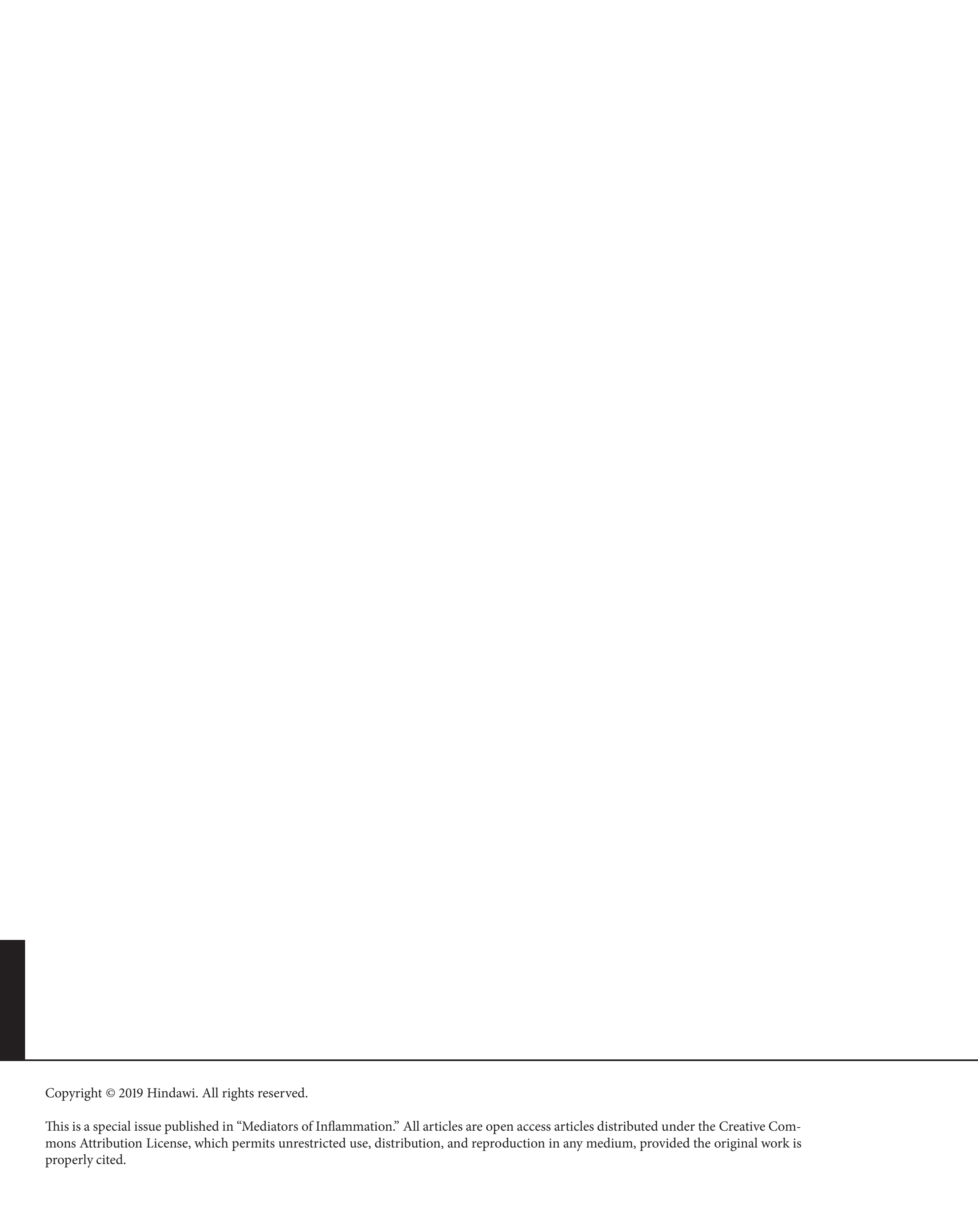

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Editorial

Inflammation as a Target in Cancer Therapy

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There is a strong association between cancer and inflammation. Dysregulated inflammatory responses play a pivotal role in tumor initiation, promotion, and progression through different pathways. Epidemiological evidence suggested that a stage of chronic inflammation, due to persistent infections like either parasites or viruses, or sterile inflammation associated with ambient factors are linked with tumorigenesis. Over the past decade, pharmacological inhibition of inflammatory cells and their products, together with the manipulation of genes involved in their functions, has been shown to participate in tumor incidence and progression. In consequence, cancer-promoting inflammation is an encouraging target of therapy in oncology. The list of tumor-promoting inflammatory cells includes tumor-associated macrophages (TAMs), dendritic cells, neutrophils, immature myeloid cells, mast cells, eosinophils, and lymphocytes. These cells are present at the tumor microenvironment and produce a variety of cytotoxic and inflammatory mediators, thus sustaining immunosuppression, tumor cell proliferation and survival, angiogenesis, autophagy, extracellular matrix breakdown, metastasis, chemoresistance, and radioresistance. Thus, understanding how inflammation in the whole tumor microenvironment can be targeted in more effective ways will ultimately lead to the development of therapeutic approaches that result in durable antitumor responses.

This special issue is aimed at encouraging the persistent effort to understand how the complex network of inflammatory circuits in the tumor microenvironment could be used to inform the development of new therapeutic modalities.

Within the tumor microenvironment, multiple mediators are secreted that contribute to the recruitment of circulating monocytes and the promotion of their differentiation into tumor-associated macrophages. Macrophages are important actors in the production of mediators and cytokines that favors inflammation, but on the other hand, could participate in wound healing and angiogenesis providing favorable conditions for tumor development. Thus, understanding the agents that are able to adjust the tumor microenvironment can be an effective way to obtain durable antitumor responses. In a research study by C. Hsieh and C.-H. Wang using an *in vitro* approach, the authors have demonstrated that aspirin inhibited the secretion of MCP-1, IL-6, and TGF-β by 4T1 breast cancer cells and regulated the expression of angiogenic and inflammation-associated cytokines in both malignant cells and macrophages. The authors postulate that aspirin increased M1 and decreased M2 polarization in macrophages, resulting in the restriction of communication in this microenvironment and reduced tumor progression.

Despite advances in understanding how inflammatory processes are involved in the development of melanoma and nonmelanoma skin cancer, surgical treatment is the gold standard therapy for basal cell carcinoma. V. Voiculescu et al. reviewed the mechanisms involved in topical therapies targeting the inflammation processes occurring in cutaneous

carcinogenesis as an alternative to nonsurgical treatment. They discussed the mechanism involved in therapies targeting Toll-like receptor-7 (TLR-7) and showing that in association with radiotherapy, chemotherapy or immunotherapy have shown a superior success rate than monotherapy with minimal adverse reactions.

During esophageal carcinoma, local infiltration of inflammatory cells favors the interruption and deletion of the local basement membrane in esophageal squamous cells, favoring cell proliferation and the activation of nuclear factor kappa B (NF-κB). The aberrant NF-κB pathway is involved in the initiation and development of many malignant tumors and regulates transcription of target genes that control cell proliferation, apoptosis, invasion, and metastasis. Therefore, the inhibition of NF-κB signaling could be an effective treatment against cancer and it could also restore sensitivity to other therapeutic options. F. Guo et al., in their experimental study, demonstrated that Grape seed proanthocyanidin (GSPE) extract inhibited the proliferation, induced apoptosis, and reduced the secretion of inflammatory cytokines in the human esophageal squamous cancer cell line. The authors postulated that GSPE activated caspase-3 and attenuated the activation of the NF-κB signaling pathway by inhibiting the phosphorylation of IκB, which could provide a potential new avenue for targeting this key pathway.

Autophagy is a regulated mechanism of the cell responsible for a self-degradative process, important for balancing sources of energy and maintaining metabolic homeostasis. Before the appearance of a tumor, autophagy helps in the degradation of damaged mitochondria that could otherwise induce oxidative stress, DNA damage, and genomic instability. In cancer, autophagy is a pathway used by tumor cells for recycling intracellular constituents, used as alternative energy sources during stressing conditions like hypoxia or nutrient deprivation. I. Cotzomi-Ortega et al. reviewed recent evidence about the interaction of autophagy with protein secretion pathways during carcinogenesis. They discussed the importance of establishing how autophagy regulates secretion from cancer cells depending upon cancer type or cancer stage, which could have implications in the use of autophagy inhibitors during clinical trials. I. Cotzomi-Ortega et al. proposed that manipulation of autophagy during cancer therapy should be used with caution since it could potentially promote malignancy and have other undesirable consequences.

CD44 is a cell surface glycoprotein mainly expressed in normal epithelial cells, and it has been proposed as a stem cell marker during tumorigenesis. By alternative splicing, human CD44 gene produces different CD44 isoforms expressed in different tissues during normal or disease stages. N. Suwannakul et al. evaluated the expression of CD44 variant 9 (CD44v9) in the liver of cholangiocarcinoma patients. They reported overexpression of CD44v9 and inflammation-related markers, in tissues from human liver fluke *Opisthorchis viverrini*-related cholangiocarcinoma. As cholangiocarcinoma is a chronic inflammation-induced cancer, they proposed that CD44v9 could be a biomarker for cancer stem cells in the progression of inflammation-related cholangiocarcinoma.

In summary, we are optimistic that the original research and review articles presented in this special issue will enhance the knowledge about the importance to understand how inflammatory pathways and mechanisms that regulate inflammation could lead to the development of better and novel biomarkers and therapies for cancer.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Review Article

Mediators of Inflammation in Topical Therapy of Skin Cancers

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Taking into consideration that the immune system plays a very important role in the development of melanoma and non-melanoma skin cancers, which have a high prevalence in immunosuppressed patients and after prolonged ultraviolet radiation, the interest in developing novel therapies, in particular targeting the inflammation in cancer, has increased in the past years. The latest data suggest that therapies such as imiquimod (IMQ), ingenol mebutate (IM), 5-fluorouracil (5-FU), retinoids, and nonsteroidal anti-inflammatory drugs (NSAIDs) have been used with success in the topical treatment of some cancers. Herein, we review the topical treatment targeting the inflammation in skin cancer and the mechanisms involved in these processes. Currently, various associations have shown a superior success rate than monotherapy, such as systemic acitretin and topical IMQ, topical 5-FU with tretinoin cream, or IMQ with checkpoint inhibitor cytotoxic T lymphocyte antigen 4. Novel therapies targeting Toll-like receptor-7 (TLR-7) with higher selectivity than IMQ are also of great interest.

1. Introduction

Melanoma and non-melanoma skin cancers (NMSCs) have known an increase in incidence throughout the years as scientists estimate that over 1.3 million new cases/year of NMSC will be identified in the US, ultraviolet (UV) radiation being the most important risk factor for this type of cancer [1]. Risk factors for developing skin cancers, beside chronic UV exposure, include human papillomavirus (HPV) infection, immunosuppression, family history of skin cancer, and light skin [2, 3]. The most common forms of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), representing 80% and 20%, respectively, of NMSC [4].

BCC's incidence is increasing by 10% every year among white people living in geographical areas with high sun exposure, like Australia [5–7]. Unlike SCC, which may be lethal, BCC is only aggressive through its local extension and has high recurrence rate if the surgical treatment is not properly carried out [8]. Although surgical treatment is the gold standard therapy for BCC, being chosen in 95% of the cases, a large range of other options has developed including topical administration of IMQ, 5-FU, IM, or photodynamic therapy [9–11].

While BCCs rarely metastasize (<1% of cases), this risk in SCCs is much higher (2-5% of cases), however still remaining remarkably lower than other types of cancer [12–16]. The earliest stage in which a SCC can be diagnosed is actinic

keratosis (AK), known to invade only the epidermis of chronically sun-exposed skin areas and having a potential of <1% to 16% per year of progression to SCC [17–19]. The American Academy of Dermatology estimates that 60% of patients of at least 40 years old, who present a predisposition, develop at least one AK [20]. Risk factors for developing an AK are immunosuppression, ageing, and fair skin [21, 22]. AK treatment includes surgical (excision, dermabrasion, laser therapy, electrosurgery, and curettage) and nonsurgical treatment (5-FU, trichloroacetic acid, tretinoin, IM, and diclofenac) [1, 23, 24].

Melanoma, the most deadly form of skin cancer contributing to 10,000 deaths per year in the United States [25], is a type of tumor strongly related to inflammatory processes, due to the high levels of secreted cytokines and the production of ROS (reactive oxygen species) and RNS (reactive nitrogen species). Recent data suggests that the secreted cytokines have a paracrine role in the tumoral microenvironment and also promote tumoral growth. The expression of IL-1 stimulates angiogenesis and promotes tumoral growth [26]. During melanoma evolution, activated macrophages produce TGF-beta (transforming growth factor-beta), TNF-alpha (tumoral necrosis factor-alpha), IL-1 alpha (interleukin-1 alpha), arachidonate metabolites, and extracellular proteases, while melanocytes express IL-8 and VEGF-alpha (vascular endothelial growth factor-alpha), inducing angiogenesis [27].

It has been shown that the immune system plays a very important role in the development of NMSC, considering the fact that it has a high prevalence in immunosuppressed patients and after prolonged UV (ultraviolet) radiation (which induces skin immunosuppression) [1, 22, 28]. UV radiation induces skin immunosuppression through various mechanisms such as the following: it stimulates natural killer (NK) cells which are implicated in the mediation of antigen-specific immune suppression, it reduces the number and functionality of Langerhans cells, and it stimulates the production of various immunosuppressive cytokines and affects genes which regulate proteins like p53 that influence the cell cycle [29–32]. UVB induces mutations of the p53 tumor suppressor gene resulting in the accumulation of keratinocytes with a mutated p53 gene, which may progress to actinic keratosis (AK) and NMSC [33–37]. Therefore, stimulating the immune system might be an efficient therapeutic strategy, with intralesional interferon already being successfully used to treat AKs, BCCs, and small SCCs [5, 38–40].

Current literature confirms the idea that cancer may develop under specific environments generated by chronic inflammation. These cells suffer intrinsic genetic modifications, and the surrounding inflammatory status influences the neoplastic growth and spread. This condition favors the development of an immunosuppressive environment by recruiting suppressor cells, like $CD4^+$, $CD25^+$, $FOXp3^+$ Treg (regulatory T cells), myeloid-derived suppressor cells, tumor-associated macrophages, and regulatory dendritic cells. Moreover, the neoplastic cells may escape the immune surveillance due to some mediators like TGF-beta and IL-10 [41].

TLRs are considered novel therapeutic drug targets, especially due to their potential role in the recognition of pathogen-associated molecular patterns (PAMPs) of different origins and generation of proinflammatory response during some inflammatory conditions. Even ssRNA-based medications targeting TLR-7 and TLR-8 have potent antitumor actions and reverse the immunosuppressive action of Tregs *via* dendritic cells and *via* inducing a Th1 immune response [42]. TLR signaling acts in two different directions regarding cancer therapy, because it appears that the tumor cells use the TLR's role in the tissue homeostasis to create proper conditions for growth and survival [43].

1.1. Imiquimod. Imiquimod (1-isobutyl-1*H*-imidazo[4,5-*c*]-quinolin-4-amine)(IMQ) is a low-molecular-weight, novel synthetic compound and member of the imidazoquinoline family that binds to TLR-7 and -8, determining high levels of interferon-alpha (IFN-α), tumor necrosis factor alpha (TNF-α), and other interleukins (IL-6, IL-8, etc.) [44–46]. Its mechanisms of action are not fully understood, but some theories may explain a part of them. It has been suggested that IMQ activates Langerhans cell migration and determines contact hypersensitivity by stimulating cytokine expression and, as a result, enhances antigen presentation [46]. IMQ is an immune response modifier, offering topical, noninvasive, and nonsurgical therapeutic options for some dermatological diseases. IMQ is also able to induce tumor cell apoptosis, which may suggest that it can be used in patients with skin tumors, especially in those with small tumors, with low-risk locations, that are not eligible for other therapies [47].

The innate immune system comprises immune cells like monocytes, macrophages, neutrophils, dendritic cells, natural killer cells, mast cells, eosinophils, and basophils and also newly identified innate lymphoid cells and mucosal associated invariant T, $\gamma\delta$T cells, NKT cells, etc., and its humoral components, meaning the circulating complement system proteins/components, cytokines, and chemokines secreted by innate immune cells along with various antimicrobial peptides [48].

The innate immune cells express a large variety of pattern recognition receptors (PRRs) including TLRs which recognize the pathogen-associated molecular patterns (PAMPs). TLRs also have an impact on the adaptive immune response mediated by different types of T cells and B cells. It has been demonstrated that TLRs play an important role in sterile inflammatory diseases, from cancer to autoimmunity (systemic lupus erythematosus, rheumatoid arthritis, spondyloarthritis, multiple sclerosis, and myositis). They are also involved in the pathogenesis of multiple human cancers such as B cell malignancies, colorectal cancer, BCC, and bladder cancer [48, 49].

It has been shown that TLR-7 can bind IMQ and virus-derived ssARN and is found in the structure of the endosomal membrane of dendritic cells, macrophages, monocytes, and mast cells [50–53]. By activating the TLRs, antigen-presenting cells (APCs) (monocytes, macrophages, B cells, and dendritic cells) are stimulated and a signaling cascade that recruits protein kinases and transcription factors is started. The result is the maturation and secretion of IL-1, IL-12, IL-18, IL-6, IL-10, and IFN-α by the target cells. These cytokines also stimulate the secretion of IFN-γ by native T cells, which leads to a Th1 lymphocyte-mediated

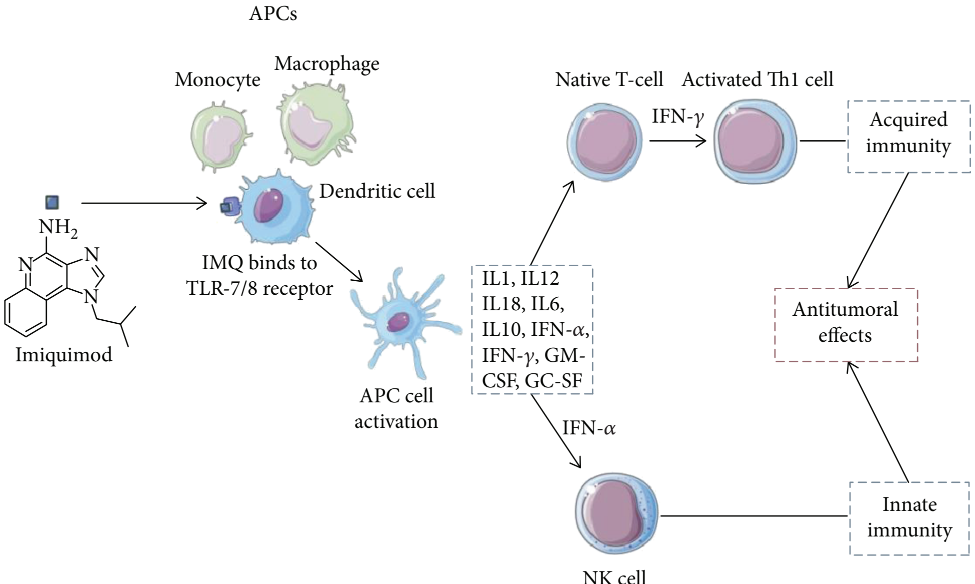

Figure 1: IMQ's primary mechanisms of action. APC: antigen-presenting cell; GC-SF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; IFN: interferon; IMQ: imiquimod; IL: interleukin; TRL: Toll-like receptor; TNF: tumor necrosis factor.

immune response and the inhibition of Th2 cells (Figure 1) [46, 54–58].

IMQ also has the ability to induce $2'5'$-oligoadenylate synthetase, leading to an activation of NK cells and perforin in cytotoxic T cells. The apoptotic effect is achieved through the activation of Bcl-2 (B cell lymphoma-2) proteins of the mitochondrial pathway [59].

Recent studies suggest that IMQ is also very useful in diseases associated with pathological neovascularization such as dysplastic nevi, melanoma, NMSCs, Kaposi's sarcoma, hemangioma of infancy, pyogenic granuloma, and angiosarcoma, as an inhibitor of angiogenesis. Its antiangiogenic activity is based on the ability to increase the secretion of IL-10 and IL-12 but also IFN's ability to decrease cellular production of some proangiogenic factors like b-FGF, IL-8, and urokinase plasminogen activator, to inhibit vascular motility and invasion, and to induce endothelial cell apoptosis [59]. Moreover, IP-10, the interferon-inducible protein 10, has an angiostatic effect [59, 60]. IL-12 inhibits endothelial proliferation and tube formation *in vitro* and angiogenesis *in vivo*, by upregulating IFN-γ, decreasing the production of VEGF and b-FGF (fibroblast growth factor), and inhibiting endothelial migration and invasion [59, 61]. The antiangiogenic mechanism of IL-10 is yet unknown, but the most probable theory is that it increases the expression of thrombospondin 1 and 2 inhibitors [49, 59].

Matrix metalloproteinases (MMP) are implicated in tumor growth, vessel formation, and metastasis [62–65]. Their role in vascular invasion and metastasis is based on their ability to cleave type IV collagen that can be found in the basement membrane [12]. This kind of activity can be stopped through MMP tissue inhibitors (TIMP), which are molecules that can bind to MMPs and inhibit their proteolytic activity, with TIMP-1 and TIMP-2 being the most important [66, 67]. It has been suggested that topical IMQ stimulates a 14-fold increase in TIMP-1 expression and a 5-fold reduction in MMP-8 [58, 59].

The advantages of the use of topical IMQ are that it is self-applied, it is a nonscarring procedure, and it is less expensive and less painful. Moreover, it can be used as an alternative on sensitive areas or lesions that involve large areas which are not susceptible to surgery [59].

Resiquimod, an imidazoquinoline, has been recently investigated as a topical adjuvant for skin cancer treatment. Although it has shown important positive results after topical treatment, the TLR-7 agonists may induce cardiac toxicity, when used at therapeutic regimens [47].

1.1.1. Imiquimod and BCC. BCC is caused by aberrant activation of the hedgehog/glioma-associated oncogene pathway, mostly due to genetic inactivation of the protein patched homolog (PTCH) gene or activation of "smoothened." Recent studies have shown that IMQ mechanisms of action include the stimulation of adenosine receptor/protein kinase A-mediated GLI phosphorylation, resulting in the inhibition of hedgehog signaling [68].

BCCs often express HLA class I molecules which will be recognized by reactive CD8 lymphocytes, but also monocytes, macrophages, and dendritic cells. The release of immunosuppressive cytokines, for example IL-10, may have an important role by impairing tumor cell recognition [47, 69].

A recent study has demonstrated that regression of BCC is associated with the activity of the innate immune response, with its origin in the macrophage-monocyte cells. Moreover, this response was associated with stimulation of apoptosis. As a result, more than 1300 genes which were differentially expressed after IMQ treatment were identified, most of them being involved in the immune response, and also a strong

upregulation of genes involved in the apoptotic signaling pathway [47, 69]. An important aspect is the decrease in Bcl-2 expression, which means that cells become susceptible to apoptosis after IMQ treatment. First, IMQ stimulates the plasmacytoid dendritic cells in the epidermis and dermis in order to release IFN-alpha and other cytokines, resulting in activation of the innate immune system cells and release of oxygen reactive intermediates and other toxic molecules, all of this leading to the apoptosis of tumoral cells. They also suggested that this mechanism is related to destruction of the overlying epithelial cells resulting in typical erosions observed during IMQ treatment. An important observation is that T cell activation occurred later during treatment, suggesting that this is not the main factor during tumoral cell elimination [47, 69].

Berman et al. observed that IMQ-induced FasR- (Fas receptor-) mediated apoptosis may contribute to the effectiveness of IMQ 5% cream in the treatment of BCC. The expression of FasR leads to apoptosis via CD95 receptor-CD95 ligand (FasL) interaction, after which a cascade of events follows, including caspase activation. On the other hand, the BCC cells normally fail to express the Fas receptor, which may be responsible for their prolonged life, escaping apoptosis. Moreover, BCC cells strongly express FasL, which is associated with apoptosis of peritumoral T lymphocytes [69, 70]. After IMQ is applied topically to the skin, it modifies the immune response by inducing IFN-α, which, in the end, upregulates the expression of FasR and at the same time continues to express FasL, making the FasR-FasL-mediated apoptosis possible. In Berman et al.'s study, they examined the expression of FasR on BCC after short-term exposure to IMQ 5% cream or vehicle, applied five times per week for approximately 2 weeks. Histology showed that BCC cells were present in all of the vehicle-treated BCCs and in 4/5 of the IMQ-treated BCCs. The FasR was expressed in three quarters of the IMQ-treated BCCs and in none of the vehicle-treated tumors. None of the vehicle-treated BCCs presented T-lymphocytes near the BCCs cells, compared to all three IMQ-treated BCCs which expressed FasR [70].

The treatment of superficial BCC implies a regimen of 5 applications/week for 6 weeks (5% IMQ cream). This application rate has proven to histologically eradicate a superficial BCC up to 82% at a 3-month follow-up and 89% at a 39-month follow-up [71–73]. A 5-year follow-up from the SINS study revealed that there were no recurrences, years after topical treatment with IMQ, in BCC lesions. One major limitation of this study is the fact that follow-up at 3-5 years was most likely made in the community by the general practitioner, who might not be as vigilant in identifying subtle changes. Regardless, this study has relevant results, considering the fact that most treatment failures are identified early, local adverse effects were not severe enough to determine withdrawal from the study and also treatment response seems to be long-lasting. In those cases in which recurrence did occur, treatment of the lesion was not influenced by the first therapeutical option [74]. An exhaustive review of the literature confirmed that cryotherapy, photodynamic therapy (PDT), topical IMQ, and 5-FU are valid alternatives for low-risk superficial BCCs [10]. Other studies show that topical IMQ 5% therapy has superior success rates than 5-FU and PDT [75, 76] even though there seems to be no link between tumor thickness and success rate regarding the three options mentioned above [77].

Studies show that IMQ is more efficient in BCCs localized on the face compared to the ones on the trunk, which is reassuring considering the high recurrence rate of facial BCC [71]. Vun et al. found no correlation between the severity of the reactions at the application site (itching, crusting) and the response rate [71]. On the other hand, Chakrabarty and Geisse observed a positive association between the dosing frequency and the response rate,and also the occurrence of local side effects. Moreover, this study showed that the occlusion of the skin after IMQ application does not enhance the efficacy, but instead it may produce severe side effects [46].

This kind of topical treatment should be seriously taken into consideration when facing a lesion with both health and aesthetic concerns. Although there are some side effects of IMQ topical therapy, they are usually mild and well tolerated [71].

Bostanci et al. have proposed the use of IMQ not only for superficial BCC, for which it is approved, but also for other histological subtypes, with good long-term cosmetic results. The authors included tumors greater than 1 cm in diameter with various subtypes, including aggressive variants (infiltrative, metatypical, and solid). A recent trial which compared the surgical results versus IMQ 5% cream in patients with nodular and superficial BCC concluded that although surgery was superior, IMQ also showed promising results. The cosmetic appearance after 3 years was superior in the IMQ group vs surgical group (60.6% vs 35.6%). The histologic clearance rate was more than 80% among nodular BCCs larger than 1 cm in diameter. However, for nasal localization of the BCC, the results were not as satisfactory, with a long-term response of only 63%. Therefore, the authors suggest IMQ treatment of nasal BCCs only if the patient cannot tolerate other types of treatment [68]. After a mean follow-up of 70 months, only 2 relapses were observed among 21 patients with complete response. These 2 relapses were diagnosed with metatypical pathology. Metatypical BCC is a rare subtype of BCC, characterized by both basaloid and squamoid differentiation. The authors suggested that IMQ treatment should be avoided in metatypical carcinoma, due to its aggressive biology. Usually, the prognosis for this type of carcinoma is worse than for the classical BCC, and the recurrence rate is higher [68]. The vast majority of recurrences of the BCC occurred within the first 12-24 months [46]. Moreover, development of SCC on 3 BCC lesions treated with vismodegib, a hedgehog pathway inhibitor, has been reported. The most probable theory is that either the initial lesion was a metatypical BCC or the hedgehog pathway inhibitor may have induced squamous differentiation in some stem cells, located in the deep epidermal layer or near the follicular bulge [68].

There is some evidence in the literature that IMQ can be successfully used in the treatment of some sclerodermiform and infiltrative types of BCC and may induce partial remission of multiple BCCs in patients with Gorlin syndrome or xeroderma pigmentosum [78].

1.1.2. Imiquimod and AK. Oyama et al. showed that AKs which responded to topical treatment with IMQ presented an increase in CD117-positive cells in the dermis. Also, it is important to note that CD117 is present in melanocytes and mast cells. Studies have also shown that the higher the inflammation induced by IMQ, the faster the AKs are eradicated [49, 79].

Therapeutic strategy is chosen based on patient preference and doctor recommendations. When facing a patient with multiple AKs, the treatment of choice is the "field treatment," using photodynamic therapy, topical chemotherapy, and immunotherapy, this way also treating subclinical AKs [1, 23, 24, 80]. A phase II study showed that topical IMQ 5%, applied 1-3 times/week, significantly reduced the number and dimension of AKs/patient. There were minimal adverse reactions, the therapy being better tolerated than other topical/surgical treatments in use. These findings accompanied by patient education might reduce the morbidity and mortality from SCC, successful treatment of AK making it hard to evolve to aggressive forms of SCC. There is still the need to further study this therapeutic option, to compare it to the gold standard treatment at the moment in order to securely use it [1]. When facing a patient with AK, studies showed that its efficacy ranges from 45.1% to 57.1%, with no significant difference between the number of applications/week (2 vs 3 applications/week) [81–83]. There are some clinical trials that showed comparable efficacy between photodynamic therapy and IMQ cream [73, 84, 85]. A recent study showed that IMQ cream 3.75% was a safe and effective treatment option for AKs, providing complete clearance of AKs in 36% of subjects in phase 3 studies [59]. However, until more information is available, Goh suggests that surgical excision or radiotherapy remains the recommended therapeutic option for such potentially aggressive tumors, because there is a risk of incomplete clearance [86]. Currently, the recommendations are two applications/week for about 16 weeks, but it may vary [11].

1.1.3. Imiquimod and SCC. Ooi et al. showed that the immune response induced by topical IMQ 5% is similar in SCCs and AKs, by increasing the number of $CD8^+$ and $CD68^+$ cells. *In situ* SCC can be really hard to differentiate from AK, and the fact that the mechanism of healing includes the same paths when treated with topical IMQ 5% means that topical therapy might be a valid alternative to surgical excision [19].

A couple of published case reports and small series have documented IMQ's off-label use in the treatment of in situ SCC, Bowenoid papulosis, extramammary Paget's disease, melanoma *in situ*, cutaneous metastases of melanoma, keratoacanthoma, and others [46].

Huang et al. studied the effects of IMQ therapy on effector T cells infiltrating human SCC, based on the theory that tumor destruction and formation of immunological memory are ultimately T cell-mediated effects. These effector T cells from treated SCCs produced more IFN-γ, granzyme, and perforin and less IL-10 and TGF-beta than the cells from untreated tumors. Moreover, the normal skin treated with IMQ presented an activation of resident T cells and a reduced production of IL-10, but no changes on IFN-γ, perforin, and granzyme, meaning that these events arise from the recruitment of different populations of T cells. An important aspect was that the blood vessels in human SCC lack E-selectin, evading the skin-homing effector T (Teff) cells and at the same time recruiting Treg cells which can suppress the immune responses. IMQ, the TLR-7 agonist, indirectly addresses both of these mechanisms. This study concluded that the IMQ-treated SCCs were infiltrated by $CD8^+$ T cells, which are associated with tumor cell apoptosis and histological signs of tumoral regression [86]. Although there was a shift in the $CD4^+/CD8^+$ cell ratio from 1 : 1 in untreated tumors to 1 : 10 in the IMQ-treated tumors, this was not due to a local proliferation, but most probably from an influx of T cells from the vascular compartment. Another interesting observation is that the treatment of cutaneous Teff cells *in vitro* with IMQ increases the activation and reduces IL-10 production, but it has no effect on IL-17 and IFN-gamma. Moreover, the T cells isolated from the human skin treated for 1 week expressed increased CD69 and decreased CD25 [86, 87].

As mentioned before, untreated SCCs do not express E-selectin and are populated by noncutaneous central memory T cells, 50% of which are $FOXP3^+$ Treg cells. IMQ induces vascular E-selectin and recruits tumor-specific CLA^+ skin-homing T cells. This will lead to a dilution of the Treg cells resident in the tumor and an activation of the tumor-specific CLA^+ skin-homing T cells within the tumor resulting in a production of IFN-γ, perforin, and granzyme and in tumor cell destruction [87].

IMQ induces the local production of IL-6 by nonregulatory Teff cells, therefore making them resistant to suppression. IMQ also reduces Teff production of IL-10 and TGF-beta, thereby reducing tonic inhibitory signals within the tumor. IMQ has an effect on the Treg cells making them reduce their ability to suppress through cytokine production (IL-10, TGF-beta) and contact suppression (CD39, CD73) [87].

Non-Treg cells in untreated SCC are an important source of IL-10, which is also produced by tumor $FOXP3^+$ Treg cells. Although some short-term trials have found that IMQ is useful in the prevention of SCC in transplant recipients, the long-term effects of IMQ in these cases is yet unknown [87].

A recent case report presented two cases of SCC treated with once daily application of 5% IMQ cream for 6 weeks. The first patient presented two months later with a subcutaneous nodule, which was histologically diagnosed as recurrent SCC, and after five months following the excision he developed metastatic SCC to a cervical lymph node. The second patient had low-grade chronic lymphocytic leukaemia with SCC in situ of the leg that failed to clear clinically at the end of the IMQ treatment, and after 4 months he re-presented with a focus of invasive SCC within the lesion. In this second case, there was a theoretical potential for failure of immune upregulation with IMQ therapy in immunosuppressed patients. Nonetheless, in the largest study to date, there was a complete clinical and histological response in 14 out of 15 patients with SCC in situ after IMQ topical treatment, once daily for 6 weeks [86].

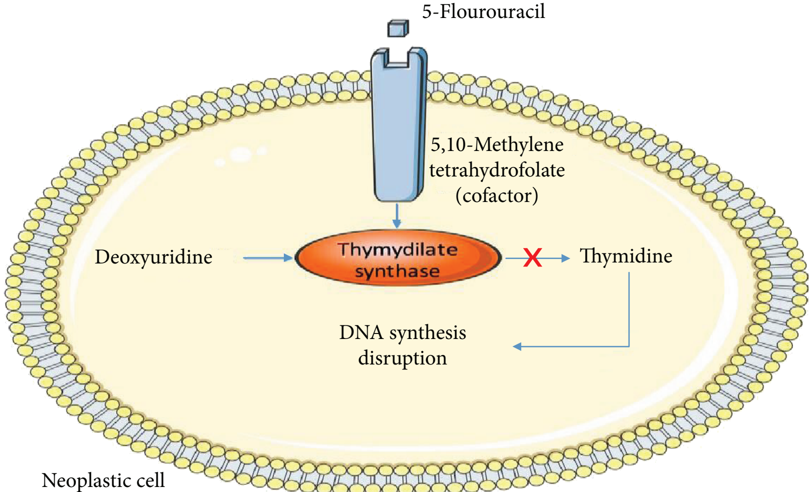

Figure 2: 5-Fluorouracil mechanism of action.

1.1.4. Imiquimod and Melanoma. It has been reported that IMQ may upregulate gene expression of endogenous angiogenesis inhibitors in melanoma tissue [59]. Off-label, topical IMQ is suggested as an alternative treatment to melanoma surgery and also as an adjunctive therapy after surgery. Topical IMQ has been used recently in the treatment of melanoma *in situ* and also cutaneous melanoma metastases [88, 89]. One case report concluded that 5% IMQ may be used in combination with topical 5-FU in cases of melanoma metastases [90].

Recent studies demonstrated the use of IMQ as an adjunctive therapy for melanoma alongside radiotherapy, by enhancing cell death through autophagy. An overexpression of the autophagy-related genes and also a large number of autophagosomes in B16F10 and B16F1 cell lines were noticed. Apparently, the autophagy was amplified via the ROS-mediated MAPK (mitogen-activated protein kinase) and NF-κB (nuclear factor-kappa B) signaling pathway. Moreover, there was an upregulation of $CD8^+$ T cells and a downregulation of Treg cells and myeloid-derived suppressor cells in the tumor lesions. Thus, this study states that IMQ may be used as a radiosensitizer and immune booster alongside radiotherapy for melanoma cases [41, 91].

IMQ alone or in combination with intralesional IL-2 may be a promising immunomodulatory treatment as adjuvant topical treatment for patients with multiple cutaneous melanoma metastases [89].

Some studies suggest that the association between IMQ and BCG (Bacillus Calmette-Guérin) vaccine induces systemic anti-melanoma immunity. The multiple pattern recognition receptor agonists present in BCG and IMQ may prove sufficient to stimulate an immune response against autologous tumor antigens [88]. There is a phase II, single-centre, randomized pilot study which started in 2017, regarding the use of topical IMQ or diphenylcyclopropenone for the management of cutaneous in-transit melanoma metastases [92].

Recent studies have suggested that it can also be used as an alternative treatment for conditions such as malignant melanocytic proliferations and Kaposi's sarcoma [59, 73].

1.2. 5-Fluorouracil. 5-Fluorouracil (5-FU) belongs to a specific drug class, anti-metabolites. It induces cellular death in cells with high mitotic activity. The main mechanism implies that 5-FU binds to thymidylate synthase through the cofactor 5,10-methylenetetrahydrofolate, causing irreversible inhibition of thymidylate synthase and preventing conversion of deoxyuridine to thymidine. Therefore, DNA synthesis in the neoplastic cells is diminished, leading to a decreased cell proliferation and promoting apoptosis (Figure 2) [93].

1.2.1. 5-Fluorouracil and BCC. Recent data suggest that 5% 5-FU cream may be used in the treatment of superficial BCC, with good cosmetic outcome, no scarring, and only mild erythema [94]. However, this treatment should be limited to patients with small tumors in low-risk locations which cannot undergo first-line therapies. Long-term clinical follow-up is recommended. The recommended regimen is two applications per day, for about 11 weeks with an average of a three-week period of follow-up [95].

1.2.2. 5-Fluorouracil and AK. There is a large number of studies which demonstrate that treatment with topical 5-FU is efficient in AKs. One study showed that 34.8% of the patients treated with 0.5% topical 5-FU and 49% of the ones treated with 5% topical 5-FU reached clinical clearance, while other studies concluded that one application/day of 0.5% for 4 weeks induced complete clearance of 47.5%-57.8% patients [96–99]. Loven and his colleagues showed that both 0.5% and 5% 5-FU have the same rate of complete clearance of 43% of patients [100]. Recent data points out that the severity of AK lesions in patients with organ transplants is significantly reduced after topical use of 5% 5-FU and 5% IMQ, although the treatment is usually longer in these subjects, because skin inflammation, which

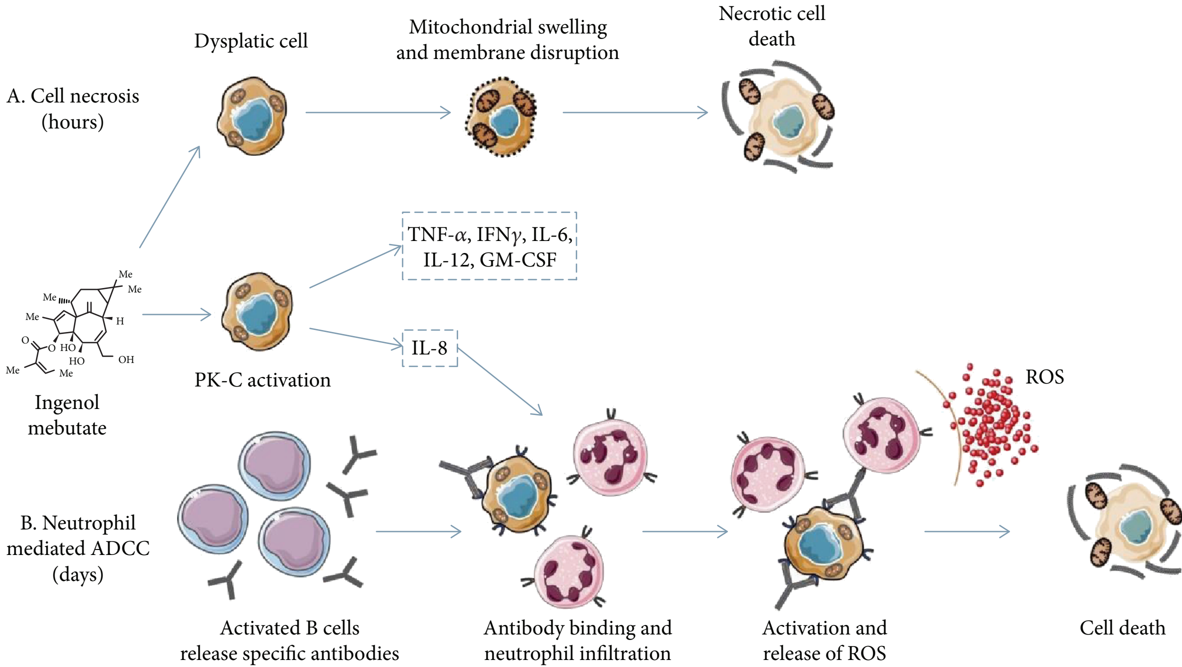

FIGURE 3: The dual mechanism of action of IM. (a) Rapid necrotic cell death occurring just hours after its application. (b) Neutrophil-mediated antibody-dependent cellular cytotoxicity occurring days after application of the drug. ADCC: antibody-dependent cellular cytotoxicity; GM-CSF: granulocyte-monocyte colony-stimulating factor; IFN: interferon; IL: interleukin; PK-C: protein kinase C; ROS: reactive oxygen species; TNF: tumor necrosis factor.

has an important role in the therapeutic effect, is usually difficult to objectify [101].

After topical use of 5-FU on AK lesions, the expression of keratin 16 was increased; a recent study suggested that proinflammatory cytokines such as IL-1 beta and TNF would be induced after the epidermal injury following 5-FU topical treatment. A two-fold increase of IL-1 beta mRNA was noticed in these cases. Moreover, MMP-1 cleaves the fibrillar type I and II collagens, major structural proteins of the dermis that can be degraded by MMP-3 and MMP-9. Also, MMP-1 mRNA was significantly increased after topical 5-FU treatment, followed by MMP-3 mRNA induction [102].

Creams and solutions are currently available in a range of concentrations, every formula containing different substances that enhance skin penetration. One of the formulas contains salicylic acid, a keratolytic agent, and also a penetration enhancer, dimethyl sulphoxide. Recent studies propose that microsponge formulations are better at depositing more products in the skin, compared to the available formulations [103]. Current treatment regimens suggest one to two applications/day, 2-4 weeks, for the 0.5% fluorouracil cream, in the treatment of AKs [11].

1.2.3. 5-Fluorouracil and SCC. Neugebauer et al. showed that even though in the long term there is no significant difference regarding SCC evolution, 5-FU is more efficient than IMQ in the short term, findings sustained by other studies [104]; therefore, 5-FU might have higher chances of stopping the progression to SCC [24]. The difference of efficiency might be due to differences in their mechanisms of action. IMQ is a synthetic immune modifier, which through TLR-7 activates the innate and acquired immune responses, while 5-FU inhibits cell proliferation and DNA and RNA synthesis, which may have a longer effect than the immune response [24, 81].

Love et al. recommend the use of topical 5-FU, twice daily for 8 weeks, but only for SCC in situ, limited to the trunk, extremities, and neck, smaller than 2 cm, if the patient cannot undergo the first-line treatment. It is not recommended for invasive SCC [95].

1.3. Ingenol Mebutate. Ingenol mebutate (IM) is an agent extracted from the sap of *Euphorbia peplus*, a plant which has been used in the past by Romans and Greeks [105], and is recently used in the treatment of various skin diseases such as warts and AK. This molecule was approved for the treatment of AK in 2012, therefore being among the newer topical therapies for skin cancer. It is suggested that there are multiple mechanisms of action, including direct cell death and a complex inflammatory response, mediated partially by PK-C (protein kinase C) activation [11, 106]. Studies have shown that there are two possible ways of inducing cancer clearance. It seems that IM stimulates the production of tumor-specific antibodies and proinflammatory cytokines, therefore inducing cellular cytotoxicity and preventing recurrence [90, 107] (Figure 3).

IM dissolves into the cell membrane and induces a rise in the intracytoplasmic calcium level which then induces mitochondrial destruction [108–110]. After topical

application, it produces a neutrophilic infiltration, due to the PK-C activation [111]. The PK-C activation stimulates proinflammatory cytokine production, expression of endothelial adhesion molecules, and tumor-specific antibody formation resulting in a neutrophil-mediated antibody-dependent cellular cytotoxicity [93]. Six hours after the first application of IM, mitochondrial swelling was observed on transmission electron microscopy, and total cell destruction was identified 24 hours after the first application [112]. The inflammatory response induced by this molecule seems to be a T cell-independent effect, with the recruitment of neutrophils which then stimulates the production of ROS [81, 107, 113–115].

The importance of neutrophils in sustaining tumor-free skin is evidenced by a study which showed that in neutrophil-depleted mice, although clearance of the tumor was achieved after 3 days of treatment, the recurrence appeared after 25 days since the treatment with IM [81, 107].

Cozzi et al. showed that topical administration of IM induces the destruction of epidermis, the new epidermis showing significant reduction in keratinocytes expressing p53 mutated gene [116]. It has also been discovered that skin which has not been exposed to UV radiation is less susceptible to develop erythema after topical administration of IM. The mechanism is unknown at the moment, but it is believed that normal skin may not be as permeable to this molecule as sun-damaged skin; also, in normal skin, mast-cell degranulation is lower than in chronic UV-exposed skin [116–118].

1.3.1. Ingenol Mebutate and BCC. IM gel therapy has proved its efficiency without important side effects in the treatment of pigmented and nonpigmented superficial BCC. These results were observed using histology and dermoscopy methods [111]. In a phase IIa trial which evaluated its use in the treatment of superficial BCC, only the highest concentration (0.05%) administered on consecutive days was statistically more efficient than the vehicle [111]. Additional trials are needed because the indications for BCC treatment are currently off-label [11].

1.3.2. Ingenol Mebutate and AK. Another recent study on the pharmacodynamics of IM, and looking at the local changes in both normal skin and in AK lesions on which they applied the drug, suggested that a strong inflammatory response was noted in both instances. There was a heavy T cell infiltration ($CD4^+$, in particular) in the papillary dermis as well as neutrophil and ICAM-1 (intercellular adhesion molecule-1) expression on the vascular endothelium of the normal skin. Also, some extravasated erythrocytes were observed in the dermis of some samples of the normal skin but, more importantly, in all of the AK lesions at the end of the treatment. Moreover, the drug modified the expression of numerous genes in both cases and, in particular, in the treated AK lesions, those involved in epidermal development being downregulated. Therefore, they concluded that IM gel 0.05% is capable of inducing epidermal cell death and also immune reactions [119]. The current treatment recommendations are one application of 0.05% or 0.015% gel/day for 2-3 consecutive days [11].

Phase 3 studies showed its efficiency in clearing AK, with sustained clearance over 12 months, using concentrations of 0.015% for face and scalp and 0.05% for trunk and extremities [111]. There is evidence to suggest that IM has higher efficacy than diclofenac 3% and IMQ 5% in the treatment of AK [120].

A case report showed full clinical remission of multiple AKs with good aesthetic outcome in a patient with organ transplant, which used IM on large skin areas. This suggests that IM may be used on large areas, even on 100 cm^2 of skin, resembling field cancerization treatment by photodynamic therapy without the systemic side effects [121]. There is also evidence that IM treats subclinical lesions present in photodamaged skin and reduces the number of tumors that develop in UV-exposed skin [106]. Treatment efficacy depends on number of consecutive days of application (2 vs 3), region (trunk vs face), and concentration (0.015% vs 0.05%), but the overall sustained clearance at 12 months ranges from 44% to 46.1% [122–124].

1.3.3. Ingenol Mebutate and SCC. Another situation in which IM may be of use is the treatment of multiple SCC in patients with organ transplant, where field cancerization is common, because the immunosuppression promotes keratinocyte tumoral formation and decreases the immunity. Nonetheless, the treatment of field cancerization is very challenging, especially in those with organ transplants [122–124].

Erlendsson et al. have concluded that repeated field-directed treatments with IM delay the development of UV-related SCC in hairless mice [125]. The authors also noticed that increased local skin reactions including erythema, flaking, crusting, vesiculation, swelling, and ulceration are associated to improved clinical outcomes. Currently, it is used off-label in the treatment of SCC [125].

1.3.4. Ingenol Mebutate and Mycosis Fungoides. A 2016 study concluded that topical IM 0.05% may be an effective alternative topical treatment for localized plaques/patches of mycosis fungoides (MF) and folliculotropic MF. It must however be taken into consideration that patients included in this trial were also receiving systemic methotrexate. The authors supposed that the mechanism of action is based on the PMN (polymorphonuclear neutrophil) oxidative burst and keratinocyte cytokine release and, nonetheless, apoptosis. No TCR (T cell receptor) rearrangement was observed in any of the biopsies [126].

Studies have shown that the adherence to IM therapy is higher than with other topical molecules, due to the shorter treatment duration [127–130].

1.4. Nonsteroidal Anti-inflammatory Agents and NMSCs. Cyclooxygenase (COX) is an enzyme which limits the production of prostaglandins from arachidonic acid. Topical therapy with nonsteroidal anti-inflammatory agents (NSAIDs) has proven to induce apoptosis, and it seems that there is a very strong link between COX2 activity and the expression of antiapoptotic proteins [131]. COX exists in two forms, COX1 and COX2; the first is constitutively expressed, while the second is expressed after inflammatory

stimuli, like ultraviolet light exposure [106, 132, 133]. The overexpression of COX2 has been revealed in numerous neoplasms, including skin cancer. Normal skin has low levels of COX2 and PGE2 (prostaglandin E2), but these levels increase with the severity of the malignancy. Recent studies suggest the importance of COX2 and its products, especially PGE2, in the development of NMSC. Studies show positive results after treatment with NSAIDs for different types of cancer. The main mechanism of action is the inhibition of angiogenesis and the stimulation of apoptosis through COX2 inhibition. Selective inhibition of COX2 in preferred due to the minimal damage to the gastrointestinal tract. In particular, celecoxib, a COX2 inhibitor, has proved its potential therapeutic effect in the prevention of skin neoplasia. Both oral and topical celecoxib have shown chemopreventive effects in animal studies by inhibiting new tumoral formation and delaying tumor latency [106]. There is a strong relation between COX2 and the expression of antiapoptotic proteins of the Bcl-2 family; therefore, the NSAID treatment may induce cellular apoptosis [11].

Diclofenac, a NSAID, reduces the production of prostaglandins by inhibiting the formation of COX2, thereby reducing dysplastic keratinocytes in cancerous lesions [106]. Other mechanisms are the induction of apoptosis by sensitizing neoplastic keratinocytes for ligand induced death, and it is also responsible for the inhibition of angiogenesis in the cancerous cells [93]. Currently, it is approved for the treatment of AK, twice-daily application, for 2-3 months. It can be used including in solid organ transplant recipients, but there are no data regarding its efficacy for BCC or SCC. Two case series have reported clearance of Bowen's disease in a total of 7 patients treated with topical diclofenac for 56 to 90 days. Further studies should be conducted before it can be recommended as treatment for NMSC [106, 134]. Diclofenac also seems to be a valid therapy option for melanoma skin metastases [11, 135].

Currently, the formula containing 3% diclofenac in 2.5% hyaluronic acid has been approved for the treatment of AK in the USA [106], its efficacy ranging from 38% to 47% complete clinical clearance of AKs in different studies [136, 137].

1.5. Immunomodulatory Benefits of Drug Associations in Skin Cancer. It has been shown that the efficacy of IMQ can be accentuated by combined therapy with checkpoint inhibitor cytotoxic T lymphocyte antigen (CTLA) 4, of which ipilimumab (a CTLA-4 specific antibody) has shown promising results in metastatic melanoma patients [138, 139]. This antibody seems to be in competition with CD28 during T cell activation [140, 141]. Associated with systemic acitretin, topical IMQ 5% seems to reduce the recurrence of superficial BCC, more than IMQ 5% cream used alone [142]. Rausch et al. showed that IMQ induces a delay in tumor growth and it does not contribute to any memory formation, but by combining it with other immune stimulants like UV-light and CD40 ligands, this inconvenience might be solved [143–145].

5-FU may be applied to the lesion alongside tretinoin cream, which enhances its actions [146].

1.6. Novel Therapies and Future Directions. 852A (N-[4-(4-amino-2-ethyl-1H-imidazo[4,5c]quinolin-1-yl)butyl]methanesulfonamide, 3 M-001), a small-molecule imidazoquinoline, similar to IMQ, which activates TLR-7 with highly selectivity, is currently being investigated for the treatment of various neoplasms, including inoperable melanoma [42].

Preclinical studies have also demonstrated that IMQ and resiquimod amplify the antitumoral effect of some vaccines by stimulating the innate immune system, but further investigation should be conducted in order to find novel therapies targeting TLR [147].

While some recent data suggest microneedling mediated delivery of diclofenac [148], another important matter is the development of better strategies for the topical delivery of the drug to AKs. Topical therapy is usually used if the tumors are present in the upper layers of the skin and for palliative reasons [59, 103]. There is some data suggesting that iontophoresis may be a good delivery method for IMQ, but the study was only conducted on mice [103].

Further directions should also be oriented towards the bacterial enzyme T4N5 endonuclease, which repairs UVA-damaged DNA. It is a local therapy which was used to treat diseases such as xeroderma pigmentosum, AKs, and BCCs, reducing the lesions [149]. This enzyme is able to minimize the production of cutaneous IL-10 and TNF-alpha and also to restore the interferon-gamma-induced ICAM-1 expression in the skin [150, 151].

2. Conclusions

As mentioned above, inflammation not only plays an important role in tumoral growth but also can be used to fight against neoplastic processes.

This analysis of current literature provides an insight into the links between inflammation and cancer.

Since inflammation is known to play a crucial role in the development of skin cancer, this review focuses on topical therapies targeting the inflammation processes occurring in cutaneous carcinogenesis. These therapies usually have minimal adverse reactions, good tolerance, and adherence to the treatment.

Currently, various associations have shown a superior success rate than monotherapy, such as systemic acitretin and topical IMQ or topical 5-FU with tretinoin cream. Another promising combination is IMQ with checkpoint inhibitor cytotoxic T lymphocyte antigen, such as ipilimumab. Novel therapies targeting TLR-7, but with higher selectivity than IMQ, are of great interest.

Conflicts of Interest

The authors declare no conflict of interests.

Authors' Contributions

All authors contributed equally to this work.

Acknowledgments

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Research Article

Grape Seed Proanthocyanidin Extract Inhibits Human Esophageal Squamous Cancerous Cell Line ECA109 via the NF-κB Signaling Pathway

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Esophageal squamous cell carcinoma is the most common type of squamous cell carcinoma. Grape seed proanthocyanidin extract (GSPE) is considered to exhibit anticancer activity against several different types of cancer. We aimed to determine whether GSPE inhibited esophageal squamous cancerous cells and the possible involvement of NF-κB in this process. The human esophageal squamous cancer cell line ECA109 was treated with GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L) for 12, 24, and 48 h. The MTT assay was used to determine cell proliferation; alterations in cell apoptosis were detected by flow cytometry; levels of inflammatory factors interleukin-6 and cyclooxygenase-2 and apoptotic proteins Bax/Bcl-2 were measured by ELISA; qRT-PCR and western blots were used to examine the activation of caspase-3 and NF-κB signaling. GSPE inhibited the proliferation of ECA109 cells and induced cellular apoptosis in a time- and dose-dependent manner. ELISA results showed that GSPE and BAY11-7082 reduced the secretion of inflammatory cytokines interleukin-6 and cyclooxygenase-2. The results of PCR and western blotting indicated that GSPE and BAY11-7082 activated caspase-3 and attenuated the activation of the NF-κB signaling pathway. GSPE induced apoptosis in ECA109 cells and inhibited ECA109 cell proliferation via a reduction in the secretion of inflammatory cytokines. This mechanism may be related to the attenuation of NF-κB activity and the sensitization of caspase-3.

1. Introduction

Esophageal carcinoma (EC), one of the most common cancers, is caused by malignant transformation of the esophagus. It is the sixth leading cause of death among malignant cancers, and the most common pathological type is esophageal squamous cell carcinoma (ESCC). The Kazakh area in Xinjiang, China, is a high-risk region for EC. Despite advances in understanding the mechanisms of cancer progression and the development of different therapeutic strategies, EC is still the leading cause of mortality in malignant tumor death among the Kazakh population in Xinjiang, particularly as a result of metastasis [1].

Chronic esophagitis is one of the most important factors for the occurrence of esophageal cancer. Murphy et al. found that non-Barrett's esophagitis increased the risk of ESCC [2]. Zhang et al. reported that local infiltration of inflammatory cells led to the interruption and deletion of the local basement membrane in esophageal squamous cells [3], which promoted cell proliferation and induced EC. Nuclear factor kappa B (NF-κB), a transcription factor that plays an important role in inflammation, is involved in the progress of

chronic esophagitis [4]. NF-κB participates in cell proliferation [5], cytoskeletal remodelling [6], cell invasion [7], and apoptosis [8]. Studies have found that NF-κB is a key factor in the development of a variety of malignant carcinomas, such as liver cancer [9], colon cancer [10], and breast cancer [11]. However, a direct connection between NF-κB signaling and EC is less certain.

Proanthocyanidins (PCs), a class of polyphenolic compounds, are widespread in plants, mostly in the epidermis and seeds. Our previous studies determined that PCs reduced oxidative damage and inflammation [12, 13]. Recent research demonstrated the anticarcinogenic activity of PCs [14], with cytotoxic effects reported in various cancerous cell lines (liver [15], colon [16], breast [17], and esophageal [18]) that were largely mediated through apoptosis and showed no adverse biological effects on normal cells. Although it was found that PCs could induce apoptosis in cancer cells, the role of NF-κB in the reversal of EC, as well as the mechanism, remains unclear. Therefore, we conducted this study to determine whether GSPE induced apoptosis in esophageal cancer cells and examined any possible involvement of NF-κB in the process.

2. Materials and Methods

2.1. Reagents. GSPE (≥95.0%) was obtained from JF-Natural Company (Tianjin, China). BAY 11-7082 and antibodies against IKK, caspase-3, and NF-κB (p65) were supplied by Abcam (Cambridge, England), and antibodies against IκB, phospho-IκB (p-IκB), and NF-κB (p100/p50) were procured from Cell Signaling Technology Inc. (Danvers, MA). Antibodies against GAPDH were purchased from Goodhere Biotechnology (Hangzhou, China). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, fetal bovine serum (FBS), and trypsin/EDTA were purchased from HyClone (Logan, Utah). 3-(4,5-Dimethylthiazol-2-yl)-3, 5-diphenyltetrazolium bromide (MTT) was obtained from Jiancheng Biotechnology Co. (Nanjing, China). The annexin V-FITC/PI apoptosis kit was procured from Multisciences (Hangzhou, China). ELISA kits for IL-6 and COX-2 were purchased from Elabscience (Wuhan, China).

2.2. Cell Culture. Human esophageal squamous ECA109 cells were kindly provided by the Department of Pathology, Key Laboratory for Xinjiang Endemic and Ethnic Diseases, Shihezi University School of Medicine (Xinjiang, China). All cells were cultured in monolayers with 90% DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed every second day.

2.3. Cell Viability Assay. The MTT assay was used to measure the viability of ECA109 cells. The cells were plated into 96-well plates at a density of 2000 cells/well in 200 μL DMEM. After incubation at 37°C overnight, GSPE (0–400 μg/mL) was added to the cells for 12, 24, and 48 h. Each treatment and time point were assayed in triplicate. After the stipulated treatment time with GSPE, MTT was added to the cells for 4 h. Subsequently, the supernatant was discarded and the formazan precipitates were dissolved in 150 μL dimethyl sulfoxide (DMSO). An automatic microplate spectrophotometer was used to measure the optical density (OD) for each well. The detected wavelength was 490 nm, and the reference wavelength was 620 nm.

2.4. Annexin V-FITC/PI Staining. Apoptosis was determined in ECA109 cells by using an annexin V-FITC/PI apoptosis kit. After treatment with GSPE (0, 25, 50, and 80 μg/mL) for 24 h, the cells were collected and washed twice with cold PBS. Subsequently, 1×10^6 cells were suspended in binding buffer, stained with annexin V-FITC and PI, and analyzed by using flow cytometry.

2.5. Cell Migration Assay. The effect of GSPE on ECA109 migration was analyzed by using a cell scratch test. Cells were plated into 6-well plates at a density of 5×10^6 cells/well in 2 mL DMEM supplemented with 10% FBS. The cells were allowed to adhere, scratched by pipette tips, and treated with GSPE (0, 25, 50, and 80 μg/mL) for 24 h. Each treatment was assayed in triplicate. After incubation at 37°C overnight, the cells were observed by using an inverted microscope.

2.6. Cell Invasion Assay. A Transwell cell invasion assay was performed. Briefly, the upper chamber of Millicell cell culture inserts was coated with 50 μL Matrigel diluted 1 : 8 with PBS. Subsequently, 4×10^5 ECA109 cells in 0.4 mL serum-free DMEM, with or without GSPE, were added to the upper chamber. The lower chamber was filled with 0.6 mL DMEM supplemented with 20% FBS as a chemoattractant to induce invasion. After incubation at 37°C for 24 h, the culture inserts were removed and the noninvasive cells on the upper surface of the culture inserts were removed by using a cotton swab. The cells that invaded through the Matrigel were fixed with methanol for 30 min and stained with 0.1% crystal violet for 10 min at 20°C. Images were captured by using light microscopy.

2.7. ELISA. Briefly, the cells were cultured with GSPE (0, 25, 50, and 80 μg/mL) and GSPE (0, 25, 50, and 80 μg/mL) + BAY11-7082 (10 μmol/L) for 12, 24, and 48 h. Supernatants from experimental cultures were collected and stored at −80°C until use. The levels of IL-6 and COX-2 in the supernatants were determined by using cytokine detection ELISA kits in accordance with the manufacturer's instructions; detection at 450 nm was conducted by using a microplate reader. The concentration of Bax and Bcl-2 in the cell culture supernatant was determined by using a Bax and Bcl-2 detection ELISA kit.

RT-PCR was performed to evaluate the mRNA expression of caspase-3, IKK, NF-κB (p50), and NF-κB (p65) after treatment with GSPE (0, 25, 50, and 80 μg/mL) and GSPE (0, 25, 50, and 80 μg/mL) + BAY11-7082 (10 μmol/L) for 24 h, as previously described [19]. The designed primers are shown in Table 1.

2.8. Western Blot Analysis. ECA109 cells were treated with GSPE (0, 25, 50, and 80 μg/mL) and GSPE (0, 25, 50, and 80 μg/mL) + BAY11-7082 (10 μmol/L) for 24 h. After treatment, the cells were collected and washed three times with PBS. The harvested cells were lysed on ice for 30 min in

Table 1: Primer of caspase-3 and NF-κB-related factor.

Name	Primer	Sequence
Homo-GAPDH	Forward	5′-TCAAGAAGGTGGTGAAGCAGG-3′
	Reverse	5′-TCAAAGGTGGAGGAGTGGGT-3′
Homo-IKK	Forward	5′-TGTACCAGCATCGGGAACTT-3′
	Reverse	5′-TCAGGAACATCACAGGCCTT-3′
Homo-IκB	Forward	5′-ACTCCCGACACCAACCATAC-3′
	Reverse	5′-CTCCGGTTTGTCAAGGTCAG-3′
Homo-NF-κBP65	Forward	5′-ACCGGATTGAGGAGAAACGT-3′
	Reverse	5′-ACGTAAAGGGATAGGGCTGG-3′
Homo-NF-κBP50	Forward	5′-TCGTTTCCGTTATGTATGTGAAGG-3′
	Reverse	5′-TGTCCTTGGGTCCAGCAGTT-3′
Homo-caspase-3	Forward	5′-ACTGGACTGTGGCATTGAGA-3′
	Reverse	5′-GCACAAAGCGACTGGATGAA-3′

100 mL of lysis buffer. The total protein was collected and quantified by using the Bradford assay. The separated proteins were transferred onto nitrocellulose membranes, which were first incubated with antibodies against caspase-3, IKK, phospho-IκB, IκB, NF-κB (p50), NF-κB (p65), and GAPDH, and then incubated with secondary anti-mouse or anti-rabbit antibodies. All western blotting studies were repeated three times.

2.9. Statistical Analysis. All values are expressed as the mean ± standard deviation (SD), and analyses were computed by using SPSS 20.0. Western blotting analysis was calculated by using Image-Pro Plus software. The comparison of the mean among multiple groups was performed with analysis of variance. Pairwise comparison among groups was performed with the least significant difference (LSD) tests. For all preplanned or a priori contrasts stipulated in the main hypotheses, a significance level of 0.05 or 0.01 was considered to indicate statistical significance.

3. Results

3.1. GSPE Inhibited the Survival of ECA109 Cells. GSPE exerted an obvious inhibitory effect on ECA109 cell survival, as shown in Figure 1. A higher GSPE dose resulted in a stronger inhibitory effect on ECA109 cells; similarly, a higher application time for a specific GSPE dose significantly decreased the survival rate of ECA109 cells ($P < 0.05$). GSPE had a significant time- and dose-dependent inhibitory effect on ECA109 cells. Through the calculation of IC_{50} after the application of GSPE for different times, we selected the treatment doses of GSPE as 25, 50, and 80 μg/mL (Table 2). In addition, our results showed that the survival rate of ECA109 was decreased by the intervention of GSPE for 24 h and 48 h, but the difference was not statistically significant.

3.2. GSPE Induced Apoptosis in ECA109 Cells. We used flow cytometry to determine whether GSPE affected the apoptosis

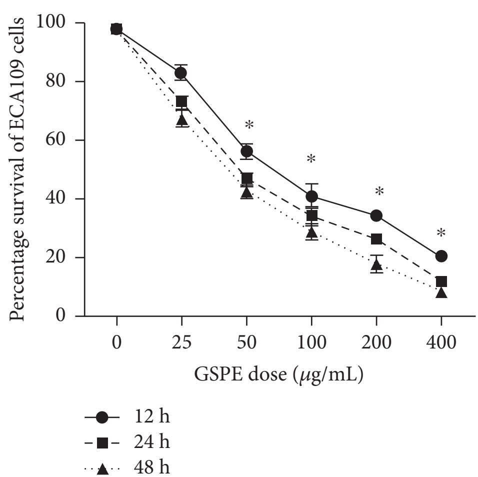

Figure 1: The effect of GSPE on ECA109 survival. The cytotoxicity of GSPE (0–400 μg/mL and 12–72 h) was detected by MTT assay. Each column represented mean ± SD of three groups of independent samples. ∗ means $P < 0.05$ compared with GSPE 0 μg/mL.

Table 2: IC_{50} of GSPE over different treatment times.

GSPE	Duration (h)		
	12	24	48
IC_{50} (μg/mL)	66.442 ± 13.54	51.713 ± 12.69	37.158 ± 13.07

of ECA109 cells. Between GSPE concentrations of 25, 50, and 80 μg/mL, the percentage of apoptotic ECA109 cells increased from 34.0% to 76.3% and the differences between each group were statistically significant ($P < 0.05$). In this experiment, we used FITC and PI double staining. In the histogram, the first quadrant represents the cells in late apoptosis and the second quadrant represents the cells in early apoptosis. We found that the application of GSPE

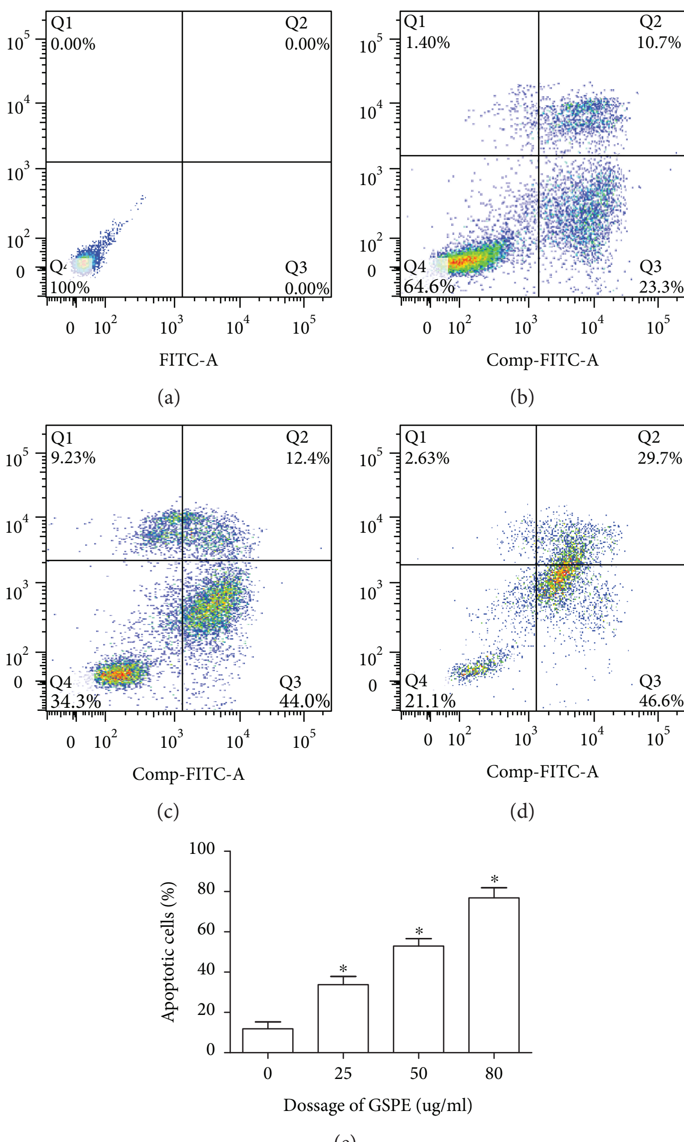

Figure 2: GSPE induced apoptosis of esophageal cancer cell ECA109 apoptosis. ECA 109 cells were treated with GSPE (0–80 μg/mL) for 24 h. Double staining with annexin V-FITC and PI was used to determine apoptosis. Values were mean ± SD of three independent samples. (a) GSPE 0 μg/mL; (b) GSPE 25 μg/mL; (c) GSPE 50 μg/mL; (d) GSPE 80 μg/mL; (e) GSPE 0–80 μg/mL. $^*P < 0.01$ compared with the GSPE 0 μg/mL group.

(25–80 μg/mL) for 24 h increased the percentage of ECA109 cells in early apoptosis and in late apoptosis ($P < 0.05$); furthermore, a dose-dependent relationship was found (Figure 2).

3.3. GSPE Inhibited ECA109 Cell Migration. Based on the results of the MTT and flow cytometry assays, we observed the change in cell migration capacity after GSPE treatment for 24 h. For 25 μg/mL GSPE, the change in cell migration distance was not obvious compared with that in the control, but at 50 and 80 μg/mL, the distance was significantly shortened (Figure 3).

3.4. GSPE + BAY11-7082 Inhibited the Invasion of ECA109 Cells. Compared with the control group, the application of GSPE (25, 50, and 80 μg/mL) reduced the number of cells that passed through the well (Figures 4(a)–4(d)). It was suggested that the inhibitory effect on ECA109 cells was elevated with the increasing concentration of GSPE, while the invasive abilities of ECA109 cells were decreased.

After the simultaneous application of GSPE (0, 25, 50, and 80 μg/mL) and 10 μmol/L BAY11-7082 to the Transwell chambers, the cultured cells were observed after 24 h (Figures 4(e)–4(h)). Compared with the control group, all concentrations of GSPE + BAY11-7082 inhibited cell movement through the Transwell chambers (Figure 4(i)).

3.5. GSPE and BAY11-7082 Inhibited Inflammatory Cytokine Levels in ECA109 Cells. In the absence of GSPE, a high level of secretion of IL-6 and COX-2 was observed in ECA109 cells.

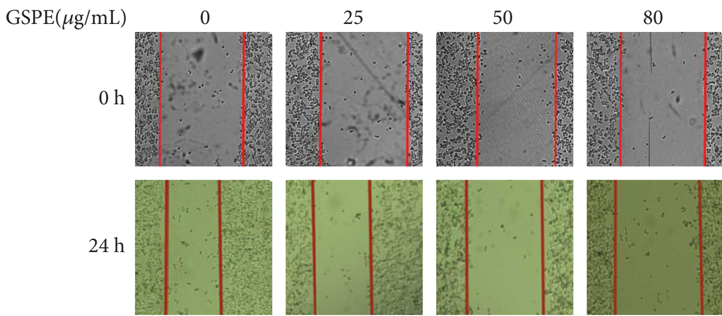

Figure 3: The effect of GSPE on the migration of ECA109. The effect of GSPE (0–80 μg/mL) on ECA109 migration was analyzed by using a cell scratch test. Each treatment was assayed in triplicate. After incubation at 37°C for 24 h, the cells were observed by an inverted microscope (magnification, ×100).

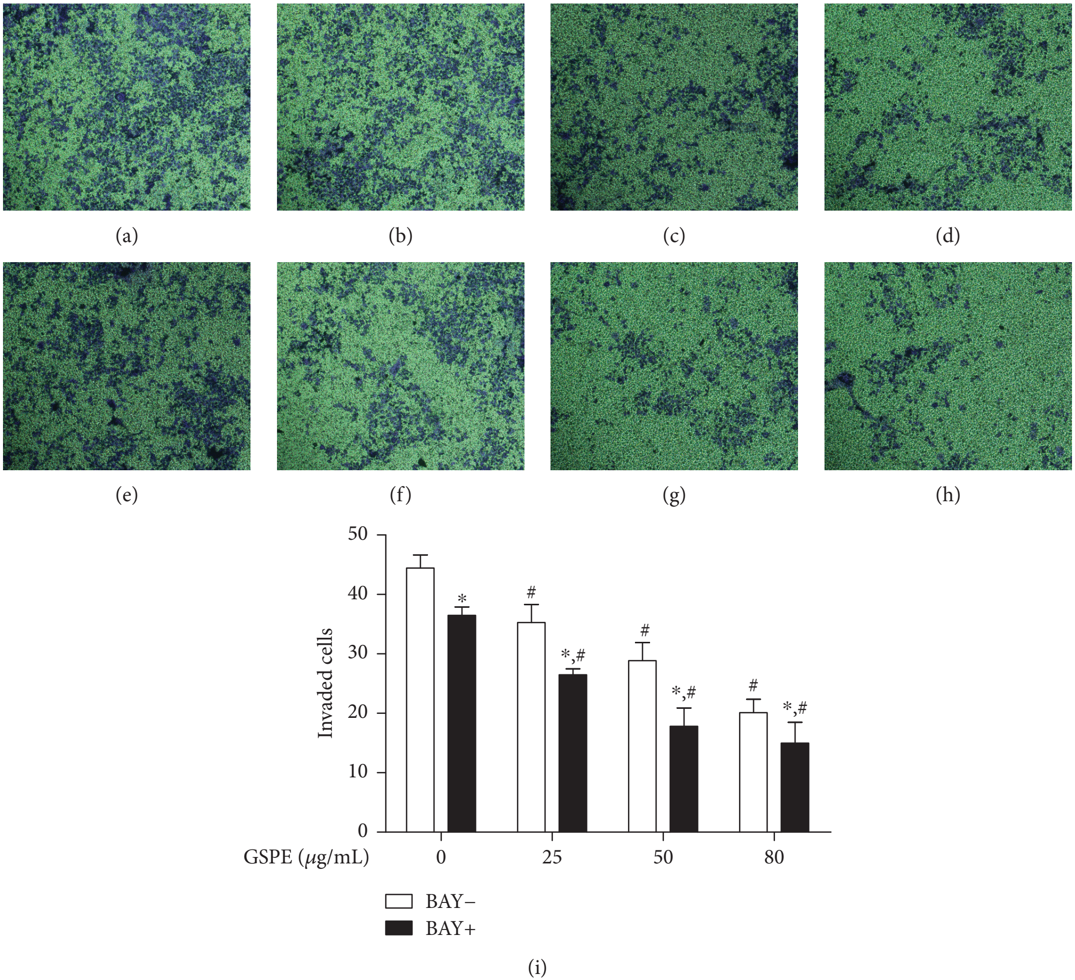

Figure 4: The effect of GSPE and BAY11-7082 on the invasion ability of ECA109 cells. The effect of GSPE on ECA109 invasion was analyzed by using Transwell assay. Each treatment was assayed in triplicate. After incubation at 37°C for 24 h, the cells were observed by an inverted microscope (magnification, ×100). (a–d) The inhibition of invasion ability in cells induced by GSPE (0–80 μg/mL). (e–h) The inhibition of invasion ability in cells induced by GSPE (0–80 μg/mL) + BAY11-7082. (i) The number of invaded cells, which was evaluated by ImageJ 2x. Each column represents mean ± SD of three groups of independent samples. $^{*}P < 0.05$ compared with the BAY11-7082 group; $^{\#}P < 0.05$ compared with the GSPE 0 group.

In the presence of GSPE, the secretion of IL-6 and COX-2 in the cells was inhibited; an increase in GSPE dose led to more obvious inhibition ($P < 0.05$) (Figures 5(a) and 5(b)). In addition, we observed the effect of the same GSPE dose applied for different times on the secretion of IL-6 and COX-2 and found that stronger inhibition occurred when

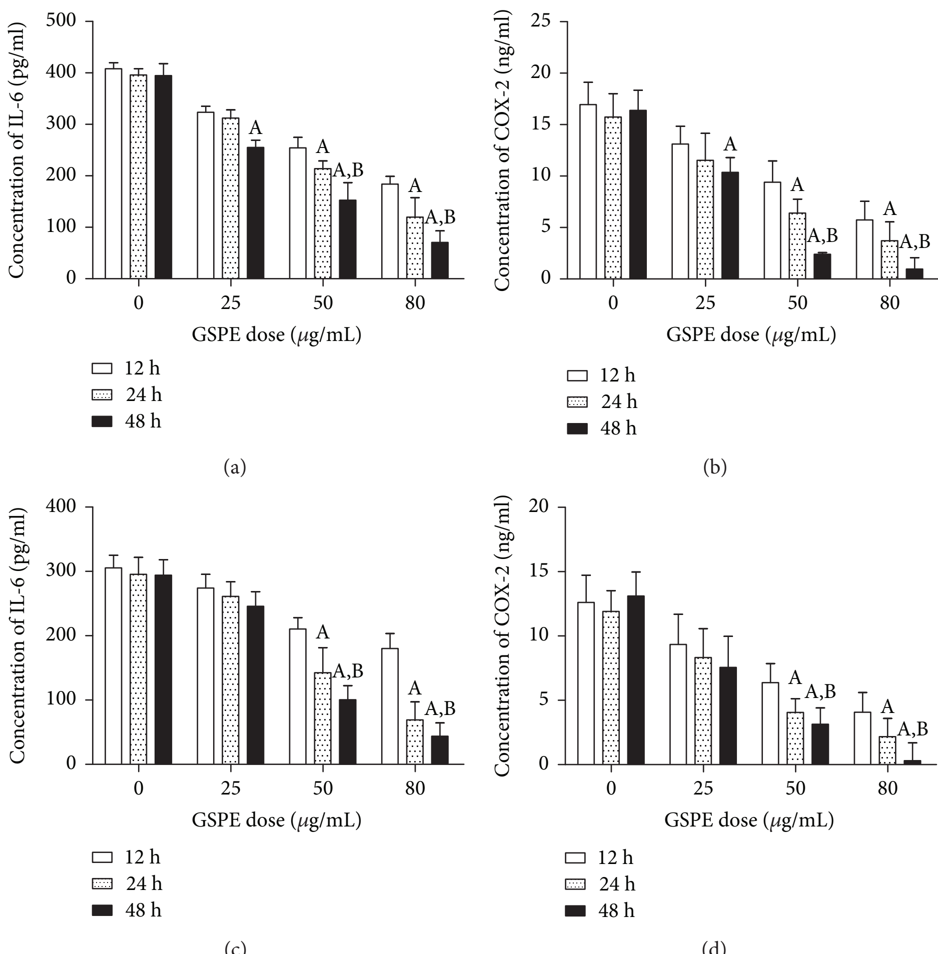

Figure 5: GSPE and BAY11-7082 inhibited the expression of inflammatory cytokines IL-6 and COX-2. (a, b) The inhibition of IL-6 and COX-2 in cells induced by GSPE (0–80 μg/mL). (c, d) The inhibition of IL-6 and COX-2 in cells induced by GSPE (0–80 μg/mL) + BAY11-7082. Each column represents mean ± SD of three groups of independent samples. $^{A}P < 0.05$ compared with the 12 h group; $^{B}P < 0.05$ compared with the 24 h group.

the same GSPE dose was applied for longer times ($P < 0.05$). The measurement of the concentration of IL-6 and COX-2 in ECA109 cells after treatment with GSPE + BAY11-7082 showed that GSPE + BAY11-7082 could inhibit the secretion of inflammatory cytokines in ECA109 cells; furthermore, the inhibitory effect of GSPE + BAY11-7082 was stronger than that caused by GSPE treatment alone (Figures 5(c) and 5(d)).

3.6. GSPE and BAY11-7082 Promoted Bax and Inhibited the Activity of Bcl-2. We investigated the effects of different times and different doses of GSPE compared with the control group. The protein levels of Bax increased and the protein levels of Bcl-2 decreased; a time- and dose-dependent relationship was observed (Figures 6(a) and 6(b)). The same changes were found when different concentrations of GSPE and 10 μmol/mL BAY11-7082 were simultaneously applied (Figures 6(c) and 6(d)).

3.7. GSPE and BAY11-7082 Activated Caspase-3. We examined the effects of GSPE and BAY11-7082 on the mRNA and protein expression of caspase-3 by using PCR and western blotting, respectively. In untreated ECA109 cells, the mRNA and protein expression of caspase-3 occurred at a relatively low level. With an increased dose of GSPE and the addition of Bay11-7082, the expression level of caspase-3 mRNA and protein increased (Figures 7(a) and 7(b)). This suggested that GSPE and BAY11-7082 promoted the apoptosis of ECA109 cells through the activation of caspase-3.

3.8. GSPE and BAY11-7082 Inhibited the NF-κB Pathway. In view of the important role of NF-κB in the regulation of cytokines and the induction of apoptosis, we studied the effect of GSPE and BAY11-7082 on the transcription factors. We used western blotting to detect the protein expression levels of various classical factors, including IKK, IκB, p-IκB, p50, and p65, in the NF-κB pathway.

In the absence of any treatment interventions, we observed that the protein expression of various transcription factors in ECA109 cells was at a high level, which indicated the activation of NF-κB signaling pathway in esophageal cancer cells. However, the mRNA and protein expression

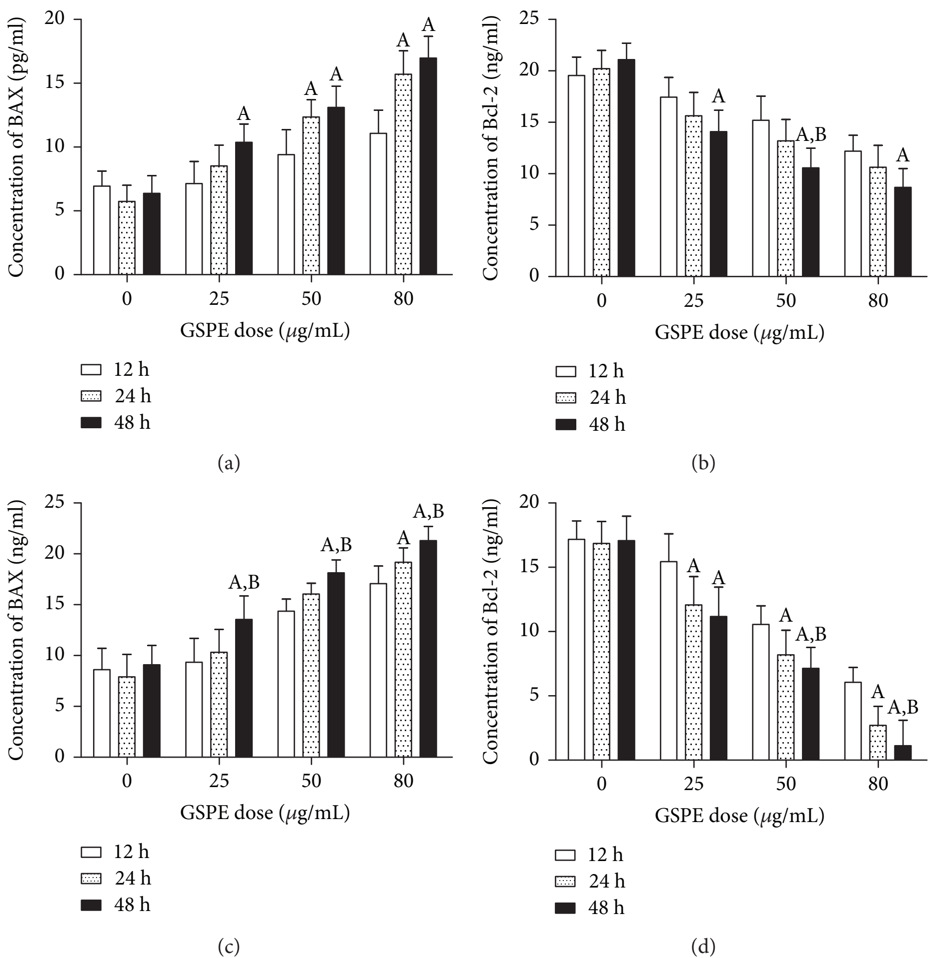

Figure 6: GSPE and BAY11-7082 inhibited Bax/Bcl-2 expression in ECA109 cells. (a, b) GSPE inhibited Bax and Bcl-2 in cells. (c, d) GSPE + Bay11-7082 inhibited Bax and Bcl-2 in cells. Each column represents the mean ± SD of three groups of independent samples. $^{A}P < 0.05$ compared with the 12 h group; $^{B}P < 0.05$ compared with the 24 h group.

levels of IKK, IκB, p-IκB, and p65 were decreased after treatment with 25, 50, and 80 μg/mL for 24 h, whereas the mRNA and protein expression levels of p50 and p65 were increased (Figures 8 and 9). Similar results were found when GSPE and BAY11-7082 were simultaneously applied to ECA109 cells. However, we found that the treatment of BAY11-7082 alone did not result in a decrease in IKK mRNA levels (Figure 8(a)).

4. Discussion

Esophageal cancer is one of the most common malignant tumors in China. The incidence of EC in the Kazakh population of Xinjiang, China, is increasing. A clinical operation is the most common treatment for this disease, but the recurrence rate is high owing to the high metastasis rate of EC [20]. Therefore, it is essential to explore effective natural plant drugs and molecular therapeutic targets that induce apoptosis and inhibit the mechanisms of cell migration and metastasis. In this study, the survival rate of ECA109 cells was determined in the presence of different concentrations of GSPE. GSPE was found to inhibit the proliferation of ECA109; as the dose increased, a stronger effect was observed on the migration and invasion of esophageal cancer cells. These inhibitory effects were accompanied by the decreased secretion of inflammatory factors such as IL-6, CRP, COX-2, and prostaglandin E2 (PGE2); Bax activation; Bcl-2 inhibition; the activation of caspase-3; and inhibition of the NF-κB pathway.

IL-6, similar to many core inflammatory factors, is increased by a large amount in the inflammatory microenvironment of cancer cells; this occurs through the induction of CRP, which activates the NF-κB pathway to reduce the activity of caspase-3 and inhibit the apoptosis of cancer cells [21]. In contrast, the activation of extracellular matrix degradation enzymes can promote the migration and invasion of cancer cells [17]. In this study, GSPE decreased the secretion of inflammatory cytokines (IL-6 and COX-2) in cells, causing the inhibition of the growth, proliferation, migration, and invasion of ECA109 cells. A high level of IL-6 and COX-2 is closely related to the growth [19], migration [22], and invasion [23] of cancer cells. COX-2 is considered to be the rate-limiting enzyme for the conversion of arachidonic acid into prostaglandin E2 (PGE2), which is often expressed in tissue damage or inflammatory response. In vitro experiments indicated that COX-2 was highly expressed in

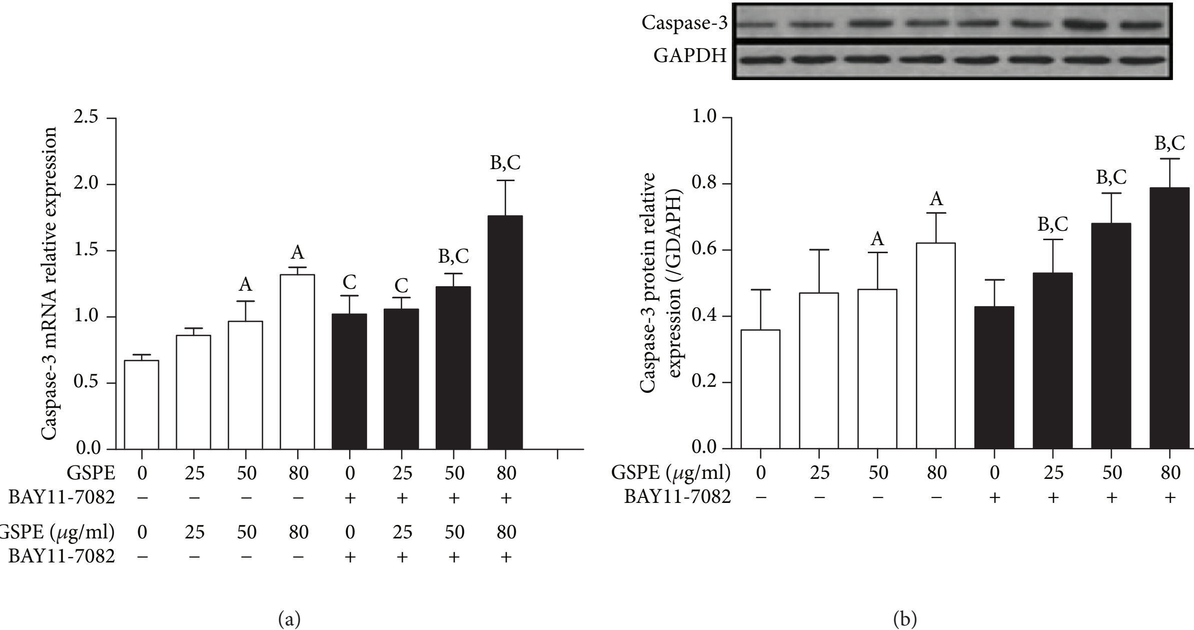

Figure 7: The effects of GSPE and BAY11-7082 on the expression of caspase-3 mRNA and protein in ECA109 cells. (a) GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L) inhibited the expression of caspase-3 mRNA; (b) caspase-3 protein was inhibited by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). Each column represents the mean ± SD of three groups of independent samples. ${}^{A}P < 0.05$ compared with the 12 h group; ${}^{B}P < 0.05$ compared with the 24 h group; ${}^{C}P < 0.05$ compared with the BAY11-7082 group.

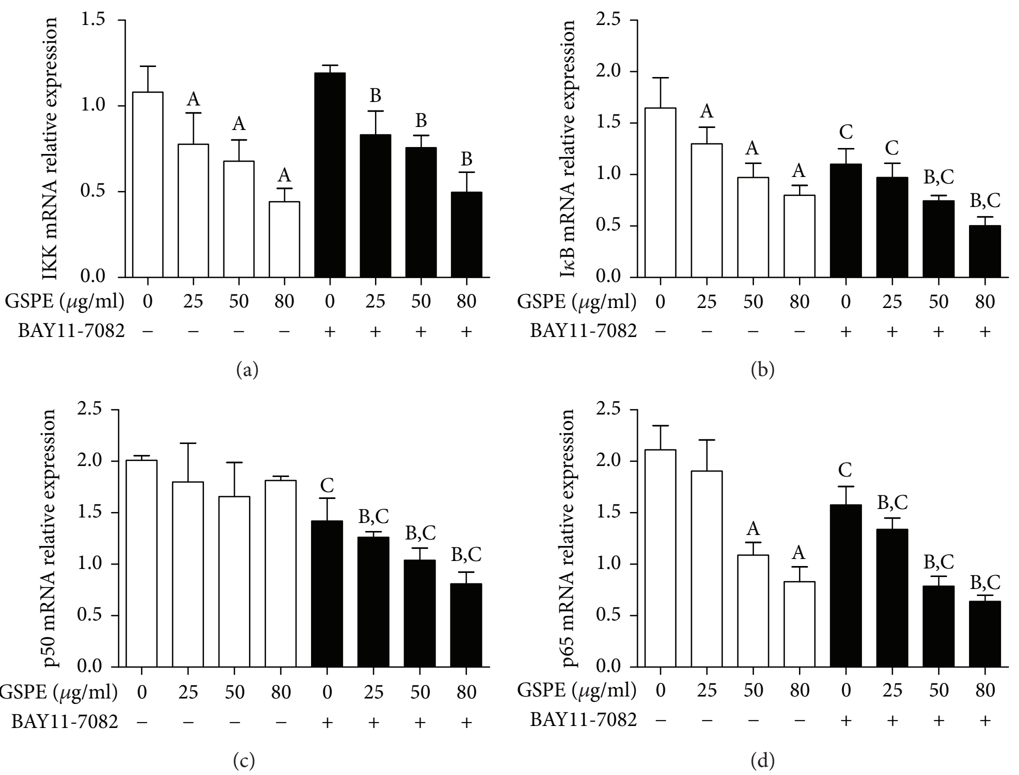

Figure 8: The effects of GSPE and BAY11-7082 on the expression of IKK, IκB, p50, and p65 mRNA in ECA109 cells. (a) The inhibition of IKK mRNA expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (b) The inhibition of IκB mRNA expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (c) The inhibition of p50 mRNA expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (d) The inhibition of the p65 mRNA by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). Each column represents the mean ± SD of three groups of independent samples. ${}^{A}P < 0.05$ compared with the GSPE 0, BAY11-7082; ${}^{B}P < 0.05$ compared with the GSPE 0, BAY11-7082+ group; ${}^{C}P < 0.05$ compared with the BAY11-7082 group.

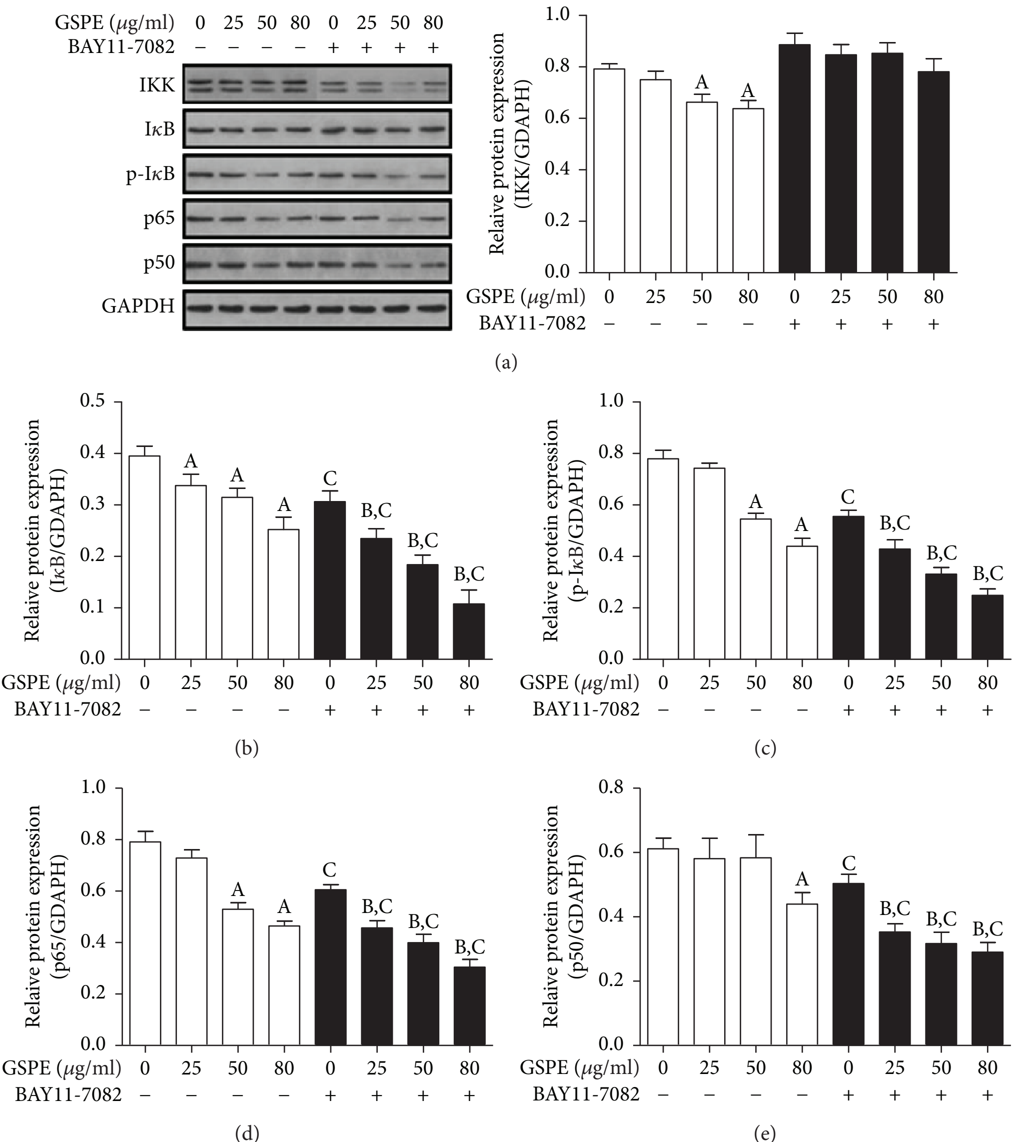

Figure 9: The effects of GSPE and BAY11-7082 on the expression of IKK, IκB, p50, and p65 protein ECA109 cells. (a) The inhibition of the IKK protein expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (b) The inhibition of IκB protein expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (c) The inhibition of p-IκB protein expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (d) The inhibition of p65 protein expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). (e) The inhibition of p50 protein expression by GSPE (0–80 μg/mL) and BAY11-7082 (10 μmol/L). The mean ± SD of three groups of independent samples are shown in each column. $^{A}P < 0.05$ compared with the GSPE 0, BAY11-7082–; $^{B}P < 0.05$ compared with the GSPE 0, BAY11-7082+ group; $^{C}P < 0.05$ compared with the BAY11-7082 group.

esophageal cancer [24], liver cancer [25], and endometrial cancer [26] and that a higher COX-2 level resulted in higher cell proliferation. In the correlation analysis of COX-2 and Bax in cancer cells of patients with renal cancer, a negative correlation was found. It is believed that COX-2 promotes the proliferation of cancer cells through the inhibition of Bax activity [27]. Therefore, we hypothesized that GSPE induced apoptosis in ECA109 cells through the activation of caspase-3 and the inhibition of Bax via the inhibition of the expression of inflammatory cytokines. This was confirmed by the measurement of the mRNA and protein levels of caspase-3.

The NF-κB signaling pathway is involved in the occurrence and development of a variety of malignant tumors [28]. NF-κB exerts antiapoptotic activity mainly by influencing the expression of various inflammatory factors, such as IL-6 and COX-2, and effectors, such as Bax/Bcl-2 and caspase-3. The study found that GSPE prominently inhibited the protein expression of p-IκB in ECA109 cells and prominently promoted IκB mRNA and protein expression, which implied that the GSPE inhibition of NF-κB may be predominantly realized through the inhibition of IκB phosphorylation. Terra et al. used procyanidins B1 and C1 to interfere with LPS-induced macrophages and found that

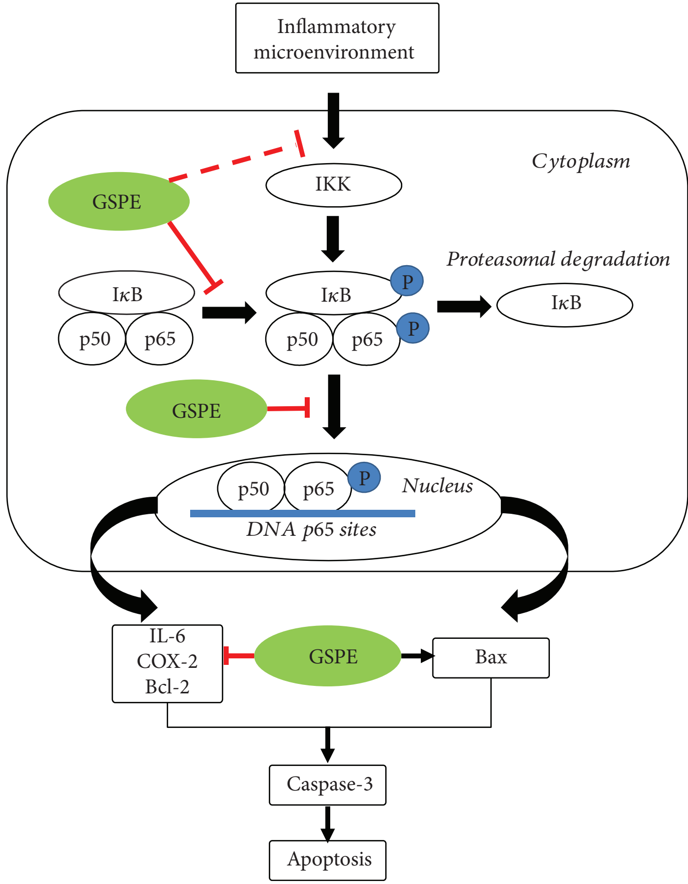

Figure 10: GSPE induced apoptosis in ECA109 cells via NF-κB signaling.

the proanthocyanidins inhibited the activation of the NF-κB pathway by inhibiting the phosphorylation of IκB [29]. However, Zhao et al. found that GSPE inhibited IκB in human ovarian cancer A2780 cells, which inhibited the NF-κB pathway and subsequently promoted apoptosis [15]. Based on the effects of GSPE, we also investigated the treatment of the NF-κB-specific inhibitor BAY11-7082 and found that GSPE + BAY11-7082 was a more effective inhibitor of the phosphorylation level of IκB compared with GSPE alone. This suggested that the inhibition of NF-κB by GSPE was achieved by the inhibition of IκB phosphorylation; a similar effect occurred with BAY11-7082, showing that GSPE and BAY11-7082 may have a synergistic inhibitory effect on the NF-κB in ECA109 cells.

In addition, we found that GSPE inhibited the expression of NF-κB p50/p65 mRNA and protein in cells. NF-κB p50/p65, the most common heterogeneous dimer in the NF-κB signaling pathway, is also an important protein for the function of NF-κB. In resting cells, NF-κB p50/p65 and IκB form complexes, which exist in the cytoplasm in an inactive form. When the cell is stimulated by an extracellular signal, the IκB kinase complex (IKK) activates the phosphorylation of IκB, and the NF-κB is exposed to the nuclear localization site. The dissociated NF-κB is rapidly shifted to the nucleus, binding to a specific κB sequence and inducing the transcription of related genes. The GSPE inhibition of NF-κB p50/p65 resulted from the inhibition of IκB phosphorylation by GSPE, which was consistent with the research of Mackenzie et al. [30]. Some studies have suggested that the ability of procyanidins to inhibit NF-κB p50/p65 expression inhibition may result from the appearance of the procyanidin dimers that may mimic the arginine residues of the NF-κB p50/p65 sequence, with respect to hydrogen bonding, to inhibit the expression of p50/p65 [31]. However, our study did not indicate whether the chemical structure of GSPE was related to the expression of NF-κB p50/p65.

In addition, we found that the mRNA and protein expressions of IKK were both inhibited by GSPE. However, there was no significant difference between the GSPE group and the GSPE + BAY11-7082 group. BAY11-7082, a specific inhibitor of NF-κB, inhibits the phosphorylation of IκB. Therefore, our findings also suggest that GSPE may directly affect IKK, inhibit the activation of IKK, and inhibit the phosphorylation of IκB; together, this inhibits the NF-κB pathway.

In general, the NF-κB signaling pathway plays an important role in the inhibition of the growth of ECA109 cells by GSPE. GSPE promotes the activation of the apoptotic proteins Bax and caspase-3 through the inhibition of NF-κB pathway activation and the inhibition of the expression of antiapoptotic proteins and inflammatory cytokines, thereby inhibiting the proliferation, migration, and invasion of the ECA109 cell line by the induction of apoptosis (Figure 10).

5. Conclusions

Our study has illustrated a possible molecular mechanism for the action of GSPE against cancer; however, the occurrence and development of cancer and the migration and invasion of cancer cells are complex and involve multiple factors. Therefore, the specific mechanism requires extensive research to explore the anticancer effect of procyanidins and provide a basis for their effective use. The results and discussion may be presented separately, or in one combined section, and may optionally be divided into headed subsections.

Data Availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

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

Acknowledgments

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Review Article

Autophagy and Its Role in Protein Secretion: Implications for Cancer Therapy

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Autophagy is a protein and organelle degradation pathway important for the maintenance of cytoplasmic homeostasis and for providing nutrients for survival in response to stress conditions. Recently, autophagy has been shown to be important for the secretion of diverse proteins involved in inflammation, intercellular signaling, and cancer progression. The role of autophagy in cancer depends on the stage of tumorigenesis, serving a tumor-suppressor role before transformation and a tumor-survival function once a tumor is established. We review recent evidence demonstrating the complexity of autophagy regulation during cancer, considering the interaction of autophagy with protein secretion pathways. Autophagy manipulation during cancer treatment is likely to affect protein secretion andinter-cellular signaling either to the neighboring cancer cells or to the antitumoral immune response. This will be an important consideration during cancer therapy since several clinical trials are trying to manipulate autophagy in combination with chemotherapy for the treatment of diverse types of cancers.

1. Introduction

Macroautophagy (referred herein as autophagy) is an evolutionary conserved catabolic and quality control process which involves the formation of double-membraned vesicles known as autophagosomes that engulf cytoplasmic proteins and organelles for their degradation in the lysosome [1]. Basal levels of autophagy are normally low but are induced upon exposure to starvation or diverse types of stress, indicating an important role for autophagy during metabolic homeostasis [2]. The housekeeping role of basal autophagy is evidenced by the accumulation of autophagy substrates like damaged proteins and organelles after genetic ablation of the process in a diversity of models [2]. Also, upon stimulation of stress-induced autophagy, the cells use their breakdown products for obtaining energy and to generate metabolic precursors for cell survival [3].

The importance of autophagy in health and disease was acknowledged by the award of the 2016 Nobel Prize in Physiology or Medicine to Dr. Yoshimori Ohsumi for the discovery of the Atg proteins, the proteins regulating the autophagic process [4]. Dr. Ohsumi's discovery led to the investigation of autophagy in different research areas and to a deeper understanding of the process and its regulators which has led to studies that suggest the possibility of therapeutically targeting autophagy for the treatment of diverse diseases.

The development of mutant mice deficient in ATG proteins demonstrated that autophagy is essential for survival during the neonatal stage of development in mammals [5]. The first mutant mice generated with knockout of an *Atg* gene were the *Atg5* knockout mice [6]. These mice showed reduced amino acid levels in tissues and plasma, died neonatally with a lack of obvious anatomical abnormalities at birth,

and presented a suckling defect. Since autophagy is massively induced after birth in response to starvation caused by the termination of the transplacental nutrient supply, the absence of autophagy together with the suckling defect of the mutant mice has been proposed to be responsible for the inability to restore nutrient supply and the observed neonatal lethality [6]. Although artificial milk feeding partially extended the survival of *Atg5*-null neonates, *Atg5*-null mice also presented defects in the clearance of apoptotic corpses and in the development of the heart and lung. More recently, it has been demonstrated that neuronal dysfunction in *Atg5* knockout mice is the main cause of neonatal lethality, since re-expression of *Atg5* in the brain was sufficient to avoid lethality in this model [7]. These findings underscore the importance of the autophagic pathway for proper organismal development and as a major generator of amino acids under starvation condition to maintain cellular and organismal viability.

A similar phenotype has been observed in *Atg3*, *Atg7*, *Atg12*, and *Atg16L1* knockout and *Ulk1/2* double-knockout mice [5, 8]. Yet, *beclin1*, *Ambra1*, and *Rb1cc1/FIP200* knockout mice are unable to produce homozygous offspring due to early embryonic lethality, suggesting that these genes have other important functions during development in addition to their participation in autophagy [5, 7].

To investigate the role of autophagy in a fully developed organism, adult mice subjected to conditional whole-body deletion of *Atg7* have been generated [9]. These mice developed tissue damage, including liver enlargement, decreased number of large pyramidal neurons and Purkinje cells, degenerative changes in muscle, and vacuolization in the pancreas. *Atg7* conditional knockout mice succumbed either to *Streptococcus* infection shortly after *Atg7* deletion or to neurodegeneration 2 to 3 months later [9]. Importantly, after *Atg7* inactivation, mice failed to survive fasting for 24 hours. Thus, adult mice are less autophagy-dependent than neonates since they can survive longer in the absence of autophagy. However, the autophagic process is necessary for adult tissue maintenance, especially neuronal maintenance in fully developed organisms and essential for organismal survival during fasting [2, 9].

The fact that the autophagic process has a central role in adult neuronal maintenance and in the removal of protein inclusions within neurons (like the ones occurring in Alzheimer's, Hungtington's and Parkinson's diseases) as well as in the removal of damaged mitochondria (like the ones accumulating in some familiar forms of Parkinson's disease [10]) has led to numerous clinical trials trying to induce autophagy by different means in neurodegenerative diseases [11]. Thus, it seems that diseases most likely to be treated soon with autophagy modulators in the clinic involve neurodegenerative diseases as well as cancer [11]. Importantly, as we will discuss later, autophagy has also been shown to be important for extracellular plaque formation and lateral transmission of the disease during neurodegeneration, underscoring the importance of considering every consequence of the manipulation of autophagy in the clinic.

Therapeutic targeting of autophagy in cancer is not straightforward, and evidence suggests that a careful selection of patients based on the characteristics of their tumor needs to be made when trying to manipulate autophagy for cancer therapy. However, most clinical trials trying to modulate autophagy for the treatment of cancer are using diverse drugs with the purpose of inhibiting autophagy [11]. Controversies in the field of autophagy manipulation for cancer treatment arise from the fact that autophagy has been implicated in several steps of the tumorigenic process where both tumor-promoting and tumor-suppressor functions for autophagy have been described [12]. More recently, autophagy has also been related to the extracellular release of cytoplasmic components, including proteins and particulate substrates in a process termed secretory autophagy [13], adding more complexity to the multiple roles of autophagy in cell homeostasis, signaling, and its alterations in disease. Here, we review recent evidence relating the autophagic machinery to cellular secretion with a special focus on carcinogenesis, cancer progression, and possible opportunities to improve cancer treatment.

2. The Autophagic Pathway

The autophagic process is regulated by a set of evolutionary conserved genes termed *ATG* or "autophagy-related" genes, and it comprises the following steps: initiation of the formation of the autophagosome, nucleation, expansion, and elongation of the autophagosomal membrane, closure, and fusion with the lysosome terminating in the degradation of intravesicular products (Figure 1). For an extensive review of this process, the readers are referred to excellent published reviews [1, 14, 15].

Briefly, the Atg1/ULK1/2 kinase complex (in mammals, formed by ULK1/2, ATG13, FIP200, and ATG101) regulates the induction of autophagosome formation. During the first step of autophagy initiation, the ULK1 complex forms punctate structures in proximity to the ER (endoplasmic reticulum), where the nucleation complex is formed. Activated ULK1/2 then phosphorylates components of the class III PI3K (phosphatidylinositol 3-kinase) nucleation complex. This complex consists of a class III PI3K (VPS34), beclin 1, VPS15, and ATG14L. This kinase complex is responsible for the production of the phospholipid phosphatidylinositol 3-phosphate (PI3P) at the site of autophagosome formation that serves as a signaling molecule for the recruitment of PI3P-binding proteins [16]. Vesicle elongation is mediated by two ubiquitin-like protein conjugation systems: ATG5-ATG12 and ATG8/LC3-PE. Both systems are necessary for autophagosome membrane expansion and consist of the following proteins: ATG12 and ATG8/LC3 (ubiquitin like proteins), ATG7 (E1-like enzyme), ATG10 and ATG3 (E2-like enzymes), ATG5 and ATG16 (modified targets), and ATG4 (protease). The ATG5-ATG12 system along with ATG16 functions in part to determine the subcellular localization of ATG8/LC3-PE conjugation. ATG8/LC3 is conjugated to the lipid phosphatidylethanolamine (PE) to form LC3II which is recruited to the autophagosomal membrane and is currently the most widely used assay to evaluate autophagy [15, 17]. LC3II also recognizes adaptor proteins like p62/SQSTM1 which binds ubiquitinated proteins and transports

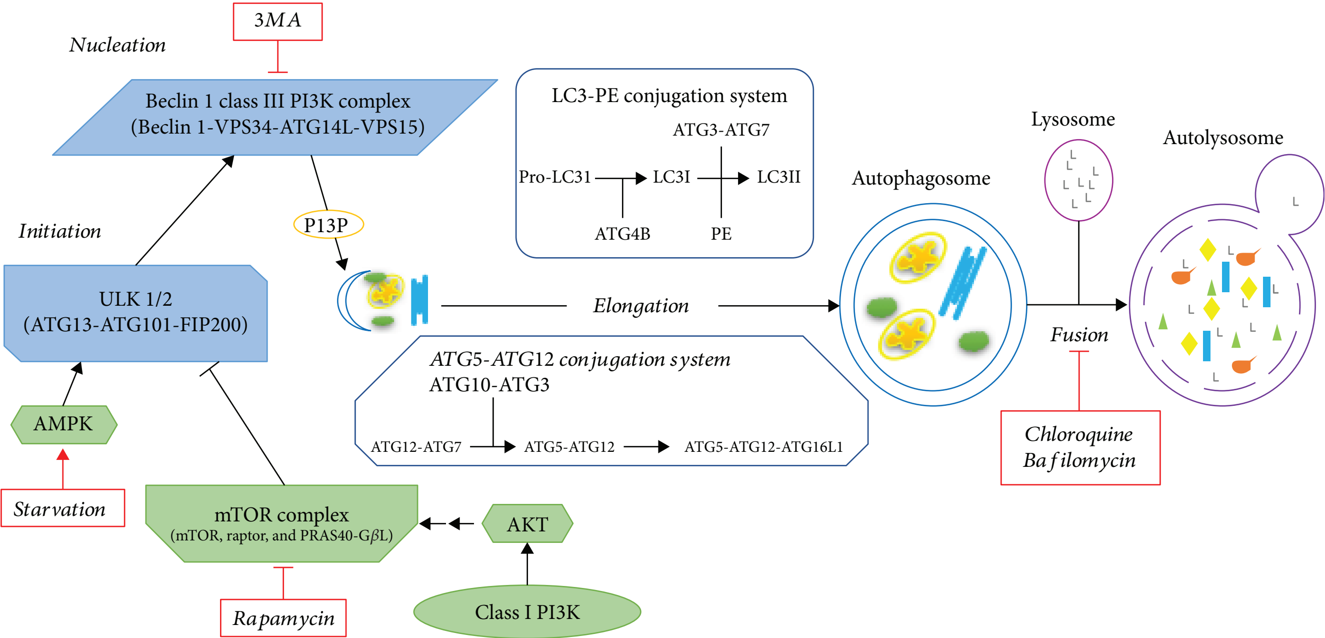

Figure 1: A general overview of the autophagic pathway and its regulators. In mammals, the ULK1/2 kinase complex regulates autophagosome initiation. ULK1/2 is regulated by nutrient sensing or stress signaling by mTOR complex 1, which inhibits autophagy in the presence of amino acids or insulin/PI3K/AKT signaling. ULK1/2 is also regulated by AMPK, which is activated by high AMP/low ATP levels. Activated ULK1/2 then phosphorylates and activates components of the class III PI3K nucleation complex responsible for the formation of PI3P and for the recruitment of PI3P-binding proteins. Vesicle elongation is mediated by two ubiquitin-like protein conjugation systems: ATG5-ATG12 and LC3-PE. Once the autophagosome is formed, it fuses with the lysosomes and their contents are degraded. The figure shows pharmacological regulators of autophagy mentioned in the text (ATG: autophagy related; mTOR: mechanistic target of rapamycin; PI3K: phosphatidylinositol 3-kinase; PE: phosphatidylethanolamine; PI3P: phosphatidylinositol 3-phosphate; AMPK: AMP-activated protein kinase; 3MA: 3-methyl adenine).

them to the autophagosome. Ultimately, autophagosomes travel along microtubules, pushed by dynein, and fuse with the lysosome and their contents are degraded. Fusion requires ESCRT (endosomal sorting complexes required for transport), SNAREs (STX17), VPS family proteins, and RAB7 [18]. Impaired lysosome function prevents complete autophagic flux. Hence, lysosomotropic agents like chloroquine or hydroxychloroquine, which impair autophagosome degradation and autophagic flux by increasing the pH of the lysosome, are used experimentally and in the clinic in several clinical trials to inhibit autophagy [11, 18].

Autophagy is regulated in response to nutrient availability as well as other cellular stress signals. A master regulator of autophagy in response to nutrient availability is the mTOR (mechanistic target of rapamycin) complex 1 (mTORC1), a serine/threonine protein kinase responsible for regulating cell growth and metabolism. In the presence of amino acids, mTORC1 is active and inhibits autophagy by phosphorylating ULK1, as well as ATG13, at multiple residues [16]. Upon amino acid deprivation, mTORC1 activation on the lysosomal surface is disrupted and both ULK1 and ATG13 are dephosphorylated, resulting in ULK1 activation and autophagy induction [16]. Another important regulator of autophagy is AMPK (AMP-activated protein kinase) which is activated by low ATP levels or an increase in the AMP : ATP ratio. AMPK can inactivate mTORC1 through its phosphorylation and can also directly phosphorylate and activate ULK1 at multiple residues inducing autophagy. Other regulators of the ULK1 complex include GSK3-TIP60, AKT, Cul3-KLHL20, and NEDD4L [16].

3. Autophagy and Cancer

Research on autophagy in the cancer biology field has led to a general consensus in which the role of autophagy in cancer is dependent on the stage of tumorigenesis [12]. In general, before the appearance of a tumor, autophagy serves a tumor suppressor function in normal cells, eliminating damaged organelles and protein aggregates which could promote genomic instability and tumorigenesis. On the other hand, once a tumor is established, autophagy serves a cell survival function in cancer cells that helps them survive hypoxia, metabolic stress, and anoikis [12]. So, the homeostatic function of baseline autophagy occurring in normal cells ensures continuous removal of superfluous, ectopic, or damaged (and potentially dangerous) entities, including organelles or proteins, operating as a quality control system that maintains cellular fitness [12]. Additionally, autophagic flux can be upregulated in response to stressful stimuli like nutritional, metabolic, oxidative, pathogenic, genotoxic, or proteotoxic stress [12]. This stimulus-induced autophagy serves a cytoprotective function by helping the cells adapt to stress and allowing them to survive.

In agreement with the housekeeping role for autophagy, cancer was the first disease that was linked to a deficiency in the autophagic pathway with the proposal that *Beclin1* functions as a tumor suppressor gene, since it was found to be monoallelically deleted in a high percentage of ovarian, breast, and prostate cancers [10, 19]. Although this proposal was recently challenged and the tumor suppressive functions of Beclin1 remain controversial [20], diverse mouse models with defects in the autophagy machinery caused by whole-body or tissue-specific, heterozygous, or homozygous knockout of *Atg* genes show increased incidence of some malignancies or increased susceptibilities to carcinogens [10, 12]. So, heterozygous deletion of *beclin1* has been associated with enhanced susceptibility to breast, ovarian, and prostate cancer in humans and increased spontaneous malignancies in mice [21]; *Atg4C* knockout mice have been shown to be more prone to develop chemically induced fibrosarcomas [22]; mosaic deletion of *Atg5* in mice induced benign tumor development in the liver [23]; and tissue-specific *Atg5* or *Atg7* knockout increased the appearance of lung carcinomas driven by $KRAS^{G12D}$ or $BRAF^{V600E}$ [24, 25], as well as $KRAS^{G12D}$-driven premalignant pancreatic lesions [26, 27]. Interestingly, mice with an *Atg7* conditional knockout in the liver developed multiple tumors in this tissue and this phenotype was reversed by *p62* knockout, indicating that p62 accumulation due to autophagy suppression contributes to tumor formation [23].

Thus, before the appearance of a malignant lesion, autophagy serves a tumor-suppressive function. The mechanism proposed involves the degradation of damaged mitochondria that could otherwise induce oxidative stress, DNA damage, and genomic instability. These elements of chronic tissue damage could also provoke an inflammatory response that could further promote tumor growth [28]. In this regard, p62/SQSTM1, one of the best characterized substrates of selective autophagy which interacts with LC3 on the isolation membrane as well as with ubiquitinated proteins, has been shown to play a role in the induction of tumorigenesis. p62 can function as a signaling hub through its interacting proteins. Among these, it can activate the TRAF6-NfκB pathway, facilitate aggregation of caspase-8, bind Keap1, a Cullin3-type ubiquitin ligase for Nrf2, and facilitate the formation of intracellular inclusion bodies [29–31]. Thus, excess accumulation of p62 due to defective autophagy leads to hyperactivation of these signaling pathways which could further contribute to protumorigenic signaling.

On the other hand, once a tumor is formed, there is ample evidence showing that tumor cells need elevated levels of autophagy to survive the stressors found within a tumor and along the metastatic process [32]. Indeed, autophagy has been shown to promote cancer cell survival under hypoxia [33, 34], nutrient deprivation [35], and anoikis [36], indicating the importance of this process for the survival of a tumor cell to the stressors to which it is exposed and suggesting a potential use for cancer therapy in combination with autophagy inhibitors. Autophagy has also been shown to be a cell survival pathway activated during chemotherapy, radiotherapy, and targeted therapies [37], suggesting promising results of clinical trials using the autophagy inhibitors chloroquine or hydroxychloroquine in combination with other therapies in different types of cancers [11]. Also, autophagy has been implicated in the development of resistance to therapy [1, 38, 39], further supporting the use of pharmacological inhibitors of autophagy in combination with traditional chemotherapy or in patients that recur. This last evidence is also in agreement with the suggestion that autophagy is an important process for the maintenance of cancer stem cells [40–43]. Nevertheless, sensitization to therapy by autophagy inhibitors might be cell type- or treatment-dependent [44, 45] and could even show antagonistic effects with chemotherapy depending on the cell type [45]. In contrast to the previous evidence that suggests a potential use for autophagy inhibition in cancer therapy, it has also been shown that autophagy inhibition in cancer cells treated with radiation [46] or immunogenic chemotherapies [47] could impair the therapy-induced antitumoral immune response. Also, there is evidence in which autophagy inhibition by itself promoted epithelial-to-mesenchymal transition in cancer cells [48]. Thus, it remains unclear if autophagy should be targeted during cancer therapy in every cancer type or what therapies should it be used in combination with.

Regarding the type of cancer cell where autophagy should be targeted, it has been shown that cancer cells with certain oncogenic backgrounds might be particularly sensitive to the inhibition of autophagy, even under nutrient-rich conditions. So, cells with activating mutations in the MAPK pathway have been proposed to be "addicted" to autophagy since they show high levels of autophagy under basal, nutrient-rich conditions and are dependent on this pathway for survival [49, 50]. So, inhibition of autophagy for cancer therapy seems to be promising for the treatment of tumors with activating mutations in KRAS or its downstream targets as BRAF like lung [25, 51], pancreas [52], brain tumors [53], or melanoma [54].

Importantly, some of the autophagy-mediated effects observed during cancer therapy seem to involve either the activation or the modulation of the antitumoral immune response [24, 55, 56]. Moreover, some of the protumorigenic effects of autophagy seem to require the release of autophagy-regulated secreted factors which could act in an autocrine or paracrine manner in cancer cells [40, 57]. Thus, a precise understanding of the secreted factors regulated by autophagy will provide important knowledge on the effects of autophagy on tumor cells as well as on the regulation of the tumor microenvironment by autophagy-competent or autophagy-deficient tumor cells.

4. Conventional and Unconventional Protein Secretion Pathways

Cell secretion is a fundamental physiological process that delivers soluble proteins and cargoes to the extracellular space. The need to expel substances from the cell serves distinct purposes including cellular growth, homeostasis, cytokinesis, defense, hormonal release, and neurotransmission [58]. In eukaryotes, classical secretion, also known as the conventional secretion pathway, involves release or exocytosis of storage vesicles or secretory granules into the extracellular

space [58]. During this process, newly synthesized proteins are translocated into the lumen of the endoplasmic reticulum (ER). Proteins secreted by classical secretion contain in their sequence a characteristic peptide with one or more positively charged amino acids in their amino terminal end followed by 6–12 hydrophobic residues [59]. The signal sequence initiates the transport of the growing polypeptide across the ER membrane into the ER lumen. Usually, classically secreted proteins are synthesized as protein precursors and the N-terminal signal peptide sequence is cleaved from the protein when the polypeptide chain is growing in the ribosome [59]. Proteins are then oligomerized and packed into carrier vesicles that exit the ER at specialized regions. The vesicles assemble into vesiculotubular structure intermediates known as the ER-to-Golgi intermediate compartments that sort proteins for further anterograde flow to the Golgi complex. In the Golgi, proteins are glycosylated to ensure proper protein structure and increased stability and to allow interactions with target proteins. In the trans-Golgi network, secretory proteins are sorted into secretory vesicles that deliver their content to the plasma membrane to result in secretion [60]. Importantly, integral plasma membrane proteins are delivered and integrated to the plasma membrane through membrane fusion by the same trafficking route [58].

Secretory vesicles and secretory granules are distinct vesicular carriers employed in constitutive and regulated secretion, respectively. While constitutive secretion is constantly undergoing in every eukaryotic cell, regulated secretion is additionally present in special types of animal cells like endocrine and exocrine cells and neurons and is exclusively triggered by extracellular stimuli [58]. Examples of regulated secretion include insulin secretion from endocrine pancreatic β-cells, secretion of zymogen from exocrine pancreatic cells to digest food, secretion of growth hormone from cells of the pituitary gland, and release of neurotransmitters at the synapse [58]. While many secreted proteins have been identified to be released by the conventional route, many other soluble proteins that are secreted into the extracellular space lack a typical signal peptide and are secreted without entering the conventional ER-to-Golgi pathway of protein secretion.

5. Autophagy and Unconventional Protein Secretion

The autophagic pathway has recently been related to the secretion of proteins from different cells. In this regard, many proteins known to be secreted by an unconventional route are known to be regulated by autophagy or their release is affected by knockdown of ATG proteins. Here, we review the proteins whose secretion has been shown to be regulated by autophagy (Figure 2, Table 1) and we later discuss the implications of the modulation of autophagy in protein secretion for cancer progression and treatment. Importantly, the term "secretory autophagy" is used to describe the process in which the canonical autophagic pathway takes part in the secretion of proteins by transporting them in the autophagosome directly to the plasma membrane, to MVB (multivesicular bodies), or to secretory lysosomes for their extracellular release. Thus, instead of inducing autophagosomal cargo degradation, secretory autophagy leads to the expulsion of the autophagosomal content to the extracellular space and it has a positive effect on protein secretion, since inhibition of autophagy reduces protein secretion (Table 1). This pathway would need specific cargo receptors as well as specific SNARE vesicular fusion proteins. On the other hand, another pathway has been described in which inhibition of autophagy leads to changes in protein secretion, particularly increased cytokine production in immune cells. In this case, autophagy has a negative effect on protein secretion since inhibition of autophagy increases protein secretion (Table 1), and this effect has been proposed to be mediated by increased mitochondrial reactive oxygen species (ROS) caused by decreased mitophagy. In the following sections, we discuss the proteins whose secretion is known to be modulated by the autophagic pathway, either because they are released through secretory autophagy or because inhibition of autophagy regulates their secretion, since both pathways would be affected by the modulation of autophagy for cancer therapy.

6. Secretory Autophagy

One of the first evidences indicating that autophagy was involved in the secretion of proteins came from studies in a mouse model of Chrohn's disease, a complex inflammatory disease of the intestine in which *ATG16L1* is one of many known risk alleles in patients [61]. So, in intestinal hypomorphic *ATG16L1* and intestinal *Atg5*$^{-/-}$ mice, autophagy deficiency mostly affected Paneth cells within the intestinal epithelium. These cells, whose normal function is to secrete both lysozyme and antimicrobial peptides, presented disorganized or diminished lysozyme-containing granules and increased lysozyme diffuse intracytoplasmic staining [61]. Thus, the process of autophagy was shown to have an important role in the maintenance of the granule exocytosis pathway in Paneth cells. More recently, lysozyme was found to be localized to autophagosomes (double-membrane, LC3^{+}/p62^{-} vesicles) of *S. typhimurium*-infected Paneth cells. These autophagosomes were not targeted for lysosomal degradation but accumulated at the apical surface of Paneth cells for lysozyme secretion, indicating an important role for autophagy in the secretion of this antimicrobial protein [62]. In this work, lysozyme secretion was impaired in the intestinal crypts of *S. typhimurium*-infected mice treated with 3MA or in mice mutant for *Atg16L1*^{T300A}, which impaired autophagy, but not by chloroquine treatment, indicating an important role for the initial steps of the autophagic pathway but not the degradation step of autophagy in the secretion of this protein. Secretory autophagy was induced by ER stress and was dependent on Myd88, a toll-like receptor (TLR) adaptor but specifically on dendritic cells. Treatment of *Myd88* $^{-/-}$ mice with recombinant IL-22 restored secretory autophagy of lysozyme in Paneth cells, indicating that Paneth cell secretory autophagy requires activation of dendritic cells to allow secretion upon ER stress in Paneth cells. Since Paneth cells are specialized intestinal cells that secrete antimicrobial proteins, including lysozyme, and since

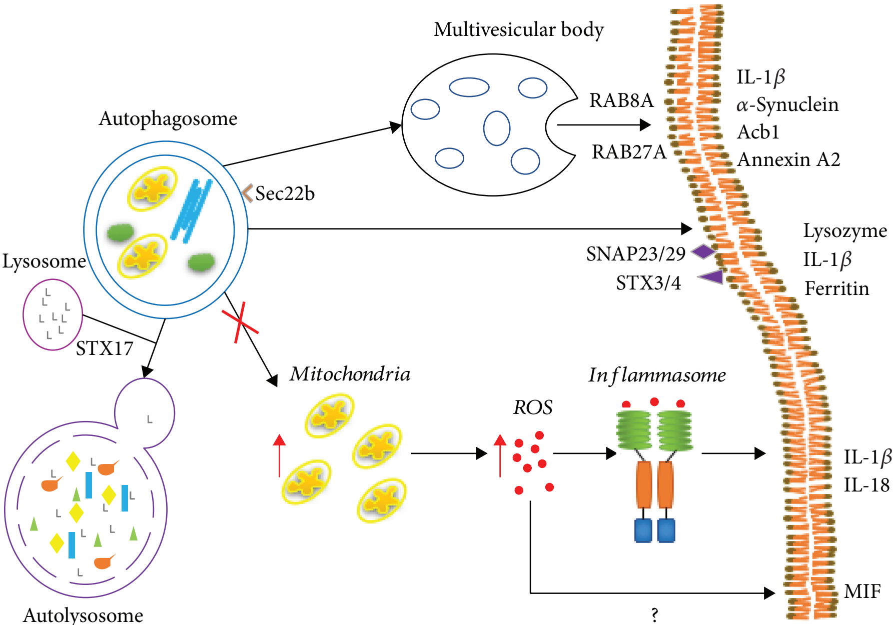

Figure 2: Overview of the different roles of autophagy in protein secretion. Three possible mechanisms of autophagy-mediated secretion have been described. In the first one, the autophagosome interacts with components of the endosomal-lysosomal system, including the multivesicular body. These interactions are mediated by Sec22b, Rab8A, and Rab27A proteins for the release of α-synuclein and annexin A2 [69, 70, 86], and only Rab8A has been characterized for the release of IL-1β [64]. It should be noted that the secretion of IL-1β and other proteins, like ferritin, can also be carried out by direct fusion of the autophagosome to the plasma membrane, mediated by Sec22b and SNAP23/29 and STX3/4 [66], and that the secretion of IL-1β is independent of proteins involved in the fusion of the autophagosome with the lysosome such as STX17 [66] probably suggesting a mechanism in which secretory autophagosomes are spared from degradation and instead are directed to the multivesicular body or the plasma membrane. On the other hand, the inhibition of autophagy prevents the degradation of damaged organelles such as the mitochondria, inducing an increase in ROS involved in the secretion of MIF, through an unknown mechanism [80]. A ROS-dependent mechanism induced by decreased mitophagy has been described for other proteins such as IL-1β or IL-18 [63, 76, 77] where mitochondrial ROS activate the inflammasome, which then induces the maturation and secretion of these proteins (ROS: reactive oxygen species; STX:, syntaxin; IL: interleukin; MIF: macrophage migration inhibitor factor; Acb1: acyl coenzyme A-binding protein).

pathogenic microbes can trigger ER (endoplasmic reticulum) stress that interferes with protein secretion, the authors suggest that during *S. typhimurium* infection, autophagy is induced in Paneth cells where the secretion of lysozyme is rerouted to an alternative secretion pathway which involves the transport of lysozyme inside a specialized secretory autophagosome which is not targeted for degradation (since it was negative for p62 which targets proteins to be degraded by autophagy), preserving the antimicrobial function of Paneth cells [62].

More mechanistic studies have been made on the role of secretory autophagy in the release of IL-1β from mammalian cells. This proinflammatory cytokine lacks an ER-localization peptide, accumulates in the cytosol in its inactive form, and is later activated by caspase-1 cleavage for secretion by an unconventional route which involves inflammasome activation and autophagy [63–65]. So, the induction of autophagy by starvation in response to conventional NLRP3 inflammasome agonists has been shown to lead to enhanced IL-1β secretion in LPS-stimulated macrophages [64] and autophagy-mediated secretion was dependent on the inflammasome components ASC and NLRP3. In agreement with the previous observation, other inflammasome-dependent cytokines, like IL-18, also showed enhanced secretion after autophagy induction [64]. Importantly, in the same study, IL-1β was found to colocalize with Rab8a and LC3 and IL-1β secretion was decreased by Cre-mediated excision of *Atg5*, by lysosomal inhibition of autophagy with bafilomycin A or by Rab8a (a regulator of polarized sorting to plasma membrane) or GRASP55 (Golgi-associated protein required for unconventional secretion) knockdown. Also, cathepsin B was found to be secreted along with the inflammasome substrates. The mentioned evidence suggests a model in which autophagosomes have a direct role in the delivery of inflammasome-activated proteins to the plasma membrane and indicates a positive role for cathepsin B in IL-1β activation and extracellular delivery by autophagy.

Importantly, specialized secretory autophagosomes involved in the secretion of IL-1β or ferritin have already been identified [66]. In this work, upon lysosomal damage, TRIM16, together with galectin-8, acted as a receptor for IL-1β targeting it to LC3II-positive autophagosomes. Fusion with the plasma membrane was dependent on Sec22b on the autophagosome and on SNAP23/29 and STX 3/4 on the plasma membrane. Importantly, the secretion of IL-1β was STX17 (a SNARE involved in the fusion with the lysosome) independent, suggesting that secretory autophagy utilizes

Table 1: Proteins whose secretion is known to be regulated by autophagy. The table shows proteins whose secretion has been shown to be regulated by alterations in the autophagic pathway, the methods used to manipulate autophagy, and the effect of autophagy on secretion: positive, if autophagy inhibition impairs secretion, or negative, if autophagy inhibition increased secretion (3MA: 3-methyl adenine; LPS: lipopolysaccharide; CQ: chloroquine; kd: knockdown; Baf: bafilomycin A1; EMT: epithelial to mesenchymal transition).

Secreted protein	Protein function	Method(s) used to modulate autophagy	Autophagy's effect on secretion	Ref.
Acb1	Acyl-CoA-binding protein involved in yeast sporulation	*ATG1*, *5*, *6*, *7*, *8*, *9*, *12*, *17* and *VAM7* mutant yeast; rapamycin	Positive; genetic inhibition of autophagy decreased and rapamycin increased secretion. Fusion of the autophagosome with the vacuole was not related to secretion.	[67, 68]
Amyloid-β peptide	Element of the amyloid plaques involved in Alzheimer's disease	*Atg7*$^{-/-}$	Positive; genetic inhibition of autophagy caused intracellular Ab accumulation and reduced amyloid B peptide secretion.	[72]
Annexin A1	Regulator of the inflammatory process	*Beclin1* kd, 3MA, and *Atg5*$^{-/-}$	Positive; genetic inhibition of autophagy or 3MA treatment decreased secretion induced by inflammasome activators. Found in screening experiments of secreted proteins regulated by autophagy.	[66, 70, 108]
Annexin A2	Ca^{2+}-dependent phospholipid-binding protein	*ATG5* kd, 3MA, and lysosomal inhibitors	Positive; genetic inhibition of autophagy or 3MA treatment decreased secretion in IFN-γ-stimulated lung epithelial cells. Found in screening experiments of secreted proteins regulated by autophagy.	[70, 86]
α-Synuclein	Aggregation-prone protein involved in Parkinson's disease	*ATG5* kd, TPPP/p25 which impaired autophagic flux at the lysosomal fusion step, trehalose, and lysosomal inhibitors	Positive; autophagy inhibition in the presence of TPP/p25 decreased secretion. Autophagosome-lysosome fusion impairment was necessary for secretion, and autophagosome-lysosome fusion impairment enhanced secretion of an LC3/p62^{+} vesicle.	[69, 70]
β-Hexosaminidase	Lysosomal enzyme, indicator of mast cell degranulation	*Atg7*$^{-/-}$ and *Atg12* kd	Positive; genetic inhibition of autophagy decreased mast cell degranulation.	[75]
Cathepsin D	Lysosomal protease	*Beclin1* kd, 3MA, and *Atg5*$^{-/-}$	Positive; genetic inhibition of autophagy or 3MA treatment decreased secretion induced by inflammasome activators. Found in screening experiments of secreted proteins regulated by autophagy.	[66, 108]
Cathepsin K	Bone resorption	*Atg5*$^{-/-}$, *Atg7*$^{-/-}$, and *Atg4*^{C74A} dominant negative	Positive; autophagy inhibition decreased secretory lysosome delivery to the plasma membrane.	[74]
CXCL8	Chemokine produced by macrophages and epithelial cells	*ATG7* kd	Positive; autophagy inhibition decreased secretion.	[100]
DKK3	Glycoprotein with angiogenesis and invasiveness-promoting roles	*ATG7* kd	Positive; autophagy inhibition decreased secretion.	[100]
FAM3C	Secreted protein inducer of EMT	*ATG7* kd	Positive; autophagy inhibition decreased secretion.	[100]

Table 1: Continued.

Secreted protein	Protein function	Method(s) used to modulate autophagy	Autophagy's effect on secretion	Ref.
Ferritin	Iron storage protein	*LC3B* kd	Positive; inhibition of autophagy decreased secretion in response to lysosomal damage.	[66]
Galectin 3	Lectin with affinity for β-galactoside glycoconjugates	*Beclin1* kd and 3MA	Positive; genetic inhibition of autophagy or 3MA treatment decreased secretion induced by inflammasome activators.	[108]
Histamine	Inflammatory response, component of mast cell granules	*Atg7*−/− and *Atg12* kd	Positive; genetic inhibition of autophagy decreased mast cell degranulation.	[75]
HMGB1	Alarmin normally present in the nucleus and released during cell death	*ATG5*, *7*, and *12* kd	Positive; genetic inhibition of autophagy decreased secretion in cancer cells treated with targeted therapy.	[93]
IL-1β	Inflammatory response	*Atg5*$^{-/-}$, bafilomycin A [64], *beclin 1* kd, 3MA [108], *ATG16L1*, *LC3B* kd [66], and *Atg7*$^{-/-}$ [91]	Positive; genetic [64] or pharmacological [108] inhibition of autophagy decreased secretion in response to inflammasome activation, lysosomal damage [66], or UVB irradiation [91].	[64, 66, 91, 108]
		Truncated *Atg16L1*, *Atg7*$^{-/-}$, and 3MA [63, 77], *Map1lc3b*$^{-/-}$ or *becn1*$^{-/-}$ [76], and *becn1* kd [77]	Negative; genetic autophagy inhibition or PI3K inhibitor treatment induced secretion in LPS primed macrophages.	[63, 76, 77]
IL-6	Inflammation	*ATG5*, *ATG7*, *ATG12*, *beclin1* kd, and *Atg7*$^{-/-}$	Positive; genetic inhibition of autophagy decreased secretion in cancer cell lines [40, 57, 89], in UVB irradiated skin [91], or in hepatitis virus infected hepatocytes [109].	[40, 57, 89, 91, 109]
		ATG7 and *Beclin1* kd	Negative; genetic inhibition of autophagy increased secretion in a breast cancer cell line but not others.	[40]
IL-8	Chemotactic factor and neutrophil activator	*ATG5* and *ATG7* kd	Positive; genetic inhibition of autophagy decreased secretion in cancer cell lines [100] or in hepatitis virus-infected hepatocytes [109].	[100, 109]
IL-18	Proinflammatory cytokine	Truncated *ATG16L1* [63] and *Map1lc3b*$^{-/-}$ or *Becn1*$^{-/-}$ [76]	Negative; genetic autophagy inhibition induced secretion in mouse models of colitis or sepsis or in LPS-primed macrophages.	[63, 76]
		3MA or bafilomycin treatment	Positive; pharmacological inhibition of both initial and degradation phases of autophagy decreased secretion in allergen-induced IL-18 secretion.	[110]
LIF	Cytokine involved in hematopoietic differentiation, stem cell development, metabolism, and growth promotion	*ATG7* kd	Positive; autophagy inhibition decreased secretion.	[100]

Table 1: Continued.

Secreted protein	Protein function	Method(s) used to modulate autophagy	Autophagy's effect on secretion	Ref.
Lysozyme	Antimicrobial protein	Hypomorphic *ATG16L1*, *Atg5*$^{-/-}$ [61] and *Atg16L1*^{T300A}, 3MA, and CQ [62]	Positive; lysozyme secretion was impaired from Paneth cells by genetic inhibition of Atg genes or 3MA but not CQ treatment.	[61, 62]
Metalloproteinase 2/9	Extracellular matrix-degrading proteases	*ATG7* and *12* kd	Positive; genetic inhibition of autophagy decreased secretion.	[57]
MIF	Proinflammatory cytokine	*Atg5* kd, *atg7*$^{-/-}$, and 3MA treatment	Negative; inhibition of autophagy increased MIF secretion in LPS-stimulated macrophages.	[80]
Neuropeptide Y	Neurotransmitter	*Atg16L1* kd	Positive; *Atg16L1* kd but not *Atg13* or *ULK1* kd decreased secretion in neuroendocrine cells.	[111]

specialized "secretory" autophagosomes that would eventually fuse with the plasma membrane and that avoid cargo degradation in the lysosomes [66].

In yeast cells, another protein has been identified whose secretion depends on autophagy [67, 68]. An acyl coenzyme A-binding protein, Acb1, is a secreted protein lacking an ER-localization sequence involved in yeast sporulation in response to nitrogen starvation. Acb1 secretion was found to be independent of the conventional secretory pathway, dependent on the presence of *ATG* genes and proteins, on Grh1 (GRASP), and was also induced by rapamycin treatment [67, 68]. Interestingly, Acb1 secretion did not require fusion with the vacuole and required components of the multivesicular body endosomal compartment, indicating that Acb1-containing autophagosomes bypass the fusion and instead they fuse with endosomes or MVBs en route to the plasma membrane [68]. Yeast mutants which failed to secrete Acb1 showed similar levels of intracellular Acb1 protein and were deficient in its secretion but not in its processing, indicating that the pathway described was a protein secretion and not a degradation pathway [67].

Autophagy-mediated secretion has also been linked to major neurodegenerative diseases. In Parkinson's disease (PD), where both the proteasome and autophagy have been involved in the degradation of α-synuclein aggregates, autophagy has also been linked to the secretion of α-synuclein, indicating its potential role for interneuronal transmission of α-synuclein and PD [69, 70]. In this regard, in a PD model involving overexpression of an aggregation-prone α-synuclein and of TPPP/p25a, a microtubule-binding protein involved in α-synuclein-aggregate formation, α-synuclein was localized to autophagosomes since it colocalized with autophagy markers LC3 and p62/SQSTM1, but these autophagosomes did not fuse with lysosomes. This study showed that TPPP/p25a impaired autophagic flux at the lysosomal fusion level and induced α-synuclein secretion, similarly to autophagic-flux inhibitor treatment. Importantly, α-synuclein secretion was decreased by *ATG5* knockdown [69]. In a similar study, in different PD models of neurons overexpressing α-synuclein, lysosomal inhibition increased α-synuclein secretion and its localization to LC3II- and p62/SQSTM1-positive extracellular vesicles [70]. Other proteins found in extracellular vesicles from bafilomycin-treated neurons were VPS35, ATP6V1A, and LAMP2 [70]. Both studies suggest an important role for autophagosome formation and autophagosome fusion with the lysosomes in the regulation of extracellular vesicle secretion. Thus, while autophagosome formation could directly deliver contents to the multivesicular body as well as to the lysosomes, autophagic flux inhibition with lysosomal inhibitors could promote enhanced delivery of autophagosomal material to vesicles and their extracellular release.

Autophagy has also been closely related to Alzheimer's disease (AD). AD brain pathology involves the formation of intracellular amyloid beta (Aβ) peptide and tau protein aggregates as well as extracellular Aβ plaques [71, 72]. Impaired autophagic flux has been described in neurons of AD mouse models, and autophagosomes have been related to the generation of the Aβ peptide [71]. In agreement with impaired autophagic flux in advanced AD, induction of autophagy by rapamycin lowered intracellular Aβ accumulation and extracellular plaque load and prevented learning and memory deficits in a mouse model of AD but only when administered prophylactically and not in mice with established plaques and tangles [73]. Moreover, amyloid precursor protein transgenic mice with conditional knockout of *Atg7* in the forebrain excitatory neurons drastically accumulated intracellular Aβ and presented reduced extracellular Aβ plaque formation due to impaired secretion of Aβ [72]. Altogether, these findings underscore the importance of autophagy for the maintenance of neuronal homeostasis but could promote AD pathology by promoting Aβ extracellular plaque formation.

Several studies have also linked the autophagic pathway to the release of secretory lysosomes in a physiological setting. For instance, autophagy-related proteins have been shown to mediate osteoclast ruffled border formation and their secretory function by directing secretory lysosomes to the plasma membrane for fusion and secretion of cathepsin K [74]. Also, secretory granules of mast cells have been found to be LC3II^{+} and CD63^{+} (a marker of secretory lysosomes) and autophagy was found to have a crucial role in mast cell

degranulation and the release of histamine and β-hexosaminidase [75].

In conclusion, secretory autophagy involves the formation of a specialized autophagosome (LC3II^{+}, double-membrane structure) which sequesters cytoplasmic cargo for secretion instead of degradation. A precise understanding of how secretory lysosomes bypass fusion with the lysosome to avoid degradation remains to be described. The discovery of specialized receptors and fusion proteins that mediate secretion which permit modulation of this secretory pathway is likely to have implications in a pathological setting.

7. Enhanced Protein Secretion Caused by the Inhibition of Autophagy

In contrast to the previous studies where autophagy induction leads to enhanced secretion of proteins, other studies have reported the opposite: pharmacological or genetic inhibition of autophagy caused an increase in protein secretion of diverse proteins, particularly proinflammatory cytokines. Of particular interest is the case of IL-1β since we have previously mentioned studies in which autophagy induction by starvation in response to conventional NLRP3 inflammasome agonists increased IL-1β secretion in LPS-activated macrophages [64, 65]. In this regard, the opposite effect has also been described: enhanced IL-1β secretion has also been described after inhibition of autophagy, also in LPS stimulated macrophages. The first report linking the autophagic pathway to the secretion of IL-1β came from Saitoh et al. in 2008 [63]. In this study, the authors found that Atg16L1-deficient macrophages showed increased secretion of IL-1β but not of other proinflammatory proteins (IL-6, TNFα, and IFNβ) to the culture medium upon LPS stimulation [63]. In this study, Atg16L1 deficiency caused accumulation of ROS after LPS exposure as well as caspase-1 activation and IL-1β cleavage [63]. Although the precise mechanism by which the production of ROS induced the activation of the inflammasome was not fully described in this work, a different group also described increased IL-1β and IL-18 but not TNF secretion after inhibition of autophagy with knockout of *Map 1lc3b* or *Becn1* in LPS-activated macrophages [76]. In this work, Nakahira et al. showed that inflammasome activation induced by autophagy inhibition in LPS-treated macrophages was dependent on the presence of increased mitochondrial ROS, decreased mitochondrial membrane potential, and mtDNA (mitochondrial DNA) release to the cytosol [76]. The authors also showed that mitochondrial ROS activated the NLRP3 inflammasome, and this activation was necessary for mtDNA release to the cytoplasm since it does not occur in NLRP3-deficient macrophages. Once in the cytoplasm, mtDNA activated the AIM2 inflammasome, which induced the secretion of IL-1β and IL-18 [76]. In agreement with the previous observations, Harris et al. described ROS-dependent IL-1β secretion after pharmacological inhibition of autophagy with 3MA or *beclin1* knockdown in LPS-activated macrophages [77]. Importantly, pharmacological autophagy inhibition with 3MA did not affect IL-6, IL-18, or TNFα secretion. The authors observed colocalization of IL-1β with GFP-LC3-stained autophagosomes which they interpret as pro-IL-1β being degraded by autophagosomes. In the same work, the authors showed that rapamycin treatment decreased IL-1β secretion in LPS-injected mice, indicating that not only the inhibition of autophagy induced the secretion of IL-1β but that its induction decreased it [77].

In this regard, oxidized mtDNA has been shown to be an important activator o the NLRP3 inflammasome [65]. The NLRP3 inflammasome is a sensor of specific pathogen, host, and environmental danger molecules which requires an initial priming signal, usually induced by TLR stimulation, required for the transcriptional induction of NLRP3 and pro-IL-1β. Upon priming, stimulation of a functional NLRP3 can be induced by a series of triggers [78]. Regarding LPS-induced IL-1β secretion induced by the inhibition of autophagy, mtDNA oxidation induced by the accumulation of damaged mitochondria due to decreased mitophagy, could be the second signal for inflammasome activation and increased IL-1β secretion. Although both works describing the role of autophagy in IL-1β secretion seem contradictory, it is important to mention that in the first case [64], Dupont et al. used conventional inflammasome agonists as nigericin to activate the inflammasome, while in the second case [63], Saitoh et al. used autophagy inhibition as the second signal for inflammasome activation. The authors also proposed that differences could be due to inhibition of basal versus starvation or mTOR inhibitor-induced autophagy [64, 79].

More recently, a similar mechanism in which inhibition of autophagy increased the secretion of a proinflammatory cytokine has been described for macrophage migration inhibitory factor (MIF) from LPS-activated macrophages. In this work, inhibition of autophagy with 3MA, *Atg5* siRNA, or *Atg7* knockout increased MIF secretion to the culture medium. This secretion occurred together with an increase in mitochondrial ROS and could be decreased with antioxidants [80]. The importance of the anti-inflammatory role of autophagy has been demonstrated *in vivo*, since Atg16L1 deficiency increased the production of IL-1β and IL-18 in a model of chemically induced colitis in mice [63] and in mouse models of sepsis where lack of autophagy caused more susceptibility to endotoxemia with increased IL-1β and IL-18 serum levels [76].

8. Autophagy and Its Interactions with the Vesicular Trafficking System

Autophagy interacts at different levels with the endolysosomal as well as with the exosome biogenesis and secretion machinery both in normal and cancer cells [81, 82]. Degradative autophagosomes can merge with the MVB to give rise to amphisomes, which later fuse with lysosomes for their degradation. This fusion depends on RAB11 [83], while RAB27a has been associated with fusion of the MVB to the plasma membrane [81]. Also, since fusion of MVBs with the plasma membrane results in the extracellular release of exosomes, induction of autophagy by starvation has been shown to decrease exosome secretion by diverting MVBs to the autophagic-lysosomal pathway for their degradation [83].

Different mechanisms of autophagy (macroautophagy and microautophagy) have been suggested to have an important role in cargo delivery to vesicles of the endosomal/exosomal system. Inhibition of autophagy has been shown to decrease the amount of cytosolic proteins in late endosomes, which are components of the MVB which can be targeted for degradation or released as exosomes. On the other hand, cytosolic proteins like GAPDH have been found to be secreted in exosomes even in the absence of autophagy, indicating that macroautophagy only partially contributes to the delivery of cytoplasmic proteins to late endosomes and that in the absence of autophagy, cargo proteins can be transported by a different pathway [84].

Thus, it has been suggested that a specialized form of autophagy has the main role in exosome cargo loading. Selective incorporation of proteins during exosome biogenesis and the mechanisms of invagination occurring during maturation of the MVB have been proposed to involve a type of endosomal microautophagy [84, 85]. Microautophagy is a type of autophagy characterized in yeast which involves direct internalization of cytosolic cargo through invaginations of the lysosomal membrane [84]. Thus, a specialized type of microautophagy, endosomal microautophagy, occurring in late endosomal MVBs has been proposed to be responsible for the delivery of cytosolic proteins to the vesicles. This process was shown to be mediated by the chaperone hsc70 and the ESCRT systems [84]. This endosomal microautophagy is a process by which autophagy contributes to the secretion of cytosolic proteins but seems to be different from secretory autophagy since it involves direct delivery of cytosolic proteins to late endosomes and is independent of ATG proteins, which participate in macroautophagy but not in microautophagy. On the other hand, delivery of proteins to the MVB during secretory autophagy requires their transport in the autophagosome and a direct interaction with the MVB as has been shown for Acb1 [67, 68], IL-1β [64], α-synuclein [69, 70], and annexin A2 [86].

Exosomes are characterized by the presence of proteins involved in their biogenesis such as Alix, TSG101, HSP70, and tetraspanins as well as cell type-specific proteins, DNA, RNA, and lipids [81]. In this regard, an important interaction of the autophagic machinery with Alix, an ESCRT associated protein, has recently been described [87]. ATG12 and ATG3 are both core autophagy components, and their conjugation (ATG12-ATG3) has been shown to be necessary for basal but not starvation-induced autophagy. This interaction is also necessary for late endosomal to lysosome trafficking and for lysosome biogenesis [87].

9. Autophagy-Mediated Secretion in Cancer

Secreted proteins are known to play important roles in supporting the hallmarks of cancer [88]. In this regard, autocrine or paracrine signaling in cancer cells is known to sustain excessive proliferation, reduced apoptosis, immune cell regulation, angiogenesis, alterations in energy metabolism, and development of resistance against cancer therapy [3, 59].

In cancer, the regulation of autophagy has been shown to have important effects on protein secretion. Perhaps the first evidence that autophagy could regulate secretion in a cancer-related setting came from a study in oncogene- (Ras-) induced senescence in human fibroblasts [89]. Cellular senescence is a state of stable cell cycle arrest which can work as a failsafe program in response to a variety of insults during transformation. In this work, autophagy was activated during senescence, and it was responsible for senescence-associated secretion of IL-6 and IL-8 through a posttranslational mechanism, since the mRNA levels of IL-6 and 8 were higher in *Atg 5/7* knockdown cells [89]. Mechanistically, it was proposed that during oncogene-induced senescence, the rough endoplasmic reticulum and autophagic vacuoles colocalized with mTOR at the trans-Golgi network in an area termed the TOR-autophagy spatial coupling compartment, TASCC [90]. Localization of mTOR to this complex was responsible for driving the synthesis of IL-6/8. In this work, amino acid depletion or dominant negative expression of Rab-GTPases decreased mTOR recruitment to the TASCC. The authors proposed that during oncogene-induced senescence, spatial coupling of the cells' catabolic (autophagic vacuoles) with the anabolic (mTOR, ER, Golgi) machinery augments their respective function and facilitates mass synthesis of secretory proteins like IL-6/8 [90]. Importantly, TASCC formation was dependent on brefeldin A [90], which blocks ER to Golgi protein transport, indicating the need for a functional conventional pathway for this secretory phenotype.

In a similar work, Lock et al. [57] described autophagy-mediated secretion of protumorigenic factors in a RAS-driven model of invasive breast cancer. In this study, autophagy was necessary for invasiveness and epithelial-to-mesenchymal transition in RAS-transformed MCF10A breast cancer cells and was also necessary for the secretion of proinvasive factors like IL-6, matrix-metalloproteinases 2 and 9, and WNT5A [57]. Also, in agreement with the proinflammatory role of autophagy, a recent work has also described autophagy-dependent inflammation (increased secretion of CSF3/G-CSF, CXCL1, IL-6, TREM1, CCL2, CCL3/MIP-1α, IL-1β, and CXCL2) in response to UVB radiation prior to tumorigenesis. Secretion of these cytokines from UVB-irradiated mice was blocked by conditional *Atg7* KO in the skin [91].

In contrast, although most of the evidence shows that autophagy is necessary for the secretion of proinflammatory cytokines like IL-6, there is also evidence showing that the inhibition of autophagy by knockdown of *ATG* genes decreased IL-6 secretion in autophagy-dependent breast cancer cell lines but increased its secretion in autophagy-independent cells [40]. This was related to the maintenance of cancer stem cells since IL-6 supplementation increased mammosphere formation in *ATG7* shRNA-expressing cells and was associated to dependence on autophagy for survival [45]. Thus, whether autophagy serves a proinflammatory or anti-inflammatory function seems to be context- and cell type-dependent.

Regarding the anti-inflammatory role of autophagy, in a mouse model of breast cancer, Wei et al. [56] found that suppression of autophagy by *FIP200*$^{-/-}$ decreased mammary tumor initiation and progression. Decreased tumorigenesis occurred together with elevated production of chemokines

in tumor cells and increased IFNγ-producing $CD8^+$ and $CD4^+$ (Th1) T lymphocytes in the tumor microenvironment [56]. In the same study, *FIP200*$^{-/-}$, $CD8^+$ T cell-depleted animals developed mammary tumors with a similar kinetics as the autophagy-competent control mice, indicating that decreased tumorigenesis in *FIP200*$^{-/-}$ mice was due to increased chemokine secretion and the promotion of an antitumoral immune response.

Other studies have linked the inhibition of autophagy with increased secretion of cytokines from tumor cells. In this regard, in a *Kras*-driven non-small cell lung cancer (NSCLC) mouse model with a concurrent deletion of *Atg7* to inhibit autophagy in the tumors, the authors found a decrease in tumor growth with accumulation of defective mitochondria. Importantly, *Kras*-driven tumors, which normally formed adenomas and carcinomas, diverted to more benign oncocytomas in the absence of A*tg7*, indicating that the functional status of autophagy determines the tumor fate [51]. Despite decreased tumor burden, mice with *Atg7*-null tumors died from pneumonia with an increased inflammatory response. Interestingly, increased overall survival in the same model was observed only when *p53* was deleted together with *Atg7* as these mice did not show extensive inflammatory responses [51]. Thus, specific mutations present in the tumor might determine the role of autophagy inhibition on tumor cell-induced inflammation. This will be an important element to be considered when manipulating autophagy, since p53 is the most frequent tumor suppressor gene mutated in human cancers with diverse and context-dependent effects on cellular function [92].

Another protein whose secretion has been shown to be regulated by autophagy is HMGB1 [64, 93] (high-mobility group B1 immune modulator protein). Of note, HMGB1 is a nuclear protein which is not secreted in normal conditions and does not need to be processed by the inflammasome [64], indicating that the autophagic process modulates secretion by regulating different cellular pathways. HMGB1 is an immunogenic stimulator that is normally present in the cell nucleus and is considered to be released together with other alarmins during necrotic cell death upon plasma membrane rupture [94]. In cancer cells undergoing cell death induced by a targeted toxin, knockdown of *ATG* proteins prevented HMGB1 release [93]. These findings indicate that the levels of autophagy in a dying cell might determine the immunogenicity of this process at least partly by regulating the secretion of HMGB1 [93]. Another alarmin whose secretion has been proposed to be regulated by autophagy is ATP [47]. In this regard, autophagy-competent cancer cells treated with immunogenic chemotherapy, induced ATP secretion and a therapeutic immune response and this effect was not observed in autophagy-deficient (*Atg5* or Atg7 knockdown) cancer cells [47].

In contrast to the above-mentioned studies which suggest that, at least in cancer therapies with immunogenic potential, cell death with autophagy could promote a better long-term therapeutic response, emerging evidence suggests that in a different setting, autophagy could have an important role in the inhibition of the antitumor immune response. In this regard, hypoxia, an imbalance between increased oxygen consumption by tumor cells and an inadequate oxygen supply caused by cancer cell proliferation and defective tumor vascularization, has been shown to be an important regulator of tumor cell adaptation to low-oxygen conditions that can reshape tumors as well as their microenvironment [95]. These responses are known to be mediated by hypoxia-inducible factors (HIFs), transcription factors that mediate gene expression networks related to characteristics of malignancy, including the induction of autophagy [95]. Hypoxia-induced autophagy has been related to resistance to therapy [96] and avoidance of immune destruction [97]. Regarding the latter, it has been shown that HIF-1α can induce PD-L1 (programmed cell death ligand-1) expression to avoid cytolytic T lymphocyte (CTL) recognition [95] as well as BNIP3/BNIP3L, which induces autophagy that has been related to the development of resistance to CTL-mediated lysis. In this regard, pharmacological or genetic inhibition of hypoxia-induced autophagy decreased STAT3 phosphorylation in hypoxic tumor cells and restored tumor cell susceptibility to CTL-mediated lysis [97]. Although this work does not explore the relationship of secretion regulated by autophagy in resistance to cell lysis, cytokine secretion is likely to have a role in this phenotype since STAT3 is known to have an important role in the regulation of inflammation [98].

Despite the possible relationship of autophagy with the antitumoral immune response that we have previously discussed, a recent work found no changes in antitumor adaptive immunity in mouse models of melanoma and breast cancer after autophagy inhibition with *Atg* gene knockdown or with chloroquine/hydroxychloroquine treatment [99]. Thus, the precise role of autophagy in mediating the immunogenicity of tumor cells remains to be established.

Finally, despite controversial results in the literature and the context-dependent role of autophagy on protein secretion, the importance of identifying secreted proteins regulated by autophagy was evidenced in a recent work in melanoma [100]. In this work, melanoma tumor cells with low autophagy had a different secretome than their high-autophagy metastatic derivatives. High-autophagy melanoma cell lines presented higher levels of IL-1β, CXCL8, LIF, FAM3C, and DKK3 with known roles in inflammation and tumorigenesis. Levels of these proteins increased after autophagy induction and decreased with *ATG7* silencing in high autophagy cells. The authors found high levels of autophagy-regulated secreted proteins in serum of patients with high autophagy and suggest that serum levels of these proteins could be used as markers of autophagy levels in tumor cells which could be targeted with autophagy inhibitors [100].

10. Discussion

Evidence suggests that whether autophagy serves an anti-inflammatory or inflammatory role in cancer seems to depend on the stage of tumorigenesis, on the cancer type, and on the secreted factor being studied. Importantly, autophagy has been related to the secretion of proteins whose release is regulated by both conventional and unconventional pathways, and autophagosomes are also closely linked with

the endosomal-vesicular pathway, indicating that it could be playing diverse or even opposing roles on protein secretion depending on the cellular context.

Indeed, autophagy has an important role in the regulation of protein secretion in several types of cells. Mechanistically, two major autophagy-mediated secretion pathways have been described. The first one, secretory autophagy [13], involves a halted autophagic flux in which autophagosomes do not fuse with the lysosome and cargo-containing autophagosomes are directed to the plasma membrane or to multivesicular bodies for secretion, as has been described for Acb1 [67, 68], lysozyme [62], IL-1β [64, 66], and α-synuclein [69, 70]. Important mediators of this pathway are proteins necessary for plasma membrane fusion like Rab8α [64, 69], Sec22b, SNAP23/29, and STX3/4 [66]; absence of STX17 [66], which is necessary for fusion with the lysosome and GRASP proteins [64, 67]; and possibly peroxisomal signaling [61, 67] (Figure 2). In the second pathway, autophagy seems to serve as an antioxidant mechanism by decreasing damaged mitochondria (Figure 2). In this case, inhibition of autophagy would increase mitochondria which would otherwise be degraded. Cytoplasmic mtDNA leaking from mitochondria with low intermembrane potential could then activate the inflammasome as has been proposed for proinflammatory cytokine secretion like IL-1β, IL-18 [63, 76, 77], or MIF [80]. If there is a specific autophagy-regulated pathway for each one of the secreted proteins described, if specific markers for the vesicles involved exist, or if there is a combination of both pathways mentioned, as has been proposed for IL-1β [64], remains to be determined.

It will thus be important to establish how autophagy regulates secretion from cancer cells, if this regulation is similar to the one observed in non-transformed cells, in what cancer types or cancer stage autophagy is regulating secretion, and if protumorigenic or immune-regulating factors are being modulated by autophagy to better target autophagy for the treatment of cancer. Importantly, many of the proinflammatory cytokines regulated by autophagy in immune cells have not been studied in models of autophagy inhibition in the context of cancer. In this regard, IL-1β has been shown to induce epithelial to mesenchymal transition in breast cancer cells [101] and IL-1 signaling has been related to inflammation and aggressiveness due to the modulation of antitumor immunity in the same type of cancer [102]. Also, MIF, whose secretion has been shown to be increased after inhibition of autophagy [80], has been found to be elevated in different types of human cancers and is known to promote tumorigenesis through stimulation of proliferation, angiogenesis, metastasis, and inhibition of the antitumoral immune response [103]. This will be an important factor to evaluate in clinical trials currently using autophagy inhibition for the treatment of several types of cancer, particularly in those types of cancer where the antitumoral immune response has an important role in patient response.

Importantly, some of the proteins that have been identified as being regulated by autophagy in cancer, e.g., IL-6 and 8, are secreted by a conventional protein secretion route and their secretion is closely related to their transcription, underscoring the importance of understanding the relationship of the autophagic pathway to conventional protein secretion routes as well as to the regulation of their transcription factors like NF-κB or STAT3, to establish how manipulation of autophagy during cancer therapy might affect the tumor microenvironment. In this regard both, IL-6 and 8 have been shown to have important roles in maintaining oncogenic signaling in cancer cells, in promoting cancer stem cell maintenance [104–106] and in the regulation of the tumor microenvironment [107]. Since autophagy inhibition has been shown to decrease IL-6 and 8 secretion, inhibition of autophagy during cancer therapy would decrease their secretion in cancer cells. However, increased IL-6 secretion has also been reported for some cancer cells [40], particularly those that are not dependent on autophagy for survival. This is an important consequence that needs to be addressed in clinical trials manipulating autophagy in those types of cancer where autophagy has not proven to be important for cancer cell survival. In these cases, autophagy inhibition could possibly induce cytokine secretion and promotion of tumorigenesis as well as escape from the antitumoral immune response.

Finally, since intercellular communication is an important feature of tumor aggressiveness and tumor cell-derived extracellular vesicles transmit oncogenic signals to the neighboring tumor cells or to the cells in the tumor microenvironment, it will be important to understand how the modulation of autophagy affects exosomal content or exosomal release from tumor cells or from the tumor microenvironment since it is likely that at least some of the effects observed by the modulation of autophagy during cancer therapy, especially in immune-competent animals, will be mediated by extracellular vesicle release.

It is probable that secretion induced by the modulation of autophagy during cancer therapy will have different and context- or tissue-dependent roles, just as the manipulation of autophagy for cancer therapy or the regulation of the antitumoral immune response. Nevertheless, since some of the consequences of the inhibition of autophagy could promote malignancy or have other undesirable consequences, it will be important to understand how autophagy modulates secretion and how manipulation of autophagy will affect secretion in order to effectively modulate autophagy and its effects on secretion for the purpose of cancer therapy as well as for the treatment of other diseases.

Conflicts of Interest

The authors state they have no conflicts of interest to disclose.

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Research Article

Overexpression of CD44 Variant 9: A Novel Cancer Stem Cell Marker in Human Cholangiocarcinoma in Relation to Inflammation

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Various CD44 isoforms are expressed in several cancer stem cells during tumor progression and metastasis. In particular, CD44 variant 9 (CD44v9) is highly expressed in chronic inflammation-induced cancer. We investigated the expression of CD44v9 and assessed whether CD44v9 is a selective biomarker of human cholangiocarcinoma (CCA). The expression profile of CD44v9 was evaluated in human liver fluke *Opisthorchis viverrini*-related CCA (OV-CCA) tissues, human CCA (independent of OV infection, non-OV-CCA) tissues, and normal liver tissues. CD44v9 overexpression was detected by immunohistochemistry (IHC) in CCA tissues. There was a higher level of CD44v9 expression and IHC score in OV-CCA tissues than in non-OV-CCA tissues, and there was no CD44v9 staining in the bile duct cells of normal liver tissues. In addition, we observed significantly higher expression of inflammation-related markers, such as S100P and COX-2, in OV-CCA tissues compared to that in non-OV and normal liver tissues. Thus, these findings suggest that CD44v9 may be a novel candidate CCA stem cell marker and may be related to inflammation-associated cancer development.

1. Introduction

Infection and chronic inflammation are important factors for carcinogenesis, and cholangiocarcinoma (CCA) is a specific type of inflammation-associated cancer. Potential risk factors of CCA are parasitic infections (*Opisthorchis viverrini* and *Clonorchis sinensis*), bile duct disorders (biliary tract cysts and hepatolithiasis), toxins, complications (diabetes, cirrhosis, and obesity), alcohol consumption, and smoking [1]. The incidence of CCA has risen globally, and the highest rate occurs in Thailand, particularly in northeastern regions such as Khon Kaen. In Khon Kaen Province, high prevalence of CCA cases is related to *Opisthorchis viverrini* (OV) infection [2]. OV infection increases inflammation and enlarges bile ducts and connective tissues, resulting in periductal fibrosis and eventually the development of bile duct cancer [3]. The diagnosis of CCA is difficult because of clinical silence and a nonspecific appearance. Discovering specific molecular biomarkers may aid early and definitive diagnosis of CCA.

CD44 is a transmembrane glycoprotein that is ubiquitously synthesized and expressed in several types of mammalian cells such as leukocytes, red blood cells, brain cells, and

epithelial cells [4]. Alternative splicing results in several isoforms of CD44 with different functions. The standard isoform of CD44 (CD44s) is generally expressed in most normal epithelial cells, and variant isoforms of CD44 (CD44v) are expressed in some epithelial-type carcinomas [5]. CD44v is involved in cell proliferation, differentiation, migration, and adhesion in normal cells and metastasis in cancer cells. Recently, CD44v was proposed as a stem cell marker for several types of cancer [6]. In particular, CD44 variant 9 (CD44v9) is overexpressed in bladder cancer, pancreatic cancer, and colon cancer [7–9]. Overexpression of CD44v9 in gastric cancer caused by chronic inflammation from *Helicobacter pylori* infection is a prognostic biomarker at an early stage and is a predictive marker for recurrence [10, 11]. These findings suggest that CD44v9 is a selective target for inflammation-related CCA.

In this study, we examined the expression of CD44v9 in OV-related CCA (OV-CCA) tissues in comparison with that in normal bile duct cells and tissues of CCA independent of OV infection (non-OV-CCA) by immunohistochemistry (IHC). S100 calcium-binding protein P (S100P) is an important mediator of cancer-related inflammation [12–14], which leads to tumor invasion and metastasis [15], and S100P was identified as a CCA biomarker in both non-OV-CCA [16] and OV-CCA [17]. Overexpression of S100P may help to predict the clinical outcome of CCA patients [18, 19]. Cyclooxygenase-2 (COX-2) is also involved in inflammation, and we analyzed both COX-2 and S100P to clarify the relationship between OV-related CCA and inflammation.

2. Materials and Methods

2.1. Tissue Samples and Clinical Data. Thirty-three human liver fluke-caused CCA tissues (26 males, 7 females) were obtained from the Cholangiocarcinoma Research Institute of Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand. The collection of CCA tissues was approved by the Ethics Committee for Human Research (HE571283), Khon Kaen University, Thailand. Informed consent was obtained from all CCA patients. Clinical data of patients were recorded including age and sex. All cancer tissues were classified using AJCC 7th edition of TMN staging [20].

Human tissue microarray slides including normal liver tissues (21 cases: 12 males, 9 females) and CCA tissues (98 cases: 56 males, 42 females) were purchased from US Biomax Inc. (LVN801 and LV1004, Derwood, Maryland, USA). CCA tissues were classified in various TNM classes and tumor stages.

2.2. Immunohistochemical Staining (IHC) and Scoring. Embedded tissue sections were deparaffinized by xylene and rehydrated using a graded series of ethanol solutions. Antigen retrieval was performed by heating tissue sections in 5% urea at 500 watts in a microwave oven for 5 minutes. Endogenous peroxidase activity was quenched with 1% H_2O_2 (Kanto Chemical, Tokyo, Japan) followed by blocking with 1% skim milk (BD Biosciences, San Jose, California, USA). The sections were treated with primary antibodies (rat anti-CD44v9 monoclonal antibody from Cosmo Bio, Tokyo, Japan; goat anti-COX-2 from Santa Cruz Biotechnology, Dallas, Texas, USA; and rabbit anti-S100P from Abcam Biotechnology, Cambridge, UK, diluted 1 : 300 each) in a humid chamber. The sections were then incubated with biotinylated secondary antibody (Vector Laboratories, Burlingame, California, USA) including rabbit anti-rat IgG, rabbit anti-goat IgG, and goat anti-rabbit IgG. All sections were incubated with an avidin-biotin-peroxidase conjugate. The immunoreaction was activated by a peroxidase stain DAB kit (Nacalai Tesque, Kyoto, Japan), counterstained with hematoxylin, and mounted with Entellan New (Merck Millipore, Darmstadt, Germany). Stained tissues were visualized under a microscope (Olympus BX53F, Tokyo, Japan). The intensity of staining was graded by an IHC score between 0 and 4 by two investigators as follows: no staining (0), weak staining (1+), moderate staining (2+), strong staining (3+), and very strong staining (4+) in cholangiocytes and CCA cancer cells. The representative images for each score are shown in Figure S1A.

Paraffin-embedded OV-CCA tissues from 18 patients were stained using a double fluorescent staining method. Primary antibodies (anti-CD44v9, S100P, and COX-2 antibodies) were used at a dilution of 1 : 200, and secondary antibodies (donkey-anti-rat IgG Alexa fluor 488 and 594, donkey-anti-rabbit IgG Alexa fluor 488, and donkey-anti-goat IgG Alexa fluor 594) (Abcam Biotechnology, Cambridge, UK) were used at a dilution of 1 : 400. Nuclei were stained using DAPI (Southern Biotech, Birmingham, Alabama, USA). Stained tissues were examined by a fluorescent microscope (Olympus BX53F, Tokyo, Japan).

2.3. Statistical Analysis. Statistical analysis was performed using an SPSS software version 23.0 (IBM Corporation, USA). To compare patient clinical data between three groups, a chi-square test was used for age distribution, sex ratio, and TNM stage. The nonparametric Kruskal-Wallis test was performed to determine significant differences in IHC scores between the three groups, followed by multiple comparison with an adjustment of p value by the Bonferroni method (a pairwise test smaller than $0.05/3 = 0.017$ was significant at the 0.05 level and $0.01/3 = 0.0033$ at the 0.01 level). Statistical analysis was considered significant at $p < 0.05$.

3. Results

3.1. Clinical Features of CCA Patients. The summary of sample data is shown in Table 1. The age distribution was younger in the subjects of normal liver tissue array samples than those of non-OV and OV-CCA patients. There was no significant difference in age distribution between non-OV and OV-CCA groups. The sex ratio was not significantly different among the three groups. Patients with OV-CCA had more severe cancer progression than non-OV-CCA patients and had a significantly higher degree of tumor grade (T; T1–2 vs. T3–4), lymph node metastasis grade (N; N0 vs. N1), and distant metastasis grade (M; M0 vs. M1). There was an increased number of higher stages (III + IV) in OV-CCA cases than in non-OV-CCA cases.

Table 1: Clinicopathological information of normal subjects and CCA patients.

Characteristics	Normal liver $n = 21$ No. (%)	Non-OV-CCA $n = 98$ No. (%)	OV-CCA $n = 33$ No. (%)	Statistical significance
Age (years)				
≤40	15 (71.4)	12 (12.2)	2 (6.1)	**
>40	6 (28.6)	86 (87.8)	31 (93.9)	
Sex				
Male	12 (57.1)	56 (57.1)	26 (78.8)	n.s.
Female	9 (42.9)	42 (42.9)	7 (21.2)	
TNM classification				
T[a]				
T1	—	2 (2.0)	1 (3.7)	##
T2	—	51 (52.0)	5 (18.5)	
T3	—	43 (43.9)	9 (33.3)	
T4	—	2 (2.0)	12 (44.4)	
N[a]	—			
N0	—	67 (68.4)	3 (20.0)	##
N1	—	31 (31.6)	12 (80.0)	
M[a]	—			
M0	—	95 (96.9)	24 (85.7)	#
M1	—	3 (3.1)	4 (14.3)	
Tumor stage[a]				
I	—	2 (2.0)	0 (0.0)	##
II	—	40 (40.8)	3 (11.5)	
III	—	27 (27.6)	7 (26.9)	
IV	—	29 (29.6)	16 (61.6)	

OV: *Opisthorchis viverrini*; CCA: cholangiocarcinoma; n.s.: not significant. $^{**}p < 0.01$ compared to that of the normal liver group. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to that of the non-OV-CCA group. [a]Patients missing clinical information are not included in the statistical analyses.

3.2. Overexpression of CD44v9 in Human CCA Tissues. Cholangiocytes in normal liver tissues had no CD44v9 staining by IHC (Figure 1(a)). In CCA tissues, CD44v9 expression was observed in the membrane and cytoplasm of cancer cells (Figures 1(b) and 1(c)). There were no stains in these tissues when the primary antibodies were omitted (Figure S1B). In normal liver samples ($n = 21$), there was no positive CD44v9 staining (0%). CD44v9 was stained in 55.1% of non-OV-CCA ($n = 98$) and 87.9% of OV-CCA cases ($n = 33$). The IHC score of CD44v9 was significantly higher in CCA tissues than in normal tissues (Figure 2(a)). Furthermore, the CD44v9 staining score was significantly higher in the OV-CCA group than in the non-OV-CCA group.

3.3. Expression of Inflammation-Related Markers in CCA Tissues. S100P staining was not detected in bile duct cells of normal liver tissues (Figure 1(d)). In contrast, S100P staining occurred in the nucleus and cytoplasm of cancer cells in CCA tissues (Figures 1(e) and 1(f)). Without the primary antibody, no staining was observed in these tissues (Figure S1B). All normal liver tissue samples were negatively stained. S100P expression was observed in 58.2% of non-OV-CCA cases and 97.0% of OV-CCA cases. CCA tissues had markedly greater IHC scores than normal liver tissues, and scores were significantly higher in OV-CCA tissues than in non-OV-CCA tissues (Figure 2(b)).

Similarly, COX-2 expression was not observed in cholangiocytes of normal liver tissues (Figure 1(g)). COX-2 was overexpressed in the cytoplasm and nucleus of bile duct cancer cells (Figures 1(h) and 1(i)). The stained tissues were not detected by excluding primary antibodies (Figure S1B). COX-2 staining was positive in 56.1% of non-OV tissues and 100% of OV-CCA tissues. COX-2 IHC scores were significantly higher in both non-OV and OV-CCA tissues compared to that in normal liver tissues, and the scores in OV-CCA tissues were significantly higher than that in non-OV tissues (Figure 2(c)).

Among the expression of these molecules in non-OV-CCA and OV-CCA samples, there are significant correlations between the staining intensities of CD44v9 and S100P or COX-2 by Spearman's rank correlation coefficient (r). The correlations were moderate between CD44v9 and S100P ($r = 0.455$, $p < 0.001$) and also CD44v9 and COX-2 ($r = 0.465$, $p < 0.001$).

3.4. Double Fluorescent Staining for Human OV-CCA Tissues. In OV-CCA tissues, CD44v9 was primarily expressed in the cell membrane and moderately in the

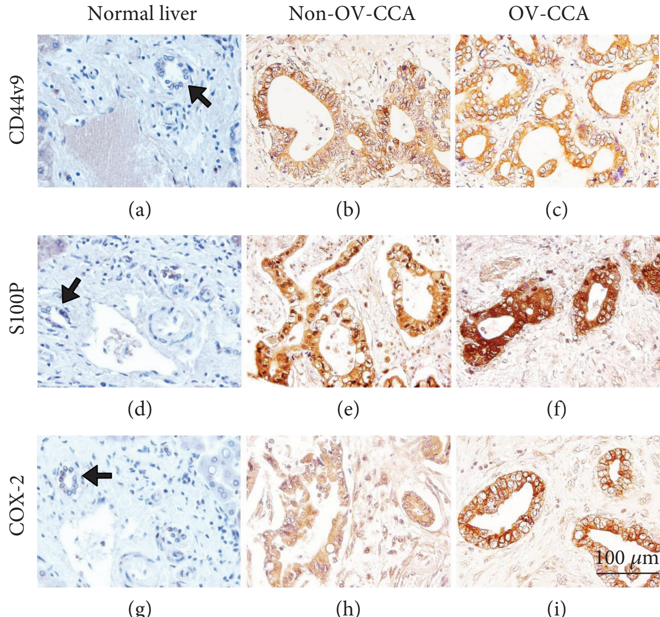

Figure 1: Immunohistochemical staining for CD44v9 (a–c), S100P (d–f), and COX-2 (g–i) in human liver tissues. Normal liver (a, d, g), non-OV-CCA (b, e, h), and OV-CCA tissues (c, f, i). Arrows indicate normal cholangiocytes.

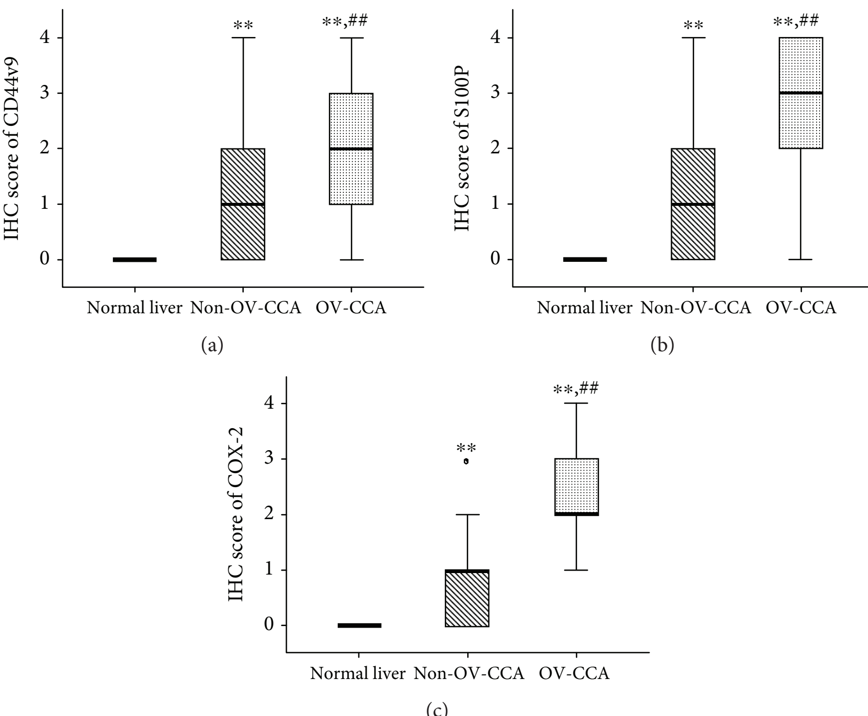

Figure 2: Box plots of CD44v9 (a), S100P (b), and COX-2 (c) staining in human liver tissues categorized by IHC score. The horizontal bold lines represent the median value, and the lower and upper boxes represent the 25th and 75th percentiles, respectively. The whiskers represent the range of data, and the circle is an outlier. A Kruskal-Wallis test was used to test for a significance difference among the three groups, and a Mann-Whitney U test was used to compare two groups with an adjustment of p value by the Bonferroni method. $^{**}p < 0.01$ compared to the normal liver group and $^{\#\#}p < 0.01$ compared to the non-OV-CCA group.

cytoplasm of CCA cells (Figures 3(b) and 3(f)), and S100P mostly appeared in the cytoplasm and nucleus of cancer cells (Figure 3(c)). Double-positive cells for CD44v9 and S100P were observed in OV-CCA tissues (Figure 3(d)). COX-2 was expressed in the nucleus and cytoplasm of cancer cells (Figure 3(g)). Both CD44v9 and COX-2 were expressed in some cancer cells of OV-CCA tissues (Figure 3(h)).

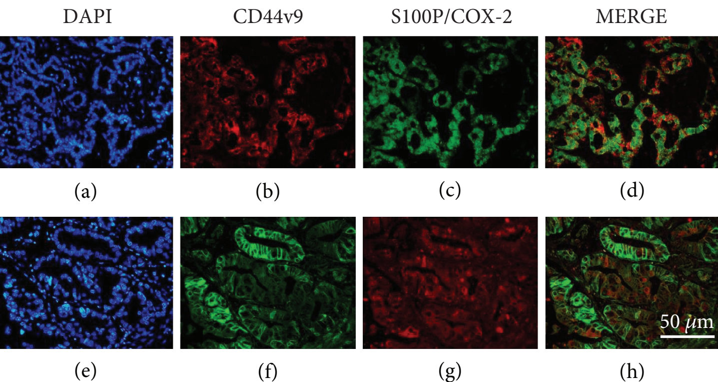

Figure 3: Representative images of double fluorescent staining in human OV-CCA tissues. DAPI staining of nuclei (a, e), CD44v9 (b, f), S100P (c), COX-2 (g), and merge (d, h).

4. Discussion

We firstly evaluated CD44v9 as a candidate biomarker of OV-related CCA by IHC. The level of CD44v9 expression was significantly higher in cancer cells of CCA patients, particularly in OV-CCA, than in normal liver tissues. We also investigated the expression of inflammatory markers, S100P and COX-2, which were overexpressed in CCA tissues, predominantly in OV-CCA.

The alternative splicing of human CD44 gene produces different CD44 variant isoforms, which are abundantly expressed in several tumors, while the standard isoform is mainly expressed in normal epithelial cells [21]. In multiple complexes of variant isoforms, the exon combination of variable regions provides a heterogeneity of CD44 molecules. In exon combination, isoforms of CD44 include a property of tissue-specific expression by distinct expression in different tissues with normal or diseased states [22]. These combinations predominantly are exhibited in specialized tissues, e.g., CD44v3-10 is a keratinocyte form and CD44v8-10 is an epithelial form [23–25]. Moreover, the various isoforms of CD44 could present a broad spectrum of physiological functions and may have defined functions [26, 27] depending on a number of exon combinations through its posttranslational modification of CD44 splicing variant, which impacted on the molecular interactions and tumorigenicity [28]. Non-Hodgkin's lymphoma (NHL) showed a different expression pattern between individual v6 and v6-containing isoforms. A single CD44v6 exon composition was expressed in low-grade NHL, while the combination of v6 exon with other variant exons was presented in high-grade NHL [29]. Different tissues express different CD44 variants with various exon combinations, and the cellular functions might relate to its variable of exon combination. Among various exon combinations, CD44v9 is involved in several exon combinations such as v1-10, v6-10, v7-10, and v8-10. In gastric adenocarcinoma, CD44v9 is contained in v6-10, v7-10, and v8-10 combinations [30]. CD44v8-10 has a role of cancer stem cell in CCA development via redox regulation [31]. Defects in mRNA splicing are an important cause of cancer, and the most common form of splicing defects are genomic splice site point mutations [32]. The present study showed the existence of CD44v9 in CCA, and our previous studies [33, 34] indicated inflammation-related DNA damage. Further study is needed to clarify the molecular mechanisms of CCA development mediated by splicing defects.

Inflammation is a fundamental cancer-promoting factor. OV infection causes the production of reactive oxygen species (ROS), such as nitric oxide and superoxide, which leads to the formation of DNA lesions, including 8-nitroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) [33, 34], indicating that OV infection is a cause of inflammation-associated carcinogenesis. Previously, we found a high level of expression of stem cell markers, CD133 and Oct3/4, and a high level of 8-oxodG in OV-CCA tissues, suggesting that stem cell mutations are involved in the inflammatory microenvironment during CCA development [35]. CD44 variant isoforms including CD44v9 mitigate ROS [31, 36], which may explain the high level of expression of CD44v9 in addition to S100P and COX-2 predominantly in the tissues of OV-CCA, a cancer driven by inflammation. CD44v9-positive cells were detected in both non-OV and OV-CCA tissues. Some non-OV-CCA tissues expressed CD44v9, which may be explained by a report that solid tumors including CCA are under inflammatory conditions including hypoxia [37]. Additionally, a correlation between the expression of S100P and receptor for advanced glycation end product (RAGE) has been reported [38] and might be involved in an inflammatory response including COX-2 induction. We postulated environmental factor-related inflammation such as OV infection and tumor-producing inflammation such as hypoxia and S100P-RAGE in the multiple steps of carcinogenesis [39]. OV-CCA may be affected by both OV infection and tumor-producing inflammation, and non-OV-CCA may be affected by tumor-producing inflammation alone. Interestingly, in this study, significant positive correlations were observed between the staining of CD44v9 and S100P or COX-2, suggesting a role of CD44v9 in inflammation. This finding raises a possibility that CD44v9 expression is associated with an inflammatory state during CCA development.

Previously, we found that prolonged oxidative stress induces stem cell properties via gene downregulation, which results in CCA genesis with aggressive clinical outcomes [40]. Qu et al. observed the coexpression of CD44 and S100P

protein in spheroid-forming pancreatic ductal epithelial cells chronically treated with cadmium, which suggests that S100P expression likely contributes to the aggressive and stemness nature of spheroids [41]. In an inflammatory microenvironment, COX-2 stimulates the development of breast cancer stem cells [42], and the proliferation of CD44$^+$ stem-like cells in gastric cancer is cooperatively stimulated by COX-2/PGE2-mediated signaling [43]. Previously, we found that COX-2 activation may be involved in inflammation-mediated stem cell proliferation and differentiation in urinary bladder carcinogenesis [44]. Although it is still unclear how inflammation affects cancer cell stemness, our results suggest a positive correlation between CD44v9 and inflammation.

Several studies found that CD44v9 expression is associated with cancer tumorigenicity. Kiuchi et al. observed a high expression of CD44v9 in the progression of pancreatic cancer cells during mitosis [8]. Additionally, CD44v9-expressed cells have apoptotic resistance and enhanced invasive properties [45, 46]. Wang et al. illustrated that a small population of CD44-positive CCA cells have properties of cancer stem cells including self-renewal [47]. Seishima et al. reported that the anti-inflammatory drug sulfasalazine reduces proliferation of CD44v9-positive cells and has a significant impact on ulcerative colitis-associated tumor cell differentiation [48]. Similarly, the formation of cancer cells in inflammation-mediated human gastric adenocarcinoma is correlated with CD44v9 expression. In addition, CD44v9-ablated or sulfasalazine-treated mice have reduced expansion of gastric tumor cells and development of premalignant lesions in the stomach [49]. We observed overexpression of CD44v9 in CCA, especially in OV-CCA. Taken together, these data indicate the importance of CD44v9-positive cancer stem cells in the progression of inflammation-related cancer. Reassessment of anti-inflammatory drugs may be a valuable approach to develop new chemotherapies. Further investigation of CD44v9-targeted cancer stem cells is needed, and the overexpression of CD44v9 in CCA may point to new anticancer stem cell therapeutic strategies.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Supplementary Figure S1: IHC scoring and negative control. (A) Representative CCA examples of negative and positive staining of CD44v9 (A–E), S100P (F–J), and COX-2 (K–O) by scoring range as follows: 0 for no staining (A, F, K), 1+ for weak staining (B, G, L), 2+ for moderate staining (C, H, M), 3+ for strong staining (D, I, N), and 4+ for very strong staining (E, J, O). (B) Representative CCA examples of tissue staining without primary antibodies. CCA tissues were stained with only secondary antibodies (VECTASTAIN Elite ABC HRP Kit), including rabbit anti-rat IgG for CD44v9 (PK-6104), goat anti-rabbit IgG for S100P (PK-6101), and rabbit anti-goat IgG for COX-2 (PK-6105). *(Supplementary Materials)*

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Research Article

Aspirin Disrupts the Crosstalk of Angiogenic and Inflammatory Cytokines between 4T1 Breast Cancer Cells and Macrophages

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The tumor microenvironment is rich in multiple cell types that influence tumor development. Macrophages infiltrate tumors, where they are the most abundant immune cell population and secrete a number of cytokines. Aspirin acts as a chemopreventive agent against cancer development. This study investigated whether aspirin regulates crosstalk between breast cancer cells and macrophages. To study these interactions in a tumor microenvironment, a conditioned media was employed using 4T1 breast cancer cells cultured in RAW 264.7 cell-conditioned medium (RAW-CM), and a cocultured model of both cells was used. When 4T1 cells were cultured in the RAW-CM, there were increases in cell viability and secretion of the cytokines VEGF, PAI-1, TNF-α, and IL-6. Treatment with aspirin inhibited 4T1 cell growth and migration and MCP-1, PAI-1, and IL-6 production. In the coculture of both cells, aspirin inhibited secretion of MCP-1, IL-6, and TGF-β. Furthermore, aspirin significantly decreased the M2 macrophage marker CD206, but increased M1 marker CD11c expression. In summary, aspirin treatment inhibited the crosstalk of 4T1 and RAW 264.7 cells through regulation of angiogenic and inflammatory mediator production and influenced the M1/M2 macrophage subtype. This highlighted that aspirin suppresses the tumor favorable microenvironment and could be a promising agent against triple-negative breast cancer.

1. Introduction

Breast cancer is the most frequently occurring cancer in women worldwide, especially in developed countries, and the incidence is increasing globally. In 2015, the World Health Organization performed a statistical analysis that revealed approximately 570,000 women die from breast cancer annually, indicating that up to 15% of all deaths in women are due to cancer [1]. Breast cancer has a heterogeneous pathology comprised of multiple components, including tumor cells and neighboring stromal cells, such as adipocytes, fibroblasts, macrophages, and other immune cells, that play fundamental roles in normal mammary development as well as breast carcinogenesis [2, 3]. Moreover, tumor microenvironment changes, such as changes in the extracellular matrix, soluble factors, and signaling molecules, stimulate carcinogenesis and resistance to the immune response [2]. These diverse microenvironments play critical roles in tumor progression and metastasis.

The complicated interactions between tumors and the immune system have attracted the attention of scientists over the past decade. Briefly, the dynamic interactions between innate and adaptive immunity play an important role in tumor progression and inhibition [4]. Mononuclear phagocytes are innate immune cells that protect individuals from harmful pathogens and repair injured tissues. However, in the tumor microenvironment, malignancies recruit circulating monocytes by producing tumor-derived chemotactic factors such as macrophage chemoattractant protein-1 (MCP-1), vascular endothelial growth factor (VEGF), and macrophage colony-stimulating factor (MCSF) and then induce monocytes to differentiate into tumor-associated macrophages (TAMs) [5]. In the tumor microenvironment, multiple mediators are secreted and contribute to cell proliferation, migration, angiogenesis, remodeling of endothelial cells [4], providing favorable conditions for tumor growth and metastasis, and suppression of adaptive immunity [6].

Macrophages that produce mediators are crucial initiators of chronic inflammation in the tumor microenvironment. Macrophage heterogeneity includes categorization into M1 and M2 macrophages based on two distinct phenotypes that are a result of macrophage polarization and the development of different characteristics [7]. M1 macrophages produce inflammatory cytokines that evoke the adaptive immune response. Conversely, M2 macrophages promote angiogenesis and wound healing and suppress the adaptive immune responses [7]. Interestingly, TAMs resemble M2 macrophages and have protumor properties in tumor microenvironments. Several studies on *murine* tumor models have shown that TAMs promote tumors [8] and produce cytokines and chemokines that sustain and amplify the inflammatory state [9]. Therefore, agents with the potential to adjust this microenvironment have been proposed as effective future cancer therapies [3, 8].

Aspirin, acetylsalicylic acid, is a nonsteroidal anti-inflammatory drug commonly used to reduce inflammation and prevent heart attack and stroke [10, 11]. However, over the past two decades, studies have shown that regular use of aspirin may have an additional promising role against cancers [12]. This chemoprevention by aspirin was reported for inflammation-associated cancers such as colorectal, breast, lung, prostate, stomach, and ovarian cancers [10]. Moreover, accumulating epidemiological evidence has revealed that aspirin has effects when used against breast cancer [13, 14]. Although aspirin is a promising chemopreventive agent, gastrointestinal side effects and optimal doses are important factors to consider for clinical applications. Therefore, alternatives using aspirin, such as lower doses or combinations with treatments, have been continually proposed.

Currently, little is known about the role of aspirin in immune regulation of tumors, especially in terms of the tumor microenvironment. The main goal of this study was to better understand breast cancer chemoprevention by aspirin, which may regulate immune responses in both malignant cells and macrophages in the tumor microenvironment, as well as interfere with crosstalk between these cells. These insights might provide potential strategies for ameliorating triple-negative breast cancer, such as 4T1 cells, which is a highly aggressive type of breast cancer with resistance to treatments [15].

2. Materials and Methods

2.1. Cell Culture and Treatments. The murine breast cancer 4T1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA), and macrophage RAW 264.7 cell line was purchased from Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Caisson, Smithfield, UT, USA) containing 10% fetal bovine serum (FBS, Genedirex, Las Vegas, NV, USA) with 1% penicillin/streptomycin/amphotericin B (Caisson) in a humidified atmosphere with 5% CO_2 in a 37°C incubator. Both cell lines were used to prepare conditioned medium and cocultures in this study. Aspirin (Sigma, St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma) to generate a stock solution. The final concentration of DMSO in the vehicle group was 0.1%, which is equivalent to the highest dose (2 mM) received by cells during aspirin treatment.

2.2. RAW-CM Preparation. RAW 264.7 cells, 2.5×10^4 cells/well, were seeded in 6-well plates containing 10% FBS/DMEM and cultured overnight. The cells were then cultured for 24 h in the presence or absence of 100 ng/mL lipopolysaccharide (LPS, Sigma) in 1% FBS/DMEM according to a previous study, with modifications [16]. Supernatants were collected, and cell debris was removed by centrifugation prior to use in experiments.

2.3. Cell Viability Assay. The 4T1 cells were seeded into 96-well plates at a density of 2×10^3 cells/well (Becton Dickinson, Franklin Lakes, NJ, USA) and were concurrently treated with 0.5, 1, or 2 mM of aspirin in media containing 20, 50, or 75% unstimulated or LPS-stimulated RAW-CM and 1% FBS/DMEM for 24, 48, and 72 h. After treatment, the cells were incubated in a 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT (Sigma) solution for 3 h. Supernatants were aspirated, DMSO was added to solubilize the formazan crystals, and absorbance was measured at 540 nm using a spectrophotometric microplate reader (BioTek, Winooski, VT, USA). The control was considered to be 100%, and cell viability of each sample is presented as percentage of control based on the formula $(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{control}} - A_{\text{blank}}) \times 100$, where A_{sample}, A_{blank}, and A_{control} refer to the absorbance of the sample, blank, and control at 540 nm, respectively.

2.4. Cell Migration Assay. Migration of 4T1 breast cancer cells was measured using wound-healing assays. To determine the optimal concentration of RAW-CM for 4T1 cell migration, 4T1 cells were cultured in media containing 20, 50, or 75% RAW-CM and 3% FBS/DMEM for 24 h. Cells were seeded in 24-well plates and incubated until 80% confluence was reached. This monolayer of cells was gently scratched using a 20 μL pipette tip, and the media was replaced with 0.5, 1, or 2 mM aspirin in fresh medium, 50% unstimulated RAW-CM, or 50% LPS-stimulated RAW-CM for 24 h. Cells were viewed and imaged through a microscope equipped with a camera (WS500, Whited, Taoyuan, Taiwan) at 100x magnification. Then, the healing in the image was measured with a microscale of image software (Whited).

2.5. Cytokine Production as Measured by ELISA. The 4T1 cells, 2×10^4 cells/well, were seeded in a 48-well plate overnight and then treated with 2 mM aspirin in complete medium or 50% RAW-CM for 72 h. Culture supernatants were collected, and levels of cytokines, including MCP-1 (BioLegend, San Diego, CA, USA), VEGF (Peprotech, Rocky Hill, NJ, USA), PAI-1, TNF-α, IL-6, and TGF-β (R&D, Minneapolis, MN, USA), were measured by ELISA according to the manufacturer's instructions. Briefly, plates were coated overnight with capture antibodies and then washed and blocked. After washing, the culture supernatants were added to the plates and the plates were incubated for 2 h. After washing, the plates were incubated first with detection

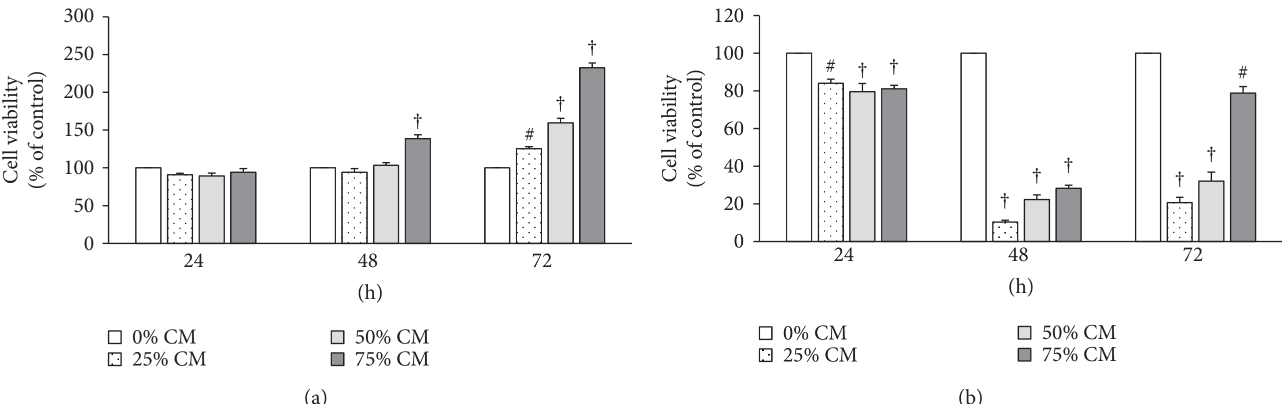

Figure 1: Viability of 4T1 cells cultured in different amounts of RAW 264.7 macrophage-conditioned medium (RAW-CM). Different concentrations of (a) unstimulated and (b) LPS 100 ng/mL-stimulated macrophage-conditioned medium (RAW-CM) at 25, 50, and 75% were used to culture 4T1 cells. Cells were cultured for 24, 48, and 72 h, and cell viability was measured using MTT assays. Data are from at least three independent experiments and presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA and least significant difference (LSD) post hoc tests. $^{\#}p < 0.01$ and $^{\dagger}p < 0.001$ versus control (0% RAW-CM).

antibodies, next with horseradish peroxidase-conjugated streptavidin, and finally with substrate solution. Absorbance was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Cytokine levels were calculated based on cytokine standard curves.

2.6. Cocultures of 4T1 Cell and RAW 264.7 Cell. To define the role of the mammary microenvironment in tumorigenesis, the experimental models consisted of 4T1 *murine* breast cancer cells cultured alone in RAW-CM or cocultured with RAW 264.7 cells. To mimic a physiological environment where macrophages infiltrate into the areas surrounding breast cancer cells, RAW 264.7 and 4T1 cells were cocultured in the same well of 6-well plates at densities of 1×10^5 and 4×10^5 cells/well. The cells were then maintained in 1% FBS/DMEM and treated with 2 mM aspirin for 72 h. Culture supernatants were harvested and stored at −20°C until cytokine levels were measured by ELISA.

2.7. RAW 264.7 Cell Characterization. Macrophages were incubated in the presence or absence of aspirin for 72 h and cultured in either control medium, the presence of LPS for the last 24 h of the incubation, or cocultured with 4T1 cells for 72 h. To assess surface marker expression, RAW 264.7 and 4T1 cells were collected after 72 h of coculturing and stained by incubating with fluorescein FITC anti-mouse CD11c and Alexa Fluor 647 anti-mouse CD206 monoclonal antibodies (Sony Biotechnology Inc.) at 4°C in the dark for 30 min. After washing, viable cells were stained with Hoechst 33342 (ChemoMetec, Allerød, Denmark) and subjected to FlexiCyte fluorescence-activated cell sorting analysis. The frequency of cells expressing each surface marker was determined by NucleoCounter NC-3000 (ChemoMetec) and analyzed using NucleoView NC-3000 software (ChemoMetec). Expression was quantified using median fluorescence intensity for the marker of interest.

2.8. Statistical Analysis. Results are presented as mean ± SEM and are a compilation of at least three independent experiments. Statistically significant differences among groups were identified by one-way ANOVA with least significant difference post hoc tests using IBM Statistical Product and Service Solutions (SPSS version 19). A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. RAW 264.7 Cell-Conditioned Media Affects 4T1 Breast Cancer Cell Viability and Migration. To mimic the physiological tumor environment of macrophage infiltrates into tumor tissues and to study the effect of macrophage mediators on 4T1 cell viability, breast cancer 4T1 cells were cultured in RAW 264.7 cell-conditioned media (RAW-CM), as shown in Figure 1. The 4T1 cells were cultured in different concentrations of RAW-CM in the presence or absence of lipopolysaccharide (LPS) stimulation, and cell viability was assessed using MTT assays. The culture condition lacking LPS stimulation mimicked macrophage infiltration into the breast cancer microenvironment, while the culture condition with LPS stimulation mimicked infiltrating macrophages that are active due to inflammatory responses.

A progressive increase in the number of 4T1 cells occurred with an increase in concentration of unstimulated RAW-CM. This increase in cell number, compared to the control (0% RAW-CM), occurred in a dose-dependent manner with the incubation time (Figure 1(a)), suggesting the macrophages present promoted breast cancer cell growth. The opposite result was observed when 4T1 cells were cultured in the LPS-stimulated RAW-CM, where 4T1 cell viability significantly decreased during incubations of 24 to 72 h ($p < 0.05$, Figure 1(b)). This suggests that mediators were secreted by active macrophages that caused toxicity, and thereby decreased cancer cell numbers.

Wound-healing assays were used to analyze cell migration, which is an indicator of cancer metastasis. Cells were grown until a confluent monolayer and scraped, and then the distance of healing by the cell layer was measured. The 4T1 cells cultured in 3% FBS/DMEM, that is, control,

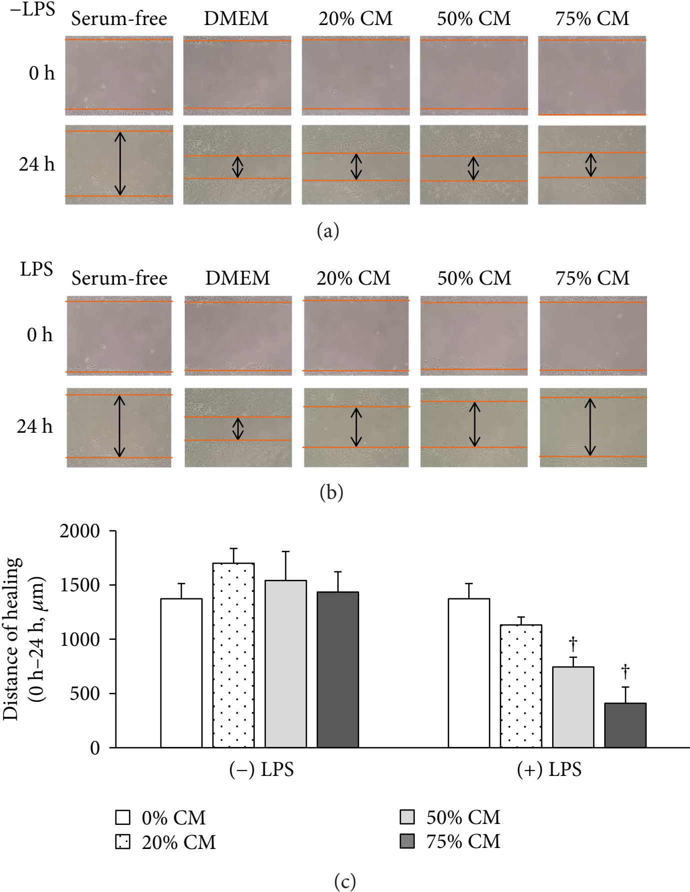

Figure 2: Migration of 4T1 cells cultured in different amounts of RAW-CM. Migration patterns of 4T1 cells were assessed in scratched areas by culturing cells for 24 h in 20, 50, and 75% (a) unstimulated or (b) LPS-stimulated RAW-CM and then monitoring wound healing. (c) Distance was measured by microscope under a microscale and presented as percentage inhibition relative to the control. Data are shown as mean ± SEM and are from three independent experiments. Statistical analysis was performed using one-way ANOVA and LSD post hoc tests. $^{\dagger}p < 0.001$ versus control (0% CM).

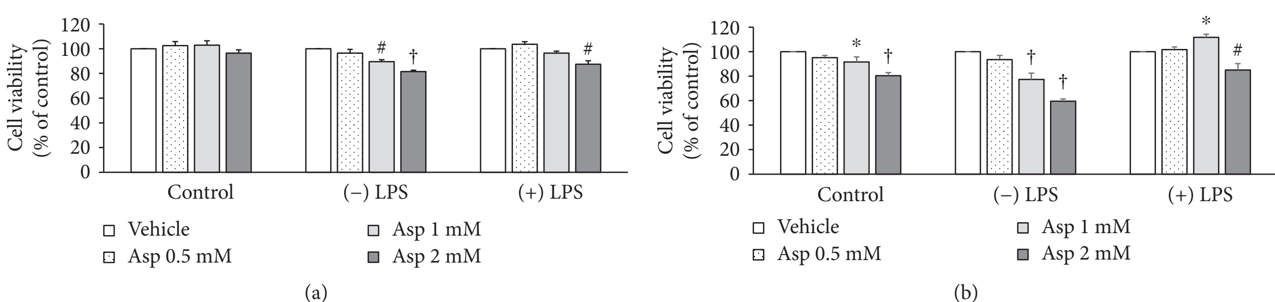

Figure 3: The effect of aspirin on viability of 4T1 cells cultured in RAW-CM. Different doses of aspirin were used to treat 4T1 cells cultured in 50% unstimulated or LPS-stimulated RAW-CM. Cells were cultured for (a) 24 or (b) 72 h, and cell viability was assessed using MTT assays. Data are shown as mean ± SEM and are from three independent experiments. Statistical analysis was performed using one-way ANOVA and LSD post hoc tests. $^{*}p < 0.05$, $^{\#}p < 0.01$, and $^{\dagger}p < 0.001$ versus vehicle control.

exhibited apparent healing, while the cells cultured in serum-free media, that is, negative control, did not. The distance of 4T1 cell migration over 24 h was measured for each treatment condition, including cells incubated in 20, 50, and 75% RAW-CM. RAW-CM was collected from cells that were not stimulated with LPS as a spontaneous condition and was found to have no effect on cell migration (Figure 2(a)). Meanwhile, RAW-CM collected from LPS-stimulated cells inhibited healing after scraping in a dose-dependent manner (Figure 2(b)). The migration distance was measured by microscope under a microscale, and the results are shown in Figure 2(c). The 50 and 75% LPS-stimulated RAW-CM

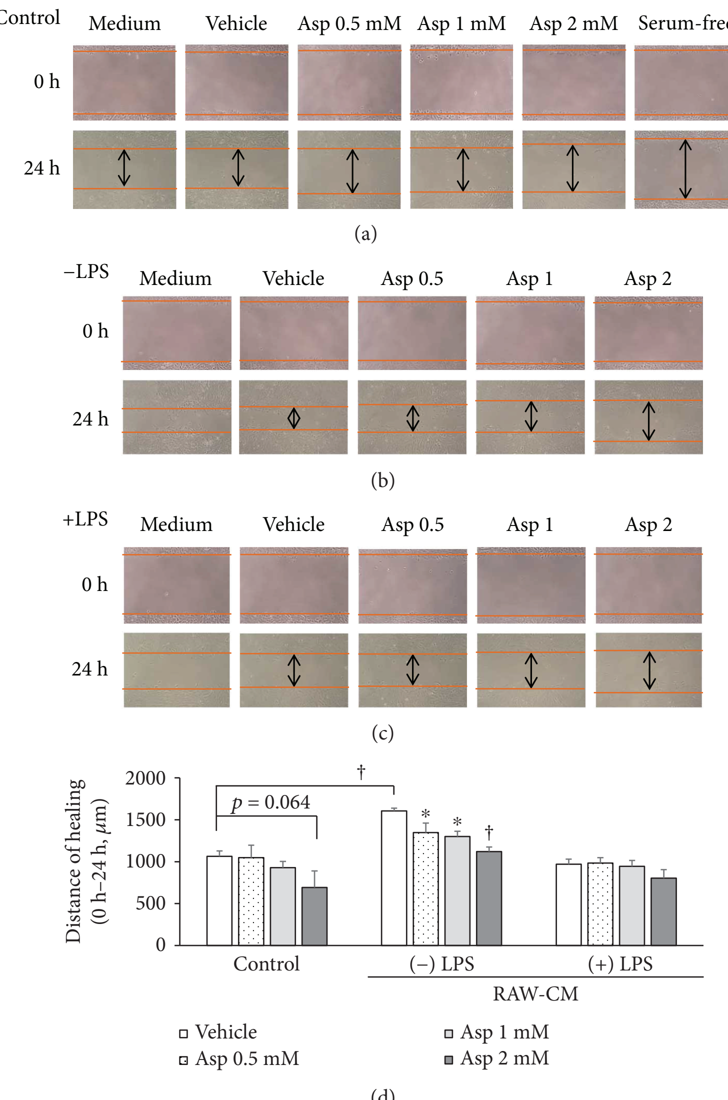

Figure 4: The effect of aspirin on migration of 4T1 cells cultured in RAW-CM. Different doses of aspirin were used to treat 4T1 cells, which were cultured for 24 h in (a) 3% FBS/DMEM, (b) 50% unstimulated RAW-CM, and (c) 50% LPS-stimulated RAW-CM for 24 h, and wound-healing assays were performed. (d) Distance was measured by microscope under a microscale and is presented as percentage inhibition relative to the control. Data are shown as mean ± SEM and are from three independent experiments. Statistical analysis was performed using one-way ANOVA and LSD post hoc tests. $^{*}p < 0.05$ and $^{\dagger}p < 0.001$ versus vehicle control.

conditions significantly inhibited cell migration ($p < 0.01$), which is consistent with the effect this conditioned media had on 4T1 cell viability.

3.2. Aspirin Inhibited 4T1 Breast Cancer Cell Growth and Migration in RAW 264.7 Cell-Conditioned Media. Subsequently, we investigated whether aspirin treatment influences 4T1 breast cancer cell growth when cultured under different macrophage-related conditions. The 4T1 cells were cultured in RAW-CM to mimic a microenvironment with macrophage infiltration into areas surrounding breast cancer cells, and then cell viability and migration were assessed. The 4T1 cells treated with 1 and 2 mM of aspirin had decreased cell viability when incubated in both unstimulated and LPS-stimulated RAW-CM for 24 h, while 4T1 cell numbers were not affected by aspirin in the complete medium (Figure 3(a)). Cell number displayed more apparent decreases of 23% ($p < 0.001$) and 40% ($p < 0.001$) in unstimulated RAW-CM compared to cells in control medium, when cells were treated for 72 h with 1 or 2 mM aspirin, respectively (Figure 3(b)). However, only the high dose of 2 mM aspirin inhibited cell viability in the LPS-stimulated RAW-CM.

To investigate the effects of aspirin on 4T1 cell migration in RAW-CM, wound-healing assays were utilized. The 4T1 cells were cultured in fresh medium (Figure 4(a)), unstimulated RAW-CM (Figure 4(b)), or LPS-stimulated RAW-CM (Figure 4(c)) to mimic the macrophage-infiltrated microenvironment. Aspirin had no effect on cell migration in the fresh medium (Figure 4(a)). In the unstimulated RAW-CM, 0.5 to 2 mM aspirin significantly delayed scratch-healing form in a dose-dependent manner ($p < 0.05$) compared to

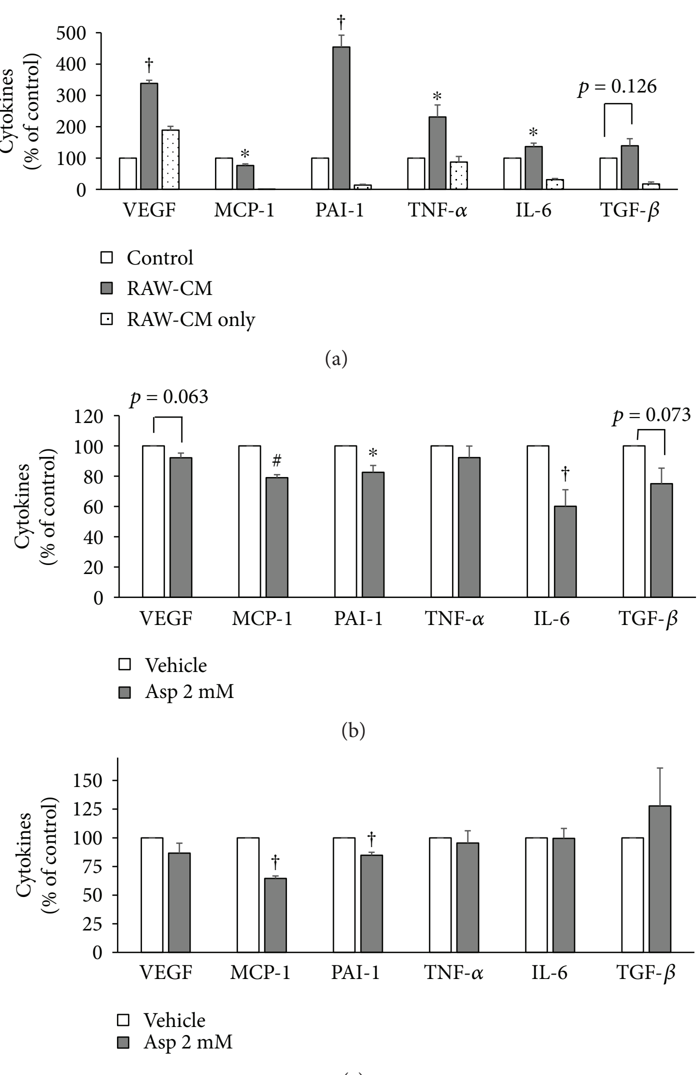

Figure 5: Effect of aspirin on carcinogenic cytokine production by 4T1 breast cancer cells cultured in control medium and RAW-CM. (a) Effect of RAW-CM on cytokine production of 4T1 cells. Cells were cultured in 50% RAW-CM for 72 h, and then cytokines in the supernatants were measured by ELISA. (b) Aspirin was used to treat 4T1 cells, which were cultured in control medium (1% FBS/DMEM) for 72 h, and cytokine levels in the supernatants were measured. (c) Aspirin was used to treat 4T1 cells, which were cultured in 50% RAW-CM for 72 h, and then cytokine levels in the supernatants were measured. Data are shown as mean ± SEM. Statistical analysis was performed using independent sample *t*-tests, where statistically significant differences are indicated as $^{*}p < 0.05$, $^{\#}p < 0.01$, and $^{\dagger}p < 0.001$ versus control.

the vehicle group (Figures 4(b) and 4(d)), while healing was not affected by aspirin in the LPS-stimulated RAW-CM (Figures 4(c) and 4(d)).

Therefore, the unstimulated RAW-CM, which mimicked the tumor microenvironment, promoted growth of 4T1 cells and was suitable to use for future experiments. Meanwhile, LPS stimulation triggered RAW 264.7 cells to exert an acute inflammatory response that inhibited growth and migration of 4T1 cells. On the basis of these studies, aspirin is an effective chemopreventive agent in the tumor microenvironment but did not exert an anticancer effect during the acute inflammatory stage.

3.3. Aspirin Inhibited 4T1 Cell Production of Angiogenic and Inflammatory Cytokines. Cytokines related to breast cancer carcinogenesis in the cultured supernatants were measured by ELISA. Cytokine levels are listed in Supplementary 1, and data are presented relative to the vehicle control in Figure 5. First, 4T1 cells were cultured in fresh medium (control) or RAW-CM and the supernatants were analyzed (Figure 5(a)). The RAW-CM only allowed background levels of mediators in the original conditioned medium to be measured. VEGF, plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor (TNF-α), and interleukin (IL-6) secretion were significantly higher when the 4T1 cells were cultured in 50% RAW-CM, suggesting that macrophage-related mediators in the conditioned media promoted carcinogenic and inflammatory cytokine production by the breast cancer cells ($p < 0.05$).

To investigate the effects of aspirin treatment on secretion of these cytokines, cytokine levels relative to tumor characteristics were analyzed (Figures 5(b) and 5(c)). As shown in

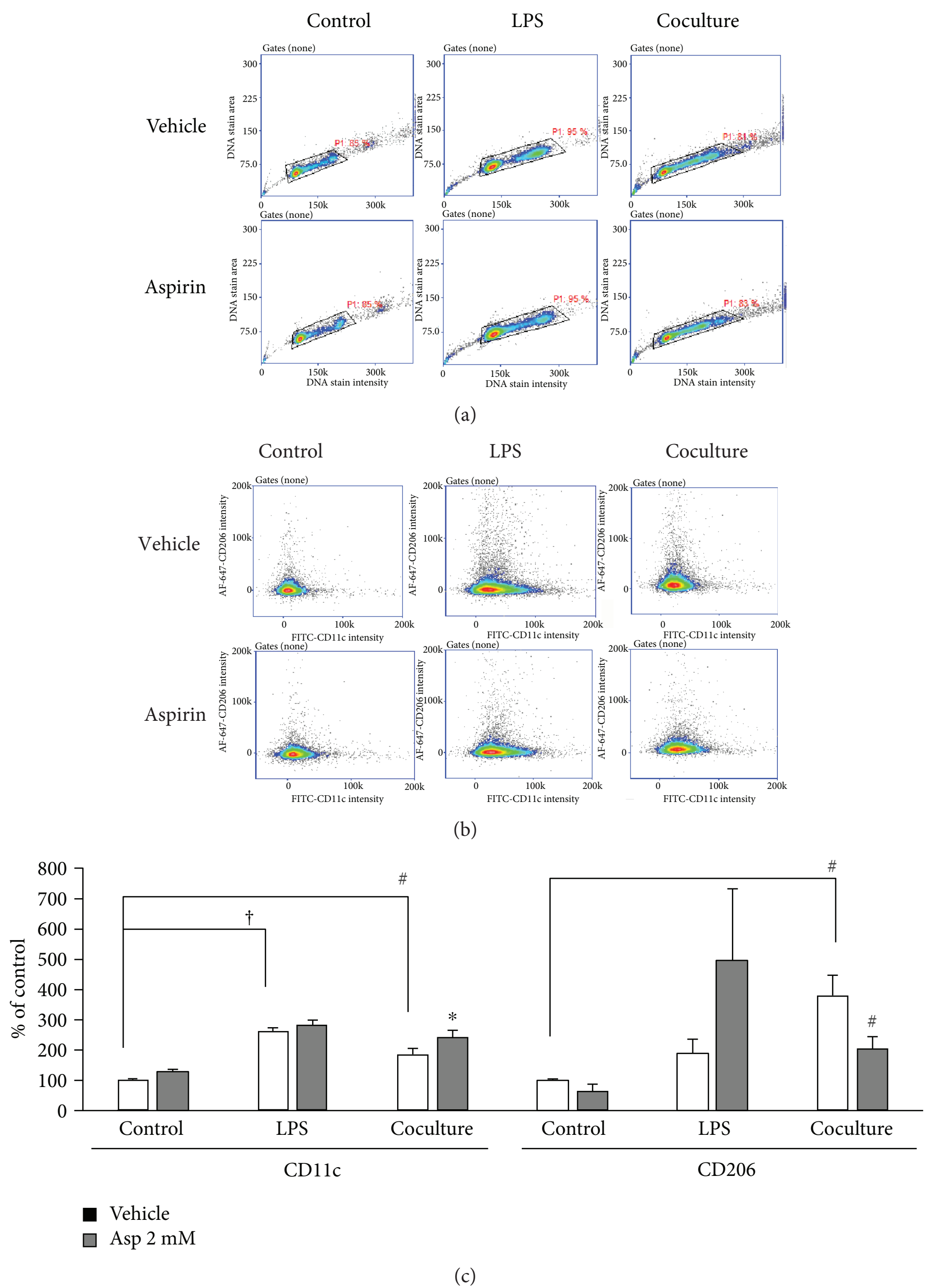

Figure 6: Effect of aspirin on M1 and M2 macrophage subtypes following LPS stimulation and coculture with 4T1 cells. Macrophages were incubated in the presence or absence of aspirin for 72 h and cultured in either fresh medium as a control, the presence of LPS for the last 24 h of the incubation, or cocultured with 4T1 cells for 72 h. (a) Histogram plots, (b) fluorescent intensity plots, and (c) quantitative data were presented. The immunofluorescent intensity of CD11c (M1) and CD206 (M2) on macrophages was analyzed using a NC-3000. Data are shown as mean ± SEM. Statistical analysis was performed using one-way ANOVA and LSD post hoc tests. The comparisons between different culture mediums were done by t-tests. Statistically significant differences are indicated as $^{*}p < 0.05$, $\#p < 0.01$, and $\dagger p < 0.001$ versus vehicle control.

Figure 5(b), when the 4T1 cells were cultured in fresh medium as a control condition, aspirin treatment significantly decreased MCP-1 ($p = 0.001$), PAI-1 ($p = 0.019$), and IL-6 ($p < 0.001$) levels and slightly decreased VEGF level ($p = 0.063$). As shown in Figure 5(c), when the 4T1 cells were cultured in 50% RAW-CM, aspirin treatment only decreased MCP-1 and PAI-1 production ($p < 0.001$ and $p = 0.004$, resp.).

3.4. Aspirin Regulated Macrophage Subtypes in Cocultures of Breast Cancer Cell and Macrophage. We determined whether aspirin treatment affects M1 and M2 macrophage

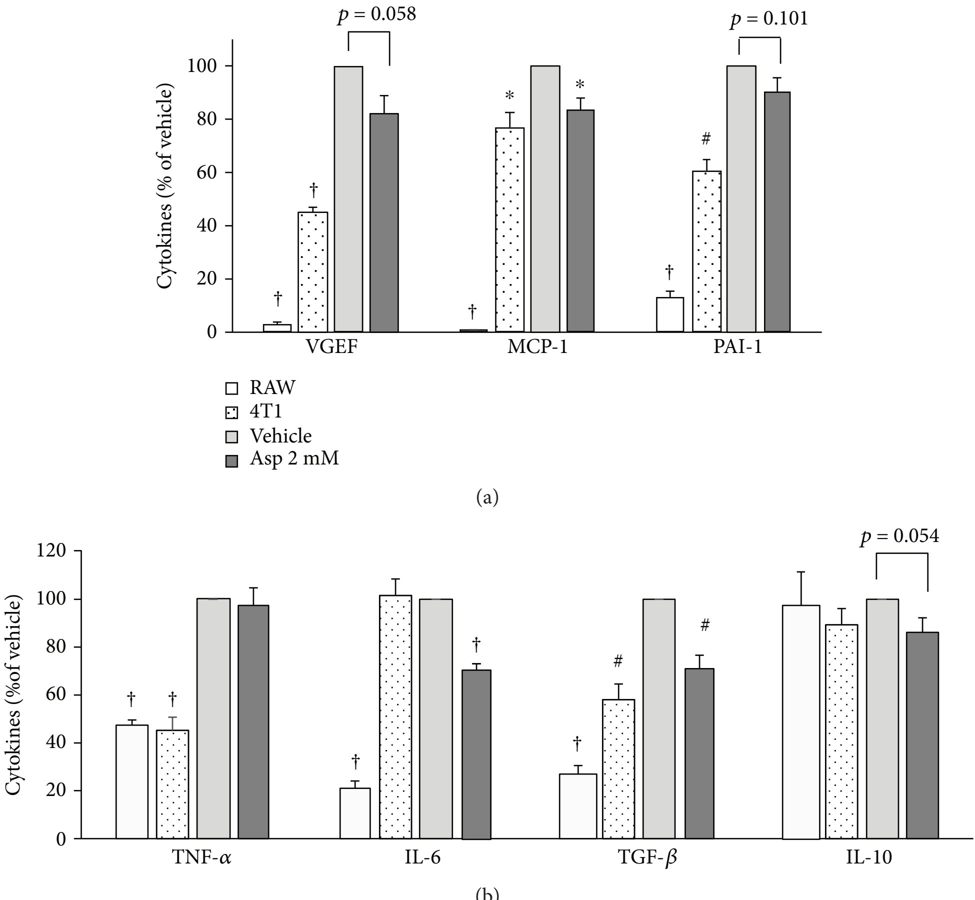

Figure 7: Aspirin inhibited angiogenic and inflammatory cytokines in supernatants of 4T1 and RAW 264.7 cell cocultures. 4T1 cells were cultured in the presence of macrophages for 72 h, supernatants were collected, and cytokine levels relative to tumor characteristics were measured by ELISA. (a) Angiogenic cytokines VEGF, MCP-1, and PAI-1. (b) Inflammation-related cytokines TNF-α, IL-6, TGF-β, and IL-10. The blank bar indicates RAW 264.7 cells only, the dotted bar indicates 4T1 cells only, the gray bar indicates cocultures containing both cells, and dark gray indicates cocultures treated with 2 mM aspirin. Data are shown as mean ± SEM. Statistical analysis was performed using independent sample t-tests. Statistically significant differences are indicated as $^{*}p < 0.05$, $^{\#}p < 0.01$, and $^{\dagger}p < 0.001$ for treatment versus co-control vehicle.

subpopulations based on surface marker expression. Cluster of differentiation (CD)11c is a marker of M1 macrophages, while CD206 is a marker of M2 macrophages. RAW264.7 cells were cultured in control medium, LPS-stimulated RAW-CM, or cocultured with 4T1 cells and then characterized. Histograms and fluorescence intensity plots are presented in Figures 6(a) and 6(b), while quantitative data is presented in Figure 6(c). CD11c expression increased by 181% in RAW 264.7 cells following LPS stimulation ($p < 0.001$), but CD206 marker expression was not affected. When RAW 264.7 cells were cocultured with 4T1 breast cancer cells, CD206 expression significantly increased by 281% ($p = 0.002$). After treatment with aspirin, CD11c significantly increased by 32% ($p = 0.012$) and CD206 decreased by 41% ($p = 0.046$) compared to the vehicle control in cocultured RAW 264.7 cells, suggesting aspirin altered the macrophage prolife when in the presence of neoplastic cells, but not the condition of LPS stimulation (Figure 6(c)).

3.5. Aspirin Inhibits Crosstalk and Production of Carcinogenesis-Related Cytokines in Cocultures of Breast Cancer Cell and Macrophage. To further confirm the production of potential mediators of interactions between cells in culture supernatants, 4T1 and RAW 264.7 cells were cocultured together to mimic the physiology of the tumor microenvironment. Cytokine levels are listed in Supplementary 2, and the data are presented relative to the vehicle control in Figure 7. Cytokine levels relative to tumor characteristics were assessed for VEGF, MCP-1, PAI-1, TNF-α, IL-6, transforming growth factor- (TGF-) β, and IL-10 by ELISA at the end of 72 h of coculture. There were only very low levels of VEGF, MCP-1, PAI-1, TNF-α, and TGF-β in the individual RAW 264.7 or 4T1 cell supernatants. When both cell types were present and treated with 2 mM aspirin, there was significant inhibition of MCP-1, IL-6, and TGF-β ($p = 0.019$, $p < 0.001$, and $p = 0.008$, resp.), and trending decreases in VEGF, PAI-1, TNF-α, and IL-10 ($p = 0.058$, $p = 0.101$, $p = 0.058$, and $p = 0.054$, resp.).

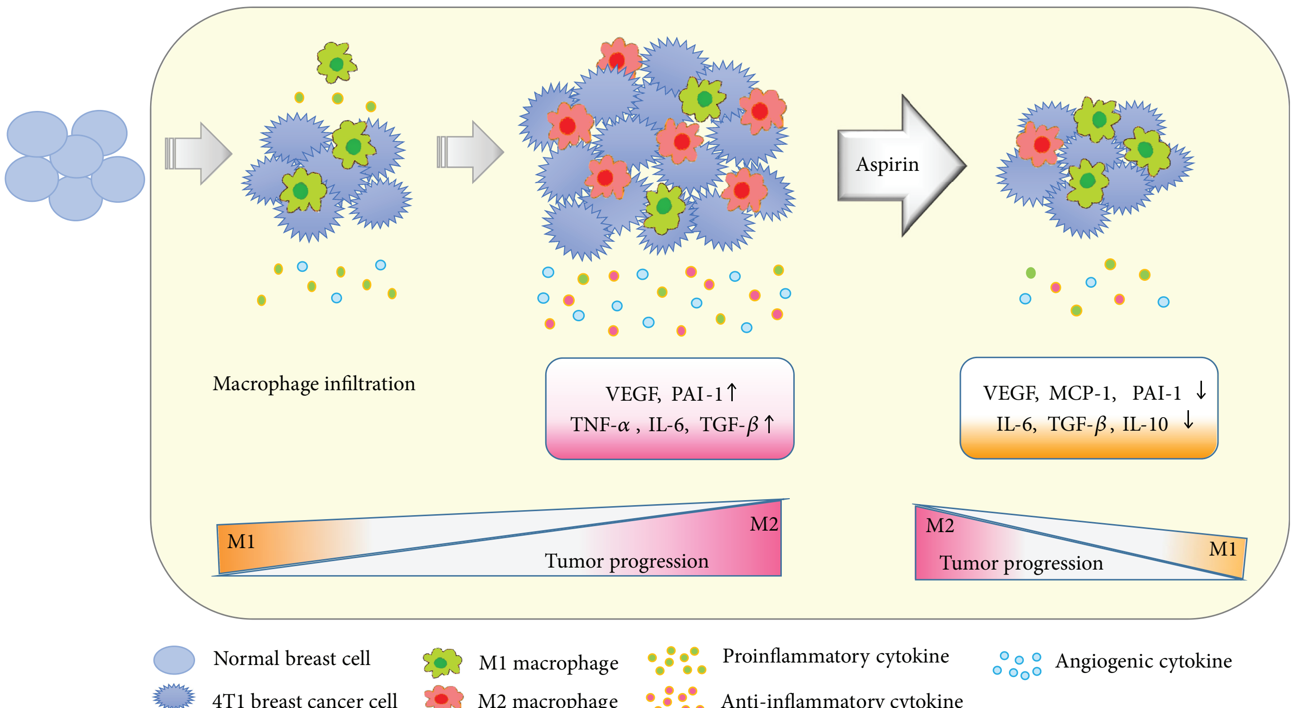

Figure 8: The schema of possible mechanism of chemoprevention of aspirin. In 4T1 breast cancer cell environment, RAW264.7 macrophage infiltration increased VEGF, PAI-1, TNF-α, IL-6, and TGF-β levels, and M2 macrophage expression, resulting to, benefit to tumor progression. Aspirin treatment decreased angiogenic and inflammation-associated cytokine VEGF, PAI-1, MCP-1, IL-6, IL-10, and TGF-β production. In addition, treatment of aspirin increased M1 expression and decreased M2 expression in macrophages, resulting to interference of the communication in this microenvironment and blunted tumor progression.

The effects of aspirin treatment in the coculture model were apparent compared to the RAW-CM model, suggesting cocultures containing both types of cells can effectively cross-talk. These data indicate that aspirin disrupted secretion of mediators associated with carcinogenesis in both RAW-CM and cocultures. A schematic of factors with a possible active role in aspirin treatment is proposed in Figure 8.

4. Discussion

Breast cancer is the most prevalent malignant tumor currently found in women. The breast tumor microenvironment includes neoplastic, neighboring stromal, and recruited immune cells, such as macrophages and lymphocytes, where crosstalk among these cells is involved in tumor progression and metastasis [2]. Interestingly, macrophages, the most abundant immune cell type present in solid tumors, infiltrate and secrete many cytokines while neoplastic cells form. This creates chronic inflammation that provides conditions in this microenvironment conducive to tumor development and angiogenesis [17, 18].

The breast cancer cell line 4T1 is triple-negative, which is a form of breast cancer associated with a poor prognosis because the cells lack effective therapeutic targets, behave aggressively, and are accompanied with overexpression of inflammation-related mediators [15]. This has motivated scientists to identify effective agents against this type of cancer. In this present study, aspirin was determined to be a potential chemopreventive agent with antiangiogenic and anti-inflammatory properties in a tumor microenvironment created using RAW-CM and cocultures of RAW 264.7 macrophages and 4T1 breast cancer cells. The results of the present study suggest aspirin interfered with crosstalk between these two cell types and, thus, inhibited cancer cell growth and migration.

Normally, macrophages have a critical role in host defense that involves connecting innate and adaptive immune responses, as well as tissue repair. Macrophages secrete multiple cytokines that participate in inflammatory responses, tissue damage, pathogen clearance, tissue homeostasis, and disease development [19, 20]. LPS, that is, bacterial endotoxin, is a common agent that activates macrophages involved in the innate immune response and causes immune cell infiltration and inflammation [21, 22]. A number of studies have shown that endotoxin may be anticarcinogenic, possibly due to its ability to recruit and activate immune cells and proinflammatory mediator production [22]. Tumorigenesis accompanies macrophage infiltration. Therefore, RAW-CM may mimic the microenvironment associated with chronic disease, including the presence of multiple inflammatory mediators [17]. In the RAW-CM model, LPS stimulation triggered RAW 264.7 cells to undergo an acute inflammatory response and, thus, inhibit 4T1 cell growth and migration, which is consistent with other evidence. LPS activates TLR4 signaling in tumor cells, leading to tumor evasion from immune surveillance and tumor growth delay

[23]. Meanwhile, unstimulated RAW-CM, which may mimic the tumor microenvironment, promoted 4T1 cell growth. This suggests that aspirin is a promising chemopreventive agent and it is not only anti-inflammatory but also anticarcinogenic. These anticancer properties have also been exhibited in human breast cancer MDA-MB-231 cells [24].

In a previously published study, mice were inoculated with 4T1 cells and implanted with sponge discs for 1 or 24 days to create acute and chronic inflammatory environments [25]. Tumor progression and circulating levels of VEGF and TNF-α were greater in the presence of chronic inflammation than acute inflammation. In addition, VEGF and TNF-α molecules are critical for the proliferation, angiogenesis, macrophage recruitment, and metastasis associated with tumor progression [25]. Populations of macrophages, dendritic cells, and lymphocytes were significantly larger in mice with chronic inflammation [25], suggesting that chronic cell infiltration is important for tumor progression. In an obesity-related breast cancer study, 4Tl cell proliferation was significantly observed when cells were cultured in adipocyte-conditioned medium without any stimulation, indicating that spontaneous adipocyte infiltration contributed to 4T1 cell growth [16].

Our previous study demonstrated that aspirin treatment significantly inhibits the proliferation and migration of 4T1 cells, as well as causes an associated decrease in MCP-1 and VEGF production [26]. In this present study, PAI-1 and IL-6 production by 4T1 cells was also inhibited by aspirin treatment. In the RAW-CM model, VEGF, PAI-1, TNF-α, and IL-6 production by 4T1 cells significantly increased, indicating there are carcinogenic mediators in the RAW-CM. After aspirin treatment, production of MCP-1 and PAI-1 decreased, suggesting that aspirin interfered with interactions between macrophages and breast cancer cells and, thus, inhibited tumorigenic signals. Moreover, in an obesity-related breast cancer study involving 4T1 cells cultured in 3T3-L1 adipocyte-conditioned medium and cocultured with adipocytes, aspirin decreased the production of MCP-1 and PAI-1 [26]. This is consistent with the data from this present study, supporting that these two cytokines have important roles in immune cell recruitment and tumor progression.

MCP-1, that is, CCL-2, is a chemokine that recruits and activates monocytes during inflammation. In tumor progression, MCP-1 plays an important role through facilitation of macrophage infiltration, which is involved in tumor progression and immunosurveillance [27, 28]. In addition, a previous study reported that blocking MCP-1 signaling notably inhibited 4T1 cell migration [29]. PAI-1 is produced by multiple cells and is involved in several pathological conditions, including aging, obesity, and inflammation, and high levels have been demonstrated to accompany tumor progression [30]. Recently, TGF-β-treated endothelial cells were reported to induce PAI-1 secretion and promote metastasis of triple-negative breast cancer cells [31], illustrating the potential of PAI-1 as a target of breast cancer therapies. In addition, IL-6 and TNF-α are conductor cytokines that mediate and have multiple physiological functions in various pathogenic inflammatory diseases, where they are involved in tumor progression, angiogenesis, and migration [32]. Recently, it was revealed that proinflammatory cytokines in serum, such as IL-6, IL-8, and TNF-α, are associated with clinical stage and lymph node metastasis in breast cancer patients [32]. The levels of these cytokines are associated with the course of breast tumorigenesis, and, thus, these cytokines have potential as prognostic cancer biomarkers.

In this present study, aspirin suppressed MCP-1, PAI-1, and IL-6 production by 4T1 cells cultured in fresh medium and RAW-CM, suggesting to inhibit proliferation and migration of breast cancer cells. In the coculture model, treatment with aspirin significantly inhibited MCP-1, IL-6, and TGF-β and slightly inhibited VEGF, PAI-1, TNF-α, and IL-10 production. Production of these inflammatory and angiogenic mediators by 4T1 cells in fresh medium, RAW-CM, and coculture models was blocked by aspirin. On the basis of these results, the suppressive properties of aspirin interfere with community-associated factors in the breast tumor microenvironment. In addition, aspirin may also act through other pathways to exert its chemopreventive properties involving inflammation, cyclooxygenase- (COX-) 2, platelets, hormones, or PI3 kinase [33]. One of the most studied aspirin anticancer mechanisms is the partially downregulated COX-2 expression in many types of breast cancer cells, including MCF-7, MDA-MB-231, and SK-BR-3, contributing to inhibition of cancer cell proliferation [34].

Macrophages can divide into two distinct phenotypes of M1 and M2. M1 macrophages are promoted by T-helper cell type 1 (Th1) cytokines and produce proinflammatory cytokines that evoke an adaptive immune response. Meanwhile, Th2 cytokines polarize monocytes into M2 macrophages that promote angiogenesis, clean injured tissues, and suppress adaptive immune responses [7]. Imbalances in M1 and M2 macrophage populations may lead to pathological changes [35]. It has been demonstrated that mice that received 7,12-dimethylbenz(a)anthracene chemical carcinogens have higher F4/80+ macrophage recruitment in perigonadal adipose tissue compared to mice that did not receive any carcinogen, especially, the higher level of CD11c + M1 type [36]. In the present study, there was a significant increase in M2 cells when RAW 264.7 cells were cocultured with 4T1 cells, suggesting that this suppressive microenvironment promoted the growth of breast cancer cells. In the tumor microenvironment, malignancies recruit circulating monocytes that have differentiated into TAMs. TAMs resemble M2 macrophages and exert protumor functions through immunosuppressive actions [5]. Therefore, modifications, such as through suppression of TAM recruitment, switching of the TAM phenotype, and production of associated mediators, have been proposed as cancer therapeutic strategies [37].

Interestingly, aspirin treatment increased M1 marker expression, but decreased M2 marker expression in cocultures of the present study, suggesting that aspirin influences the macrophage profile in the neoplastic microenvironment away from a suppressive immune response, thus contributing to breast cancer cell suppression. Recently, it was demonstrated that macrophage phenotypes are regulated by aspirin

in a model of RAW 264.7 cells cultured in pancreatic cancer cell line Panc02-conditioned medium. Aspirin significantly decreased protein and RNA levels of the M2 marker CD206 and prevented pancreatic carcinogenesis [38]. Burnett and colleagues reported that aspirin upregulates IL-10 gene expression in THP-1 cells, but not in cocultures of MCF-7 and THP-1 cells [39]. In a clinical trial on breast cancer patients, TGF-β expression was lower during the early stages of disease, but higher and associated with CCL2 levels during late stages. Moreover, TGF-β stimulated CCL2 expression and then induced monocytes/macrophages to secrete Th2-attracting chemokines into a breast cancer MDA-MB-231 cell tumor microenvironment [40]. In the present study, aspirin inhibited TGF-β expression in the coculture model, resulting in decreases in MCP-1 production and Th2 accumulation that dampened downstream communication in the microenvironment.

Clinical trials have revealed that aspirin is an effective chemopreventive agent. Observational studies have shown that regular aspirin use reduces the incidences of several cancers, as well as distant metastases of these cancers [41]. Meta-analyses and systematic reviews have also proposed that aspirin's chemopreventive properties can be used to fight breast cancer [13, 14]. In cardiovascular subjects of five large randomized trials, aspirin use decreased the risk of cancer mortality and metastases [33]. Recently, a larger cohort study that included 13 prospective studies with 857,831 subjects revealed that long-term (>5 years) regular use of aspirin 2 to 7 times/week prevented breast cancer [42]. Based on previous findings, regular use of aspirin (75 to 350 mg/day) reduces the incidence of and mortality from breast cancer in epidemiologic experiments [13, 14, 33, 42]. Researchers need to pursue a comprehensive understanding of aspirin treatment-associated issues, such as gastrointestinal side effects, optimal doses, duration, and combinations with other compounds, to facilitate the use of aspirin as a cancer therapy.

5. Conclusions

Based on accumulating evidence, macrophages play a crucial role in the tumor microenvironment, which includes intricate crosstalk involving a series of inflammatory chemokines and cytokines and angiogenic mediators secreted from neoplastic cells and infiltrating macrophages. The findings of this study indicate that aspirin has chemopreventive properties that function through both 4T1 breast cancer cells and macrophages. Aspirin interfered with the connection between various cells by decreasing communication through proinflammation and angiogenic mediators and modulating M1/M2 macrophage subtypes, suggesting that aspirin is a promising agent to prevent tumor progression.

Data Availability

The data used to support the findings of this study are all provided in the manuscript and supplementary file.

Conflicts of Interest

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

Acknowledgments

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Supplementary Materials

Supplementary 1: effect of aspirin on carcinogenic cytokine production by 4T1 breast cancer cells cultured in control medium and RAW-CM. Supplementary 2: aspirin inhibited angiogenic and inflammatory cytokines in supernatants of 4T1 and RAW 264.7 cell cocultures. *(Supplementary Materials)*

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