

**INFLAMMATORY MECHANISMS OF CHEMOKINE RECEPTOR 7 EXPRESSION IN
METASTATIC SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN)**

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The microenvironment of aerodigestive cancers contains tumor promoting inflammatory signals often involved in innate immunity. SCCHN is an epithelial malignancy characterized by the secretion of inflammatory mediators that can promote tumorigenesis and lymph node metastasis. The chemokine receptor CCR7 is a key molecule whose aberrant expression in SCCHN has been linked to pro-survival, invasive and metastatic pathways. Indeed, the selective upregulation of CCR7 in metastatic SCCHN tumors has been previously described. However, the mechanisms of CCR7 expression have not yet been elucidated. Inflammatory cytokines are known to upregulate CCR7 in immune cells through downstream NF- κ B dependent mechanisms. In addition, antimicrobial peptides such as human β -defensin 3 (HBD3) are capable of promoting an inflammatory microenvironment and may possess tumor-promoting properties. Given the frequent overexpression NF- κ B in SCCHN and its association with a more aggressive SCCHN phenotype, I hypothesized that NF- κ B may be a key mediator of invasive and metastatic disease by promoting CCR7 expression in SCCHN tumors. Indeed, I identified and studied four potential NF- κ B binding sites in the promoter region upstream of the CCR7 gene and report on their relative contribution to CCR7 expression in metastatic SCCHN. Furthermore, I demonstrate that HBD3 induces CCR7 expression in dendritic cells as well as primary SCCHN tumors in an NF- κ B-dependent fashion. Interestingly, HBD3 stimulation provides anti-apoptotic signals to SCCHN cells, as evidenced by tumor resistance to cisplatin-induced cell death.

As presented in this dissertation, these findings suggest that HBD3 represents a novel, NF- κ B-regulated mediator of CCR7 expression and anti-apoptotic pathways, which may be exploited by developing SCCHN tumors to enhance their growth, survival and evolution into a metastatic phenotype. NF- κ B appears to be a key regulator of basal and inducible CCR7 expression. The observed NF- κ B induction of CCR7 and its subsequent downstream pathways provide clinically important therapeutic targets to control the progression and metastasis of SCCHN tumors.

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1.0 INTRODUCTION

1.1 SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

1.1.1 EPIDEMIOLOGY

Over 90% of tumors in the head and neck region are squamous carcinomas of epithelial origin and include carcinomas of the oral cavity, pharynx and larynx. Worldwide, SCCHN is a significant public health concern with over 500,000 new cases annually (1). In the US, SCCHN is the sixth most common malignancy with over 40,000 new cases diagnosed annually. Patients often present with advanced stage disease and despite advances in combinatorial therapy the prognosis remains poor. The average 5-year survival rate is only 30-50% primarily due to the presence of multiple primary cancers and lymph node metastases (2). Therefore, early detection of head and neck cancers is essential for improved prognosis.

Chronic tobacco and alcohol use remain the most important risk factors for developing SCCHN. Studies have shown that 85% of head and neck cancers are associated with tobacco use. Smokers and drinkers face an additive risk that is up to 40 times greater than in those who neither smoke nor drink (3). Furthermore, mutations in p53 correlate significantly with smoking and drinking habits (4). In addition, environmental factors such as viral infection have also been implicated in SCCHN pathogenesis. Epstein-Barr Virus (EBV) infection is associated with nasopharyngeal carcinoma (5). Human Papilloma Virus (HPV) type 16 and 18 infections are implicated in the development of oropharyngeal carcinomas (6, 7). Indeed epidemiologic studies attribute the increased incidence of oral cancers to increasing prevalence of HPV infections (8). Other environmental factors that increase the risk of developing head and neck carcinoma include exposure to wood dust, asbestos, paint and gasoline fumes (9).

1.1.2 CLINICAL FEATURES & PATHOLOGY

Head and neck carcinomas develop from the squamous epithelium and present as precancerous or cancerous lesions that are typically asymptomatic. However, when the lesions develop into palpable masses, symptoms may occur such as ulceration of oral mucosa, persistent sore throat, hoarseness, difficulty swallowing, or ear infection. Studies show that the progression of head and neck cancer is a multistep process from normal histologic features to hyperplasia, dysplasia, invasive carcinoma, and eventual metastasis. At each stage of progression, underlying genetic instabilities promote the amplification or deletion of oncogenes and tumor-suppressor genes (including p53, cyclin D1, EGFR, COX2, VEGF, MMPs, CXCR4, and CCR7) all of which are involved in the process of transformation and malignancy (10).

Studies show that the most important prognostic factors for SCCHN are the presence of locoregional metastasis, vascular or lymphatic invasion and the extracapsular spread of tumor cells into the neck tissue (11). These are typically late stage disease markers, emphasizing the need for early detection. However, a significant proportion of patients present nodal metastasis at diagnosis, thereby highlighting the need to understand the underlying mechanisms of tumor progression in SCCHN and identify new predictive and prognostic markers that can form the basis for clinical therapeutic targets.

The metastasis of tumor cells is a complicated multi-step process involving several mediators between tumor cells and their surrounding microenvironment (12). In SCCHN, the predictable pattern of lymph node metastasis has led to an interest in examining the role of chemokine receptors in facilitating the observed organ-specific homing. Previous reports have identified CXCR4 and CCR7 as two key chemokine receptors expressed by SCCHN tumors that may be involved in facilitating tumor metastasis (discussed in chapter 1.2.3). The schematic in Figure 1-1 represents our understanding of CCR7-directed lymph node metastasis in SCCHN.

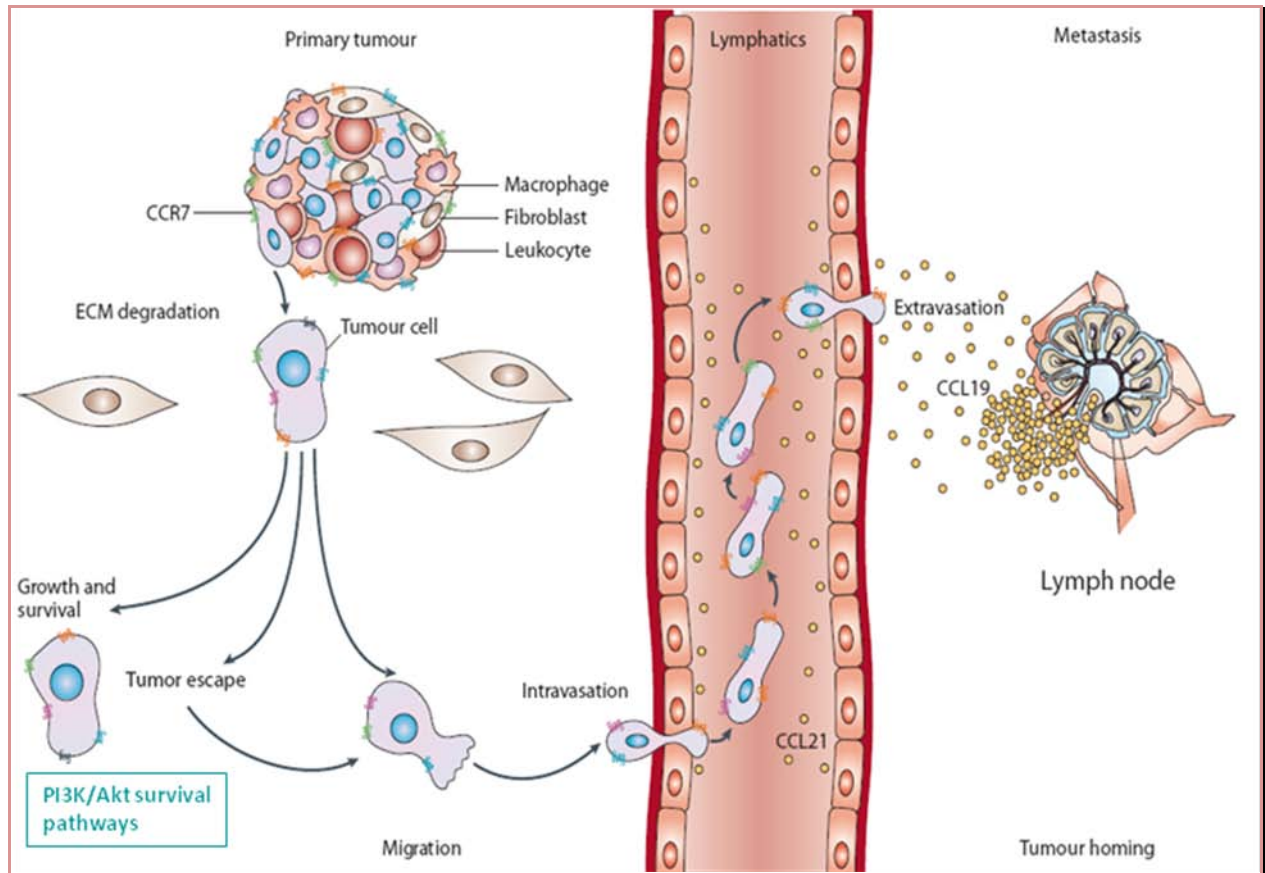


Figure 1-1: CCR7-directed lymph node metastasis in SCCHN. Metastasis is a process that is highly dependent on the interactions between tumor and the surrounding microenvironment. A tumor growing at the primary site engages in productive interactions with an inflammatory cell infiltrate that may include leukocytes and macrophages. The release of inflammatory cytokines and peptides (such as defensins – discussed in chapter 1.3) can potentially upregulate CCR7 expression on tumor cells. Subsequent processes of tumor proliferation and survival, inflammatory cytokine secretion, extracellular matrix degradation and tissue remodeling result in the escape of the CCR7 expressing tumor from the primary tumor bed. Interstitial CCR7 signaling propels the tumor cell to migrate directionally toward its chemoattractants (CCL19 and CCL21) which are highly expressed within the lymphatic epithelium and lymph nodes. This chemotactic migration of CCR7-positive tumor results in secondary metastases in the lymph nodes. (Schematic adapted from Nature Reviews Cancer, 2007: 7, 79-94)

1.1.3 THERAPEUTIC STRATEGIES AGAINST SCCHN

A major hurdle of cancer therapy is maximizing the treatment outcome while minimizing collateral damage to surrounding tissues. In the anatomically complex head and neck region, this is particularly challenging since the cancer and its treatment affect critical organs and functions, including speech, swallowing, breathing and facial appearance. As previously mentioned, majority of SCCHN patients present with locally advanced disease with some nodal involvement. Often this requires combination therapy that includes surgical resection, radiotherapy, chemotherapy and/or targeted agents.

Surgery is most effective for early stage, low-grade tumors. Surgical techniques have advanced over the last several years to minimize invasiveness while maintaining complete tumor removal. Surgery also provides prognostic information such as histology and nodal involvement which is helpful in choosing a secondary form of therapy. Often radiation therapy is combined with surgery or chemotherapy for stage III – IV tumors as this combinatorial therapy has been shown to maintain locoregional tumor control as well as organ preservation (2). Recently, targeted therapies using agents such as monoclonal antibodies (cetuximab, panitumumab) or tyrosine kinase inhibitors (erlotinib) in combination with radiotherapy have found widespread use as first line therapy (13, 14). For patients with recurrent unresectable metastatic disease, curative therapy may no longer be an option. Therefore palliative care through control of symptoms as well as cytotoxic drugs (cisplatin, paclitaxel) and systemic chemotherapy may achieve measurable tumor reduction (15).

Unfortunately, despite the advancements in these treatment modalities, head and neck cancers have a high mortality rate. Abstinence from tobacco and alcohol use is still the best approach to prevent disease occurrence. Advances in detection techniques may enable clinicians to diagnose and treat these tumors earlier thereby improving outcome. Furthermore, improving our understanding of the mechanisms of tumor progression and metastasis remains the cornerstone to developing new therapeutic options for the treatment of SCCHN.

1.2 INFLAMMATION & CANCER

1.2.1 INTRODUCTION

Over the course of evolution, the immune system has tailored its response to protect its host against infection and tissue damage by recognizing danger signals from either exogenous sources (invading pathogens) or endogenous sources (cytokines, heat shock proteins, reactive oxygen intermediates – that are released by injured tissues undergoing stress, damage or abnormal death). When tissues are wounded, danger signals are released mobilizing cells of the immune system to route towards a localized region of tissue damage and mount an inflammatory response that is generally responsible for clearing the original insult. Mechanisms of cell proliferation and angiogenesis are enhanced in order to facilitate tissue regeneration and homeostasis. These proliferative and acute immune responses are only resolved after the insulting agent has been cleared and tissues repaired. Therefore, controlling the extent of this immune response is critical for successful pathogen clearance as well as maintaining tissue integrity. If the immune response is inadequate, the infection/injury prevails and may eventually kill the host. On the other hand, if the immune response persists due to an inexorable insulting agent, then excessive proliferation and chronic inflammation ensues, which can result in chronic disorders, autoimmunity and malignant transformation of affected tissues (16). Indeed, reports by Haddow and Dvorak that wound healing and carcinogenesis involve similar processes controlled by growth factors, angiogenic signals and inflammatory agents have shaped the notion that tumors are wounds that do not heal (17, 18).

Historically, the apparent association between inflammation and cancer was first reported by Rudolf Virchow in 1863 when he observed the presence of leukocytes within tumors and suggested that the neoplasms arose at the sites of chronic inflammation (19). For over a century, this hypothesis was

abandoned but it has gained resurgence in the last decade as our understanding of the interactions between tumors and their surrounding inflammatory immune infiltrate has advanced. Based on several lines of evidence and characterization of the cellular and molecular pathways involved, a role for inflammation in tumor development is now widely accepted. Epidemiological studies have revealed that chronic infections and inflammation increases predisposition towards various cancers (20). For instance, *Helicobacter pylori* infection increases the risk for gastric adenocarcinoma and gastric mucosal lymphoma; inflammatory bowel disease is associated with increased risk for colon cancer; prostatitis is associated with prostate cancer; chronic pancreatitis is associated with pancreatic adenocarcinoma; and papilloma and hepatitis viruses are associated with cervical and hepatocellular carcinomas respectively (21-23). In addition, population-based studies have confirmed that the use of anti-inflammatory agents (such as aspirin, cyclooxygenase-2 inhibitors, and non-steroidal anti-inflammatory drugs) decreases the incidence of several cancers (24-28). As discussed later in this chapter, the recruitment and activation of inflammatory cells, cytokines and chemokines is observed in all malignancies, and their therapeutic targeting has been found to decrease the incidence and spread of cancer. In fact, chronic inflammation plays a role in tumorigenesis, including cellular transformation, promotion, survival, proliferation, angiogenesis, invasion and metastasis. Thus, there is now substantial evidence in the literature supporting the link between inflammation and cancer.

Since inflammation typifies a host defense mechanism against infection and injury, the presence of a rich immune cell infiltrate at tumor sites was generally thought to represent an attempt by the immune system to eradicate malignant cells. However, the finding that leukocytes can promote, rather than restrict, tumor development is somewhat paradoxical and highlights the complex nature of the interactions between immune cells and developing tumors (29). This chapter examines the cellular and molecular mechanisms that link inflammation and cancer and how these pathways suppress effective anti-tumor immunity.

1.2.2 INFLAMMATORY CYTOKINES IN CANCER

Cytokines are small immunomodulatory molecules secreted by leukocytes that are responsible for cellular signaling and communication. They represent a key component of the inflammatory response as they are capable of enhancing both pro- and anti-inflammatory responses. Indeed, cytokine signaling influences the very processes that are dysregulated in cancers, including cell proliferation and survival, angiogenesis and tissue remodeling, invasion and metastasis, and the subversion of adaptive immunity (30).

Tumor Necrosis Factor α (TNF α) is the best studied pro-inflammatory cytokine that plays a major role in carcinogenesis. Ironically, it was originally identified as a cytokine that induced the hemorrhagic necrosis of tumors, hence its name (31, 32). However, later studies confirmed that TNF α is a pleiotropic cytokine with regulatory functions in immune and inflammatory processes, including pro-tumorigenic capabilities (33-35). The pro-tumoral functions of TNF α stem from its abilities to promote the processes of leukocyte recruitment, tumor growth, angiogenesis, invasion and metastasis. Studies show that tumor-derived TNF α promotes local tumor growth, enhanced proliferation and reduced cell death (36). Indeed, TNF α has been shown to be secreted by a wide variety of tumor cells, including B cell lymphoma, cutaneous T cell lymphoma, breast carcinoma, colon carcinoma, lung carcinoma, pancreatic carcinoma, squamous cell carcinoma and ovarian carcinoma (37-43). There is also evidence suggesting that TNF α secretion by tumor associated macrophages (TAM) promotes tumor invasiveness by inducing matrix metalloproteinases, which are involved in remodeling of the extracellular matrix. Moreover, TNF α -induced matrix degradation releases vascular endothelial growth factor (VEGF), a key angiogenic factor required in the vascularization of tissues (44-47). Studies show that chronic TNF α exposure promotes tumor development in gastric lesions and colonic mucosa of patients with *H. pylori* infection and inflammatory bowel disease, respectively (48, 49). In an experimental fibrosarcoma metastasis model, the expression of endogenous and exogenous TNF α was found to enhance metastasis (50). Further evidence that TNF α has a carcinogenic role has come from studies demonstrating that mice deficient in TNF α or TNF α receptor are resistant to skin, liver and colorectal carcinogenesis (51-55). TNF α antagonists have

been tested in the therapy of various human malignancies including ovarian, renal cell and pancreatic carcinomas (56-58). Collectively, the findings show that the expression of TNF α confers an invasive, transformed phenotype to tumor cells, and the inhibition of TNF α signaling significantly reduces tumor progression.

The expression of various inflammatory interleukins within the tumor microenvironment has also been linked to carcinogenesis. These include IL-1, IL-6, IL-8 and IL-17. Studies show that melanoma, breast and prostate cancer tumors grown in IL-1 β -deficient mice did not metastasize, suggesting that IL-1 β promotes angiogenesis, invasion and metastasis of these tumors (59). Using mouse models and human tumors, IL-1 β was found to induce angiogenesis and production of matrix metalloproteinases thereby sustaining new blood vessel formation, tumor growth and invasion (60-62). In pancreatic carcinoma, the autocrine secretion of IL-1 β promotes growth and confers chemoresistance (63). Besides its direct effects on the survival and proliferation of endothelial cells, IL-1 β can also promote the induction of other proangiogenic factors, including TNF α , angiopoietin 1 and VEGF A (64, 65). Autocrine and paracrine IL-6 has been shown to enhance cell proliferation and growth of multiple myeloma, non-Hodgkin's lymphoma, colorectal carcinoma and renal cell carcinoma (66-69). In addition, increased levels of IL-6 have been detected in the serum and tumor biopsies of patients with colorectal and gastric carcinomas (70, 71). In tumors characterized by oncogenic ras mutations, IL-6 has been shown to promote angiogenesis and tumor growth (72). The expression of IL-8 (also known as CXCL8) by melanoma and ovarian carcinomas correlates with disease progression and metastatic potential (73-75). The secretion of IL-17 in the tumor microenvironment promotes tumor growth, survival and angiogenesis (76, 77).

1.2.3 INFLAMMATORY CHEMOKINE NETWORKS IN CANCER

Chemokines are small (8-14 kDa) proinflammatory “chemotactic cytokines” that mediate the selective recruitment and directional migration of leukocytes to inflammatory sites. They are typically induced by inflammatory cytokines, growth factors and pathogenic stimuli and signal through seven-transmembrane G-protein-coupled (chemokine) receptors (78). Chemokines (and their receptors) are structurally classified into four highly conserved groups, based on the position of the first two cysteine residues adjacent to the N-terminus (C, CC, CXC, CX₃C) (79). Recent studies show that chemokines and their receptors are expressed by several cell types, including neoplastic cells, where they control physiological and pathological processes. During inflammation-associated carcinogenesis, chemokines are involved in de novo tumorigenesis by promoting the recruitment of an inflammatory immune cell infiltrate, enhancing angiogenesis, tumor growth, invasion and metastasis (80, 81). Recent evidence shows that mice deficient in D6, a decoy scavenger receptor responsible for the resolution of inflammation by sequestering CC chemokines, have increased susceptibility to squamous cell and colitis-associated cancers (82).

1. *Regulation of tumor growth:* In melanoma, studies show that autocrine signaling of CXCL1, CXCL2, CXCL3 and CXCL8 promotes tumor growth and proliferation (83-85). Indeed, blocking the corresponding chemokine receptors to these ligands was found to attenuate melanoma cell proliferation (86). Interestingly, knockdown of CXCR4 expression or treatment with a CCL5 antagonist was found to inhibit growth of breast carcinomas (87, 88). In pancreatic carcinomas, CCL20 is overexpressed and not only stimulates growth of these tumors but also enhances the infiltration of tumor associated macrophages (89). CXCR2 and its ligands have been shown to have autocrine roles in the growth of pancreatic, head and neck, and non-small cell lung carcinomas (90-92). Autocrine signaling of CXCR4 induces proliferation of ovarian cancer cells through transactivation of the epidermal growth factor receptor (EGFR) (93).

2. *Regulation of angiogenesis:* Studies have established that chemokines within CXC family display contrasting angiogenic activity depending on the presence or absence of a three amino acid (Glu-Leu-Arg/ELR) motif on the N-terminus. CXC chemokines that are ELR⁺ display potent angiogenic activities and stimulate endothelial cell chemotaxis, whereas ELR⁻ chemokines are angiostatic (94). Thus, the net biological balance between the angiogenic and angiostatic chemokines regulates overall angiogenesis and neoplastic cell physiology. Studies have shown that ELR⁺ chemokines bind to CXCR1 and CXCR2, while the ELR⁻ chemokines bind to CXCR3, CXCR4 and CXCR5 (95). Stimulation by ELR⁺ chemokines mediates the upregulation of metalloproteinases that are involved in extracellular matrix degradation and release of angiogenic factors such as VEGF (96). The expression of ELR⁺ chemokines correlates with tumor angiogenesis in pancreatic, prostate, renal, ovarian and lung carcinomas (97-101). Interestingly, the expression of ELR⁻ chemokines is associated with inhibition angiogenesis and spontaneous tumor regression (95, 102-104).

3. *Regulation of invasion and metastasis:* The predilection of a tumor to metastasize depends not only on the characteristics of the neoplastic cells themselves, but also on the microenvironment of the target organ (105). The “chemoattraction theory” of metastasis postulates that malignant cells expressing functional chemokine receptors respond to organ-specific chemoattractant molecules to set up metastasis at new sites. Indeed, the observation that organs representing key sites of metastasis also happen to be abundant sources of the chemokine ligands for certain tumor-associated chemokine receptors lends support to this theory (106). Several lines of evidence support a role for chemokines in the invasion and metastasis of cancer. In breast cancer, CXCR4 and CCR7 are highly expressed and preferentially direct metastases to organs that are rich in their respective ligands (107). Transfection of melanoma tumors with CXCR4, CCR7 and CCR10 enhances tumor cell metastasis to the lung, lymph nodes and skin, respectively (108-110). The expression of CXCR3 has been associated with the pulmonary metastases of murine breast cancers, as well as the lymph node metastases of murine melanoma and colon cancer (111-114). The expression of CXCR5 and CCR6 in

colorectal carcinoma promotes liver metastases (115-117). In melanoma, the expression of CCR9 is associated with intestinal metastasis and CCR10 is linked to lymph node dissemination (118-120). Finally, recent studies show that the expression of CX3CR1 promotes bone metastases in prostate cancer, and perineural invasion in pancreatic adenocarcinomas (121, 122). Interestingly, the above studies also show that the neutralization of these chemokine receptor – ligand interactions results in decreased metastasis of tumors, suggesting that the targeting of this axis might be a useful therapeutic option in metastatic cancer (123).

1.2.3.1 Chemokine expression patterns in SCCHN

In oral squamous cell carcinomas (SCC), a high degree of invasion and metastases to the regional cervical lymph nodes is observed. The mechanism(s) of this site-specific metastasis has been the subject of numerous studies. Emerging evidence indicates that chemokines and their receptors play key roles in the proliferation, vascularization, survival and metastasis of these tumors, given that both the tumor stroma and the lymph nodes are rich sources of ligands for oral cancer tumor-associated chemokine receptors.

Of the chemokine ligands that have been identified in oral carcinomas, studies show that CXCL1 is overexpressed in 40% of the cases examined, and its expression correlates with tumor angiogenesis, leukocyte infiltration and nodal metastasis (124). Additionally, CCL2 expression in esophageal SCC is important for macrophage infiltration and the enhancement of tumor vascularity (125). When syngeneic primary and metastatic SCCHN tumors were compared by microarray analysis, studies show that CXCL5 and CXCL8 were significantly upregulated in the metastatic tumors (126). Selective downregulation of CXCL5 in these tumors resulted in decreased proliferation, impaired invasive and migratory capabilities *in vitro*, and decreased tumor formation *in vivo* (127). On the other hand, CXCL8 induced the upregulation of matrix metalloproteinase 7, which facilitates degradation of the basement membrane and enhances oral SCC invasion and migration (128).

Several groups have reported the expression of the chemokine receptors CXCR4 and CCR7 in SCCHN. Compared to normal epithelium, CXCR4 is upregulated in oral SCC and is associated with tumor progression and poor prognosis (129-131). When its expression was examined alongside a panel of clinicopathological factors, CXCR4 upregulation was linked to enhanced invasion, lymph node metastasis and tumor recurrence (132). Furthermore, transfection of CXCR4 into an oral SCC tumor cell line with poor metastatic potential generated a highly metastatic cell line that was found to frequently metastasize to the cervical lymph nodes of an orthotopic mouse model (133). Remarkably, the establishment of these nodal metastases could be inhibited by inhibitors to mitogen activated protein (MAP) kinase or phosphatidylinositol 3 kinase (PI3K), suggesting that the CXCR4/CXCL12 signaling axis was required. Indeed, CXCL12 (ligand to CXCR4) is frequently expressed in several oral SCC tumors and has pro-tumorigenic properties. These findings highlight the role of autocrine CXCR4-CXCL12 signaling in the acquisition of an aggressive metastatic phenotype. A recent study reported that the 5-year survival of SCCHN patients with CXCL12-positive tumors was significantly lower than that of patients with CXCL12-negative tumors (134). Moreover, when a CXCR4-positive tumor cell line was transfected to express CXCL12 and implanted into an orthotopic mouse model, it was found to seed more metastatic foci and heavier lymph node metastases than a CXCL12-negative tumor. Notably, use of a CXCR4 antagonist inhibited *in vitro* tumor mobility, significantly reduced metastatic nodules *in vivo* and prolonged survival of tumor-bearing mice. The CXCR4-CXCL12 signaling axis in SCCHN is also credited with increased cell adhesion, activation of matrix metalloproteinase 9, and enhanced tumor invasion, all characteristics that promote an aggressive metastatic phenotype (135, 136).

1.2.3.2 Role of CCR7 in SCCHN

The distinct migration pattern of oral cancers into regional tumor-draining lymph nodes raises a particular interest in examining the role of CCR7 and its ligands in metastasis. A recent study on dendritic cell (DC) migration indicates that CCR7 appears to be a stronger mediator of lymph node homing than CXCR4 (137). In fact, CCR7 expression has been associated with the lymph node metastasis of several malignancies including breast cancer, melanoma, gastric cancer, esophageal cancer and head and neck SCC (107, 109, 138-141). High CCR7 expression in esophageal SCC tumors has been correlated with clinicopathological characteristics such as tumor depth, TNM stage, lymphatic invasion and lymph node metastasis. As such, esophageal carcinoma patients with CCR7-positive tumors have significantly worse prognosis than those with CCR7-negative tumors (139, 140).

When the expression of various chemokines was examined in head and neck SCC cell lines and tumor tissue, CCR7 was found to be consistently upregulated in metastatic tumors compared to the autologous primary tumors (141). This study also showed that primary (non-metastatic) tumors were CCR6-positive while their metastatic counterparts were CCR6-negative. This receptor expression pattern is reminiscent of the DC response to inflammatory stimuli: peripheral immature DCs express CCR6 but lose this receptor upon antigen uptake and maturation, and upregulate CCR7 which facilitates emigration from the peripheral tissue sites and subsequent trafficking through the lymphatics into the lymph nodes. That these head and neck SCC tumors can mimic the differentiation and migration pattern of DCs is noteworthy and reflects the uncanny ability of tumors to co-opt existing immune mechanisms to foster their escape from immune recognition and progression into aggressive metastatic derivatives.

Interestingly, when CCR7 expression was examined in three highly metastatic subclones, derived from serial passages of metastases in a nude mouse model, studies show that the level of CCR7 expression was significantly increased in the metastatic derivatives as compared to their poorly metastatic parental cell line. These findings therefore suggest that CCR7 expression is quantitatively associated with increasing metastatic potential.

Following recent evidence that CCR7 expression can protect circulating CD8⁺ T cells from apoptosis (142), a study was undertaken to examine whether there was a similar protective effect in SCCHN tumors. Indeed, CCR7 expression in head and neck SCC was found to mediate pro-survival and invasive mechanisms through activation of PI3K pathways (143). Recent data suggest that CCR7-mediated pro-survival pathways may be responsible for the resistance of head and neck SCCs to platinum-based chemotherapies, thereby associating CCR7 expression with aggressive, recurrent tumors. Furthermore, these head and neck SCC were found to secrete CCL19 and CCL21, the ligands to CCR7, suggesting the existence of an autocrine (and paracrine) signaling loop that promotes tumor aggressiveness by maintaining the basal activation of survival and invasive pathways (144). Certainly, the elevated expression of the CCR7 ligands is correlated with the presence of CCR7-positive tumors (145, 146).

The *in vivo* role of the CCR7-CCL19/21 signaling axis was investigated using paucity of lymph node T cells (*plt*) mutant mice. These mice are spontaneously occurring null mutants for CCL19 and the lymphoid (serine) form of CCL21, thereby resulting in an inability to recruit CCR7-positive cells to the secondary lymphoid organs (147, 148). When a CCR7-positive murine oral SCC cell line was implanted in *plt* mice, there was a lower rate of tumor growth and a lower ultimate tumor burden as compared to Balb/c littermates (144). However, whether this *plt* mutation leads to decreased lymphatic invasion or fewer lymph node metastases has not yet been clarified. However, the data support the involvement of the CCR7-CCL19/21 signaling axis in the survival and proliferation of oral SCC tumors.

1.2.4 INFLAMMATORY TRANSCRIPTION FACTORS IN CANCER

1.2.4.1 Role of NF- κ B in tumorigenesis

Nuclear factor (NF)- κ B is a key transcription factor that orchestrates innate and adaptive immune responses by regulating the inducible expression of genes involved in the processes of cell survival and proliferation, stress response, inflammation and immunity. Since these pathways are also important in tumorigenesis, NF- κ B has intrinsic oncogenic potential. Several studies show that NF- κ B is activated by and induces the expression of pro-inflammatory cytokines. Additionally, NF- κ B activates the expression of other immune mediators such as chemokines, adhesion molecules and extracellular processes. These findings coupled with the constitutive activation of NF- κ B in a majority of human malignancies suggest that NF- κ B represents a molecular bridge between inflammation and cancer (149, 150).

The NF- κ B family consists of five members of the reticuloendotheliosis (Rel) family (Figure 1-2): RELA (p65), c-REL, RELB, p105/p50 and p100/p52. Each has a structurally conserved N-terminal Rel-homology domain (RHD) which contains the dimerization, nuclear-localization and DNA-binding domains. RELA, c-REL and RELB all have a non-homologous transactivation domain at their C-terminus and exist in their mature form. On the other hand, p105 and p100 are characterized by C-terminal ankyrin repeats and they require cleavage and proteolytic processing to generate p50 and p52 proteins, respectively. Except for RELB which can only form heterodimers, the other members of the NF- κ B family can form both homodimers and heterodimers with each other.

The classical NF- κ B molecule is a heterodimer of p50 and p65 subunits that exist in the cytoplasm as an inactive complex bound to inhibitory I κ B proteins. Activation of NF- κ B involves phosphorylation, ubiquitination and subsequent degradation of I κ B proteins, which releases NF- κ B p50/p65 allowing it to translocate to the nucleus and activate gene transcription. This classical pathway is triggered by proinflammatory stimuli, genotoxic stress and DNA-damaging agents. The alternative

pathway is triggered by TNF α family members and results in the phosphorylation and cleavage of p100 to form p52, which dimerizes with RELB to form an NF- κ B p52/RELB complex (151).

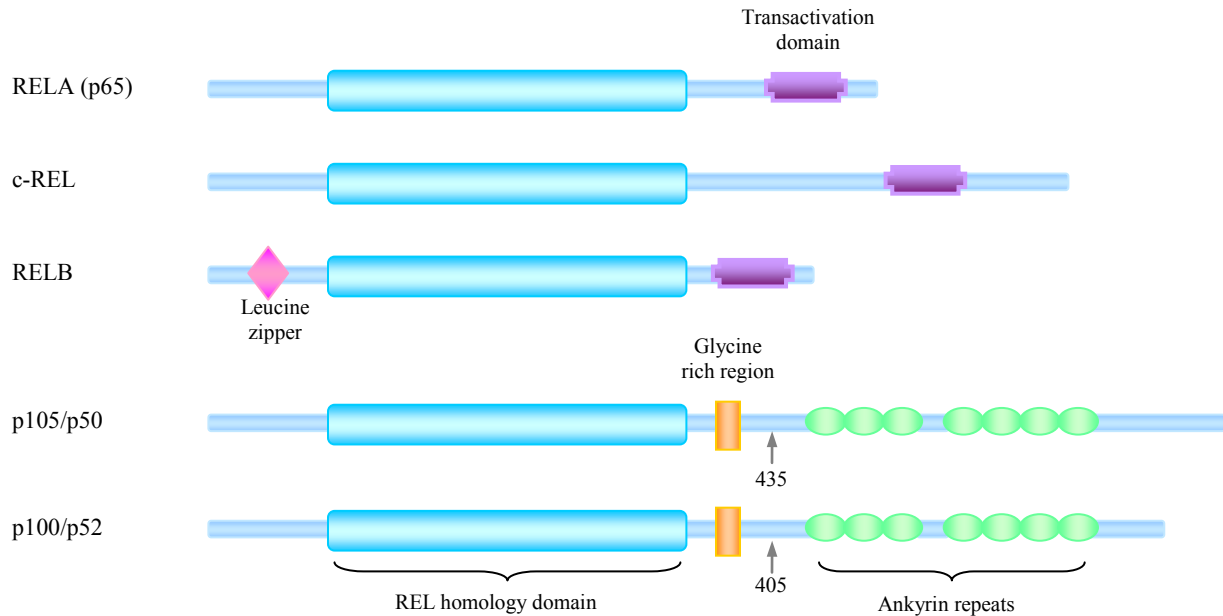


Figure 1-2: Schematic of the mammalian NF- κ B family members. The five members of the NF- κ B family are shown, along with the distinguishing structural features. Arrows in p105 and p100 indicate cleavage residues on each protein.

Emerging evidence indicates a role for constitutive NF- κ B activity in the transformation, enhanced survival and metastasis of a variety of cancers (152, 153). Recently, direct evidence for the role of NF- κ B in inflammation-associated tumorigenesis has emerged. Using a colitis-associated murine cancer model, studies show that deletion of the IKK β gene, and by extension inactivation of the IKK/NF- κ B activation pathway, resulted in dramatic reduction in tumor incidence (154). A separate study utilizing a mouse model of spontaneous hepatitis that develops into hepatocellular carcinoma, showed that the inhibition of NF- κ B activation induced apoptosis and blocked the progression from hepatitis to hepatocellular carcinoma (155). Furthermore, deletion of IKK β in hepatocytes and hematopoietic-derived Kupffer cells resulted in decreased hepatocarcinogenesis (156). Additional studies using a co-culture system of macrophages with breast and ovarian cancer cells showed that the inhibition of NF- κ B resulted

in decreased invasiveness of the tumors (157). These studies support the hypothesis that NF- κ B is essential in promoting inflammation-associated tumorigenesis.

Interestingly, NF- κ B has been shown to promote tumor invasion and metastasis by regulating the expression of matrix metalloproteinases and chemokines. Using a murine lung carcinoma model, inhibition of NF- κ B resulted in downregulation of the pro-metastatic matrix metalloproteinase 9, the urokinase-like plasminogen activator, and heparanase. Indeed, there was a reciprocal up-regulation of anti-metastatic tissue inhibitors of matrix metalloproteinases 1 and 2 and plasminogen activator inhibitor 2 (158). Studies have also shown that NF- κ B regulates chemokine expression in various malignancies, including CXCR4 in breast cancer (159), CCL5 in melanoma and breast cancer (160, 161), CXCL8 in melanoma (162, 163), and CCR7 in Hodgkin's disease (164, 165). Thus, the use of NF- κ B inhibitors appears promising in the therapeutic inhibition of tumor cell growth, invasion and metastasis (166).

1.2.4.2 Other transcription factors in tumorigenesis

Other transcription factors have also been implicated as molecular links between inflammation and cancer. The cyclooxygenase (COX)-1 and -2 enzymes are required in the synthesis of inflammatory lipid mediators such as prostaglandins. Studies show that aberrant cyclooxygenase expression is implicated in the processes of cellular proliferation, antiapoptotic activity, angiogenesis and metastasis, and correlates with poor prognosis in various human malignancies, including colon, gastric, breast, lung, prostate, and oral carcinomas (167-174). Epidemiologic studies examining the use of non-steroidal anti-inflammatory agents (which are well known to inhibit cyclooxygenase activity) in colorectal carcinomas have demonstrated inhibition of tumor growth and reduced mortality (175-177). In mouse studies, several groups have shown that the overexpression of COX-2 increases susceptibility to tumor growth (178-180). Conversely, COX-2 deficient mice are more resistant to tumorigenesis (181-183).

Hypoxia inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of an alpha and beta subunit. HIF-1 α is oxygen sensitive and is expressed under hypoxic conditions whereas HIF-1 β is constitutively expressed (184, 185). Studies show that HIF-1 α prevents the hypoxic apoptosis of neutrophils and T cells thereby promoting chronic inflammation (186, 187). Furthermore, HIF-1 α is activated by pro-inflammatory cytokines (TNF α and IL-1 β) via NF- κ B and COX-2 dependent pathways thereby supporting a link between inflammation and carcinogenesis (188, 189). HIF-1 α also promotes tumorigenesis by enhancing the transcription of the potent angiogenic factor, VEGF (190, 191). Finally, the expression of HIF-1 α has been found to correlate with metastasis in gastric adenocarcinoma (192).

The signal transducers and activators of transcription (STAT) proteins have also been shown to link an inflammatory microenvironment to tumor progression. STAT3, which is found constitutively overexpressed in numerous malignancies, supports oncogenesis through the activation of genes that are crucial for cell survival, proliferation, angiogenesis and metastasis (193, 194). In addition, STAT3 displays immunosuppressive activities that promote tumor development (195, 196).

1.3 ANTIMICROBIAL PEPTIDES

1.3.1 INTRODUCTION

The ability of animals to coexist harmoniously with the microbes around us is a remarkable tribute to the continuous surveillance of the immune system, its ability to distinguish between self and pathogen, and mount an appropriate response to effectively rid the host of intruding microorganisms. A homeostatic balance must be maintained in the mucosal linings between tolerance to normal commensal flora and inflammatory immune activation to destructive microbial invasion. In fact, infections of the respiratory, gastrointestinal and urogenital tracts are far less common than would be expected given the millions of bacteria that line these epithelia (197). This immune protection is achieved through a variety of mechanisms, including the constitutive and inducible production of anti-microbial peptides (198-204).

The detection of a ‘danger signal’ due to microbial invasion prompts the immune system to generate an acute anti-microbial response primarily by activating components of *innate* immunity. This involves the recognition of pathogenic stimuli by host receptors specialized to recognize certain pathogen-associated molecular patterns, the recruitment of inflammatory mediators and the production of anti-microbial peptides by the affected epithelia (205, 206). The rapid generation and release of anti-microbial peptides is crucial for the direct killing of invading pathogens as well as for the recruitment and activation of other elements of the immune system, including *adaptive* immunity (207, 208). As such, anti-microbial peptides act as nature’s antibiotics and are critical for maintaining cutaneous, airway and mucosal defense mechanisms, particularly when the physical barriers to infection (such as skin, mucosa) are compromised (209).

Although there are numerous forms of anti-microbial peptides, in mammalian systems most are classified in two main categories: cathelicidins (210) and defensins (211). These form a diverse group of multifunctional host molecules of *innate* immunity that are 12-50 amino acids in length, cationic in nature and therefore, well suited to interacting with negatively charged bacterial membranes (212, 213).

1.3.2 CATHELICIDINS

The cathelicidins contain a putative N-terminal signal peptide that targets intracellular storage, a highly conserved cathelin (cathepsin L inhibitor)-like domain and a variable C-terminal microbicidal domain (Figure 1-3). The cathelin-like domain is generally believed to neutralize the microbicidal domain thereby keeping the molecule inactive and preventing intracellular cytotoxicity (214). Cathelicidins are typically stored in neutrophil granules in their inactive proform (215). Upon stimulation and degranulation of activated neutrophils, cathelicidins undergo proteolytic processing to release the mature C-terminal anti-microbial peptide.

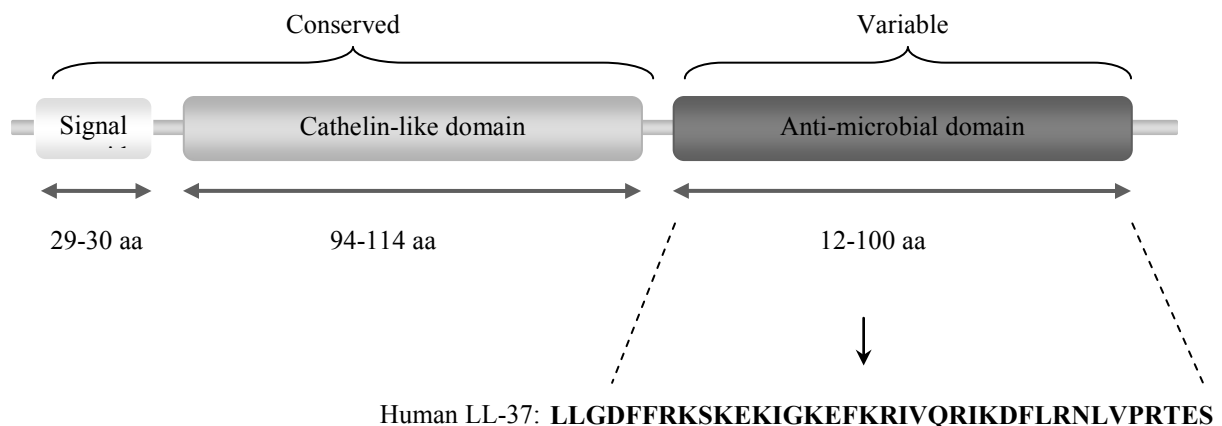


Figure 1-3: Structure of the human cathelicidin hCAP18/LL-37. The N-terminus is highly conserved and contains the signal peptide and the cathelin-like domain. The C-terminus is variable and contains the antimicrobial domain. Upon cleavage, the C-terminal antimicrobial domain (LL-37) is liberated.

At least 35 different cathelicidins have been identified from various mammalian species. However, humans produce only one known cathelicidin: hCAP18, whose C-terminal is cleaved off by appropriate proteases and liberated to form the anti-microbial peptide LL-37 (so named because the peptide begins with two leucine residues and is 37 amino acids long) (216-219). Besides the expression on neutrophils, hCAP18/LL-37 is also expressed by monocytes, mast cells, B cells, NK cells, $\gamma\delta$ T cells, epithelial cells and keratinocytes in response to inflammatory stimuli caused by tissue injury or bacterial components (220-223).

The synthesis and release of cathelicidins by airway and mucosal epithelia suggests an important role in maintaining host defenses and anti-microbial immunity (224, 225). Animal studies have provided direct evidence that cathelicidins are important components of host innate immunity. Deletion of the cathelicidin gene in mice resulted in a susceptibility to Group A *Streptococcus* infections (226). It is worth noting however, that cathelicidins lose some of their antimicrobial properties in serum and tissue conditions but still retain their immunomodulatory properties, suggesting their involvement in a variety of functions in infection and inflammation (227, 228). The multifunctional roles of cathelicidins in the immunomodulation of host defenses as well as the control of inflammation and disease are elaborated below.

1.3.2.1 Role of Cathelicidins in Immunity

1. *Direct anti-microbial effects:* The microbicidal effects of cathelicidins have a broad spectrum of activity against parasites, fungi, viruses, gram-positive and gram-negative bacteria (229-238). These effects are achieved by directly killing the pathogen, typically through membrane disruption which lyses target cells, or indirectly by inhibiting DNA and protein synthesis and by inducing the degradation of proteins required for metabolic processes (239, 240).

2. *Neutralization of bacterial endotoxins:* The microbicidal effects of cathelicidins are also achieved by binding and neutralizing endotoxins thereby mitigating the biological effects of infection such as septic shock. Studies have shown that LL-37 can bind to bacterial LPS and prevent the upregulation of genes involved in inflammatory processes (241, 242) modulate TLR responses (243, 244), inhibit cytokine secretion (245) and vascular nitric oxide production (246).

3. *Chemotactic activity:* Cathelicidins are involved in the recruitment of circulating lymphocytes to inflammatory areas. The human cathelicidin LL-37 is chemotactic for neutrophils, eosinophils, monocytes, T cells and mast cells (247-250). This activity is mediated through binding to the formyl peptide receptor-like 1 (FPRL1). In myeloid and epithelial cells, LL-37 stimulates the production of IL-8 (251, 252) and the macrophage chemoattractant proteins (MCP-1, MCP-3) (253).

4. *Angiogenesis and wound healing:* Cathelicidins are also involved in the restoration of tissue homeostasis through healing processes that include neovascularization, cell migration and proliferation (254). In human corneal epithelial cells, LL-37 is upregulated and modulates wound healing by stimulating migration and cytokine production (255). Injured skin has increased levels of LL-37, which return to normal once the wound is healed suggesting involvement of cathelicidins in cutaneous wound healing (221). In atopic dermatitis and chronic epithelial ulcers, lower levels of LL-37 are reported and studies inhibiting LL-37 show impairment of re-epithelialization (256, 257). Further studies have demonstrated that LL-37 stimulates increased proliferation and angiogenesis which is mediated by the FPRL1 receptor expressed on endothelial cells (258). Indeed, studies using mice deficient in the murine homologue of LL-37 (CRAMP) show decreased vascularization and delayed wound repair. Thus, in skin and airway epithelial cells, LL-37 induces cellular proliferation, revascularization and wound healing (257, 259).

5. *Regulation of inflammation and immunity*: Cathelicidins facilitate immune responses through their interactions with both host tissue and immune cells. A recent review outlines the role of LL-37 in mediating lung inflammation and immunity during infection (260). In other studies, LL-37 has been reported to preferentially promote the apoptosis of infected airway epithelium, thereby promoting the clearance of respiratory pathogens (261, 262). In immunity, LL-37 can modulate monocyte differentiation into DCs by upregulating endocytic capacity, phagocytic receptor and costimulatory receptor expression as well as the generation of Th1 cytokines (263). In immature DCs, LL-37 is taken up through endocytic pathways, accumulates in cytoplasmic compartments and stimulates the upregulation of HLA class II and CD86 costimulatory molecules (264). These effects on antigen presenting cells are responsible for linking innate and adaptive immune responses. Furthermore, in antigen presenting cells, LL-37 modulates the effects of IFN- γ through the suppression of cell activation, proliferation and production of inflammatory cytokines (265). LL-37 can also increase the expression of Toll-like receptors on mast cells and switch their function to direct them towards innate immunity (266). LL-37 also suppresses neutrophil apoptosis thus aiding host defenses during microbial infection by prolonging neutrophil lifespan (261, 267).

Evolutionary host-pathogen interactions suggest that various micro-organisms have devised strategies to counter the activity of cathelicidins and evade immune clearance (268). Indeed, pathogenic microbes, such as *Neisseria gonorrhoeae*, can downregulate the production of LL-37 in enteric infections thereby ensuring their own survival (269, 270). In the upper respiratory tract, bacteria are able to evade LL-37 anti-microbial action by mimicking host membrane lipids through the increase of phosphorylcholine lipid expression on their cell surface (271). Other bacteria directly inactivate LL-37 by secreting proteinases that degrade it (272-274). *Streptococcus pyogenes* excrete the protein SIC (streptococcal inhibitor of complement) which inactivates LL-37 activity (275). The bacteria *Vibrio cholerae* and enterotoxigenic *Escherichia coli* secrete the virulence proteins cholera toxin and labile toxin, respectively. These toxins transcriptionally downregulate the expression of LL-37 in intestinal epithelial cells (276).

1.3.2.2 Role of Cathelicidins in Inflammation and Disease

Despite the beneficial immunomodulatory effects of cathelicidins during pathogenic infection, their overexpression and dysregulation has been linked to the pathogenesis of some inflammatory disorders and malignancies. For instance, LL-37 expression is upregulated and exacerbates the symptoms of chronic nasal and skin inflammation (277, 278). In cystic fibrosis, the overexpression of LL-37 is associated with increased bronchial inflammation and deterioration of lung function, hence contributing to disease severity (279). Elevated levels of LL-37 have also been reported in the colon mucosa from patients with ulcerative colitis, although this has not yet been linked to the disease pathogenesis (280). In recent fascinating studies, LL-37 has been shown bind to self-RNA, facilitating the activation of TLR7 and TLR8 in DCs, thereby triggering an auto-inflammatory immune response that drives autoimmunity in psoriasis (281, 282).

As previously mentioned, cathelicidins are involved in angiogenesis, tissue repair and wound healing, processes that are typically dysregulated in tumorigenesis. Furthermore, elevated LL-37 levels are associated with persistent inflammation which can result in a pro-tumorigenic environment. Indeed, recent studies describe a role for dysregulated cathelicidin expression in promoting tumor development. In breast cancer, LL-37 is highly expressed and promotes tumor growth and metastasis (283, 284). LL-37 is also overexpressed and acts as a growth factor for lung cancer cells (285). In ovarian cancers, LL-37 expression is significantly increased and stimulates proliferation of tumor cells, enhances their migration and invasion, and facilitates the recruitment of pro-angiogenic tumor stromal cells (286, 287).

Interestingly, contrasting observations have been made on the role of cathelicidins in malignancy. A role for LL-37 in NK-cell-mediated suppression of tumor growth has been recently described in melanoma (288). In the immunotherapy of ovarian cancers, co-expression of LL-37 along with CpG oligonucleotides results in enhanced activation of NK cells (289). High dose LL-37 was also found to induce apoptosis of oral squamous cell carcinomas (290).

1.3.2.3 Regulation of Cathelicidin signaling and expression

The signaling of cathelicidins is mediated through various receptors. In humans, at least four different receptors have been identified for LL-37-induced effects: FPRL-1 (formyl peptide receptor-like 1) (247), P2X₇ receptor (291), EGFR (endothelial growth factor receptor) (292) and GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) (293). For FPRL-1 and P2X₇, activation occurs by direct binding of LL-37 to the cell surface receptor. However, activation through EGFR occurs by a transactivation mechanism whereby LL-37 stimulates the release of membrane-bound EGFR ligands that subsequently bind and activate EGFR. Previous observations indicating intracellular trafficking of LL-37 to the perinuclear region of epithelial cells suggested that an intracellular receptor may be involved in LL-37 binding (294). Indeed, GAPDH was recently identified as an intracellular receptor for LL-37 in monocytes.

Recent studies have revealed that the MAPK pathway is involved in the regulation of cathelicidin expression (295) although there may be differential regulation based on cell type and stimulus. The LL-37 gene has binding sites for several transcription factors including NF- κ B, AP1 and CREB, which have been shown to regulate LL-37 expression in different cell types (296-298). HIF-1 α has also been shown to govern the expression of LL-37 in keratinocytes (299).

1.3.3 DEFENSINS

The defensins are a family of cationic anti-microbial peptides consisting of six highly conserved cysteine residues forming three pairs of disulfide bridges that assume a conserved structural fold (Figure 1-4) (300). Based on the length between cysteine residues and their topology, defensins are classified into three subfamilies: α -defensins, β -defensins and θ -defensins. While the α - and β -defensins have been identified in various mammalian and plant species, the θ -defensins have so far only been identified in primate leukocytes. The θ -defensins are cyclic, derived from two α -defensin-like precursors, and appear to be inactivated in humans due mutations that encode a premature stop codon (301, 302). The location of α - and β -defensin genes on the human chromosome 8p22-23 suggests that they evolved from a common ancestral gene (303). In general, defensins are abundantly expressed in cells and tissues that are involved in host defense against microbial infections, including the cutaneous and mucosal surfaces (304).

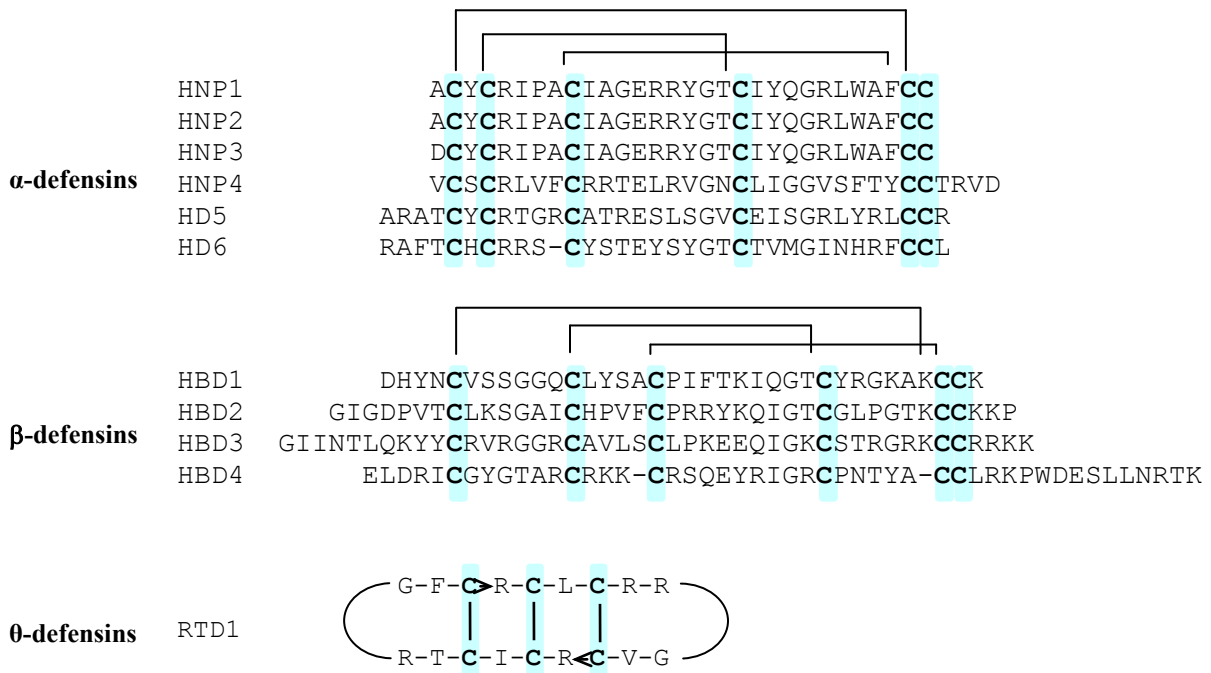


Figure 1-4: Disulfide pairing of cysteines in α -, β -, and θ -defensins. Conserved cysteines involved in intrachain bonding are highlighted. The arrows represent the peptide bonds formed in the cyclization of θ -defensins. HNP: Human neutrophil peptide; HBD: Human beta-defensin; RTD: Rhesus theta-defensin.

1.3.3.1 Human alpha (α) defensins

The cysteines in α -defensins are linked 1-6, 2-4 and 3-5. There are six known α -defensins, which include the neutrophil peptides HNP1-4 and the epithelial-derived HD5-6 (305). HNPs are synthesized by neutrophil precursors in the bone marrow. The mature peptides are expressed constitutively by neutrophils and macrophages, where they are stored in granules and released by degranulation. HD5 and HD6, known as enteric α -defensins, are produced by the intestinal Paneth cells where they contribute to host antimicrobial defense by lining the mucosal epithelial layer (300). There are various ways through which HNPs modulate immunity and inflammation. In general, neutrophil defensins act intracellularly by killing engulfed microorganisms within phagolysosomes. Recent findings show that macrophages can acquire HNPs by ingesting apoptotic neutrophils, thereby enhancing their antimicrobial activity against the intracellular pathogen *Mycobacterium tuberculosis* (306). HNPs also promote the uptake of pathogenic material by neutrophils and macrophages by inducing the aggregation of bacteria and viruses (307, 308). HNPs can also selectively chemoattract immature DCs, naïve T cells and mast cells to inflammatory sites (309, 310). In human airway epithelial cells, HNPs stimulate the secretion of IL-8 (311). In a transgenic mouse model, expression of human HD5 protected mice against lethal intestinal infection with *Salmonella typhimurium* further suggesting that locally secreted defensins can act as natural antibiotics (312). Recent studies suggest that the enteric α -defensins modulate intestinal homeostasis by adjusting the balance between bacterial populations (313). Indeed, studies show that deficiency of enteric defensins can compromise host mucosal immunity and contribute to the pathogenesis of Crohn's disease (314).

1.3.3.2 Human beta (β) defensins

The cysteines in β -defensins are linked 1-5, 2-4 and 3-6. At least 28 different human β -defensins have been identified using gene-based searches (315, 316), but only four (HBD1-4) have been studied in some detail (Table 1). The large number of β -defensin genes found is likely due to gene duplication events and the random selection of positive mutations (317, 318). In general, β -defensins are secreted by epithelial cells either constitutively (HBD1) or in response to cytokines, bacterial or viral products (HBD2-4).

Defensin	Cellular source	Tissue expression	Regulation	Receptor
HBD1	Epithelial cells, monocytes,	Epithelial cells of the oral and nasal mucosa, urogenital tract,	Constitutive or inducible by IFN- γ , LPS, peptidoglycans	CCR6
HBD2	macrophages,	kidneys, lungs, skin, eyes,	Inducible by viruses, bacteria, cytokines (TNF α and IL-1 β)	CCR6
HBD3	dendritic cells, keratinocytes	mammary glands		TLR1/2 CCR2
HBD4	Epithelial cells	Skin, stomach, lung, kidneys, urogenital tract	Constitutive or inducible by bacteria and PMA	*ND

*ND: Not determined

Table 1-1: Cellular sources and tissue distribution of human β -defensins (HBD1-6)

The first human β -defensin, HBD1, was purified from the plasma of renal disease patients undergoing hemofiltration for the elimination of uremic toxins (319). The hemofiltrate was analyzed as a pre-purified source of peptides from human plasma and a novel peptide was identified that bore homology to bovine defensins. Thus HBD1 was identified and characterized as the first human member of the β -defensin family. Since then, numerous studies have shown that HBD1 is constitutively expressed in epithelial cells of the oral mucosa, urinary and respiratory tracts (320-323). Due to its constitutive expression, HBD1 controls the microbial flora on the epithelial surfaces on which it is secreted.

Interestingly, HBD1 expression can also be modulated by inflammatory mediators such as LPS, IFN- γ and heat-denatured *Pseudomonas aeruginosa* (324).

HBD2 was isolated from psoriatic skin lesions (325). Harder *et al* speculated that antimicrobial peptides might be involved in protecting patients with psoriasis from opportunistic infections. They isolated and purified peptides from psoriatic scale extracts and identified a peptide antibiotic whose consensus sequence was homologous to bovine tracheal β -defensins as well as the previously identified human β -defensin 1. Similar studies have shown that the secretion of HBD2 into the epidermal layers of psoriatic skin provides crucial bactericidal activity thereby enhancing resistance against opportunistic microbial infections such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Indeed, studies have shown that reduced levels of HBD2 and LL-37 secretion can explain the observed higher levels of bacterial, viral and fungal infections in patients with atopic dermatitis compared to patients with psoriasis, even though both diseases are characterized by a defective skin barrier (256). Subsequent studies have identified HBD2 expression in epithelial cells of the foreskin, cornea, lung and trachea. The synthesis of HBD2 in the skin is induced in response to bacterial products or proinflammatory cytokines such as TNF α and IL-1 β through NF- κ B dependent processes (326-330).

HBD3 was also purified from lesional psoriatic scales (331), where it was found to possess a broad spectrum of anti-microbial activity against several Gram-positive bacteria, Gram-negative bacteria and yeast. Studies using functional genomic analysis identified HBD3 expression on various epithelia, including oral and nasal mucosa, gastrointestinal, respiratory and reproductive organs, as well as non-epithelial tissues such as leukocytes, heart and skeletal muscle (332-334). Studies have shown that the inducible expression of HBD3 varies depending on the site of expression. However, in general HBD3 is inducible in response to microbial products and proinflammatory cytokines such as TNF α , IL-1 β and heat-denatured *P. aeruginosa*. In keratinocytes, HBD3 expression is strongly induced by IL-22 and IFN γ through STAT3 activation, and phorbol 12-myristate 13-acetate (PMA) through protein kinase C

activation (335, 336). Unlike HBD1, HBD2 and HBD4, HBD3 has been shown to maintain its antimicrobial properties in the presence of sodium chloride at physiological concentrations.

HBD4 was first described using bioinformatics and functional genomics studies (337). It was later isolated from lung tissue whereby its expression was increased during lower respiratory tract infections (338). In fact, studies have shown that stimulation of human respiratory epithelial cells with heat-inactivated *P. aeruginosa* or *S. pneumoniae* increases the levels of HBD4 expression. In keratinocytes, HBD4 is strongly induced by PMA (336). HBD4 expression has been reported on reproductive, respiratory, gastric and kidney epithelia, with HBD4 displaying strong anti-microbial activity against both Gram positive and Gram negative bacteria.

1.3.3.3 Role of Defensins in Immunity

Innate Immunity

Defensins are involved in innate antimicrobial and antiviral immunity through the direct killing or inactivation of invading microorganisms (339, 340). However, with the exception of HBD3, the antimicrobial activities of most α - and β -defensins are vastly reduced in the presence of physiological salt concentrations. As such, their *in vivo* effects occur either in phagocytic vacuoles or on the surfaces of skin and mucosal epithelia (341). The relative selectivity of defensins for microbial membrane over that of the host cells is based on differential lipid composition, in which pathogenic membrane is rich in cholesterol and negatively charged surface groups that can bind electrostatically to the cationic anti-microbial molecules (316). The bound defensins then act either by damaging the integrity of the pathogen membrane via different mechanisms that include membrane depolarization, activation of cell-wall lytic enzymes and membrane permeabilization, all of which impair vital membrane functions, or by neutralizing endotoxins thereby alleviating the septic shock associated with the presence of microbial

products (342, 343). Studies have shown that in order to kill *E. coli*, the α -defensins (HNP1-HNP3) sequentially permeabilize the outer and inner microbial membrane resulting in the cessation of DNA, RNA and protein synthesis and eventual death of the bacteria (344). Furthermore, HNP1 is reported to exhibit anti-viral immunity against enveloped viruses by binding to their membranes and preventing viral entry (345), and by modulating intracellular pathways required for viral replication and protein synthesis (346). HNP1-HNP3 also inhibit HIV-1 infection in primary CD4⁺ T cells by blocking nuclear import and transcription of the viral genome (347, 348). Both HNP1 and HBD2 possess anti-endotoxin properties by binding to LPS and lipoteichoic acid (LTA) on Gram-negative and Gram-positive bacteria, effectively neutralizing these toxins (349). On the other hand, the bactericidal activity of HBD3 is achieved by perforating the cell wall of *S. aureus* (331) and *C. jejuni* (350), and by binding and neutralizing LPS (351). The induction of HBD2 and HBD3 expression by oral mucosa has also been shown to be important for the inhibition of HIV-1 replication. Indeed, recent studies show increased levels of HBD2 and HBD3 mRNA in the serum of HIV-1 exposed but uninfected individuals (352). This immune protection is achieved not only by directly binding to the virus, but also by downregulating the CXCR4 co-receptor for HIV infection (353, 354).

Besides their direct antimicrobial activity, defensins can also regulate the antimicrobial immune response through their influence on other components of the innate immune system. Several studies have shown that defensins can promote the recruitment of monocytes, neutrophils and mast cells, enhance the production of pro-inflammatory cytokines and regulate complement activation, all of which promote innate host inflammatory responses to microbial invasion (355). For instance, HBD2-4 can induce the activation and degranulation of mast cells, resulting in the release of inflammatory mediators such as histamine and prostaglandins (356-359). Moreover, HNP1-3 can indirectly promote the recruitment of neutrophils at inflammatory sites by stimulating bronchial epithelial cells to secrete IL-8 which is a potent neutrophil chemotactic factor (360). HNP1-3 also stimulate the production of TNF α and IL-1 by monocytes, thereby amplifying local inflammatory responses (361). The α -defensins can also bind to C1q and enhance or suppress the classical pathway of complement activation (362, 363).

Adaptive Immunity

In addition to acting as effectors of innate immunity, α - and β -defensins can also engage various mechanisms of cell-mediated and humoral adaptive immunity, as described below:

1. *Chemotactic effects*: Human α -defensins are chemotactic for monocytes, immature DCs and naïve T cells, thereby recruiting these cells to inflammatory regions and facilitating long-term cellular and humoral immunity (309, 364). Indeed, early studies using severe combined immunodeficiency (SCID) mice reconstituted with human peripheral blood lymphocytes reported that the subcutaneous administration of HNP1-2 resulted in significant recruitment of T cells to the sites of injection (365). On the other hand, HBD2 has been shown to selectively chemoattract immature DCs and T cells through its binding to CCR6 (366) and recent studies show that HBD2 and HBD3 can chemoattract macrophages and monocytes via binding to CCR2 (367).
2. *Enhancing DC maturation*: HNP1 and HBD1 are not only chemotactic for monocyte-derived DCs, but also are reported to promote the activation and maturation of DCs, as well as enhancing the secretion of the proinflammatory cytokines TNF α , IL-6 and IL-12 (368). Recent studies also show that HBD3 induces the expression of CD40, CD80 and CD86 costimulatory molecules on monocytes and myeloid DCs in a TLR1/2 dependent manner (369). The enhancement of DC maturation amplifies antigen uptake, processing, presentation and the eventual induction of an antigen-specific immune response.
3. *Adjuvants for cell-mediated & humoral immune responses*: Studies have shown that the co-administration of defensins with antigen results in an enhanced adaptive immune response. When the α -defensins HNP1-3 were simultaneously administered with ovalbumin (OVA) intranasally into mice, there was an enhanced IgG antibody response to OVA, as well as enhanced generation of IFN γ ,

IL-5, IL-6 and IL-10 by OVA-specific CD4⁺ T cells (370). In a periodontal disease study, the co-administration of HNP1, HBD1 or HBD2 with OVA resulted in enhanced OVA-specific IgG responses, which hindered disease pathogenesis (371). Furthermore, the intraperitoneal administration of HNP1-3 along with a B-cell lymphoma idiotype antigen not only increased the levels of antigen-specific IgG, but also enhanced the resistance of immunized mice to tumor challenge (372). In a murine DNA-vaccine study, mouse defensins (MBD2 and MBD3) were fused to non-immunogenic B cell lymphoma antigens and used to immunize mice. The findings showed that not only did the mice elicit potent humoral responses to an otherwise non-immunogenic antigen, but they also developed protective anti-tumoral immunity against lymphoma (373). The mechanism underlying these effects involved the enhanced uptake of 'defensin-antigen' complexes by antigen presenting cells (APC). These studies suggest that defensins can act as adjuvants that augment the generation of T cell-dependent cellular immunity as well as antigen-specific humoral immunity.

Undoubtedly, these chemotactic, stimulatory and adjuvant abilities of α - and β -defensins facilitate the initiation of adaptive immune responses by recruiting and activating the cells involved in these processes.

1.3.3.4 Role of Defensins in Inflammation and Disease

Since they are an integral part of host immunity, defensins are implicated in a wide variety of diseases (reviewed in (304)). In the gastrointestinal tract, studies have shown that chronic intestinal inflammation that characterizes diseases such as Crohn's disease and ulcerative colitis arises due to a disturbance in the relationship between the host and commensal bacteria lining the mucosa (374). In healthy subjects, normal defensin levels maintain a healthy balance between microbial flora and host cells by acting as an initial barrier against infection of mucosal surfaces. However, a deficiency in the levels of paneth cell-derived HD5 and HD6, and epithelial-derived HBD2 has been associated with the pathogenesis of

Crohn's disease primarily due to loss of tolerance to the resident bacterial flora and eventual dysregulation of the gut immune response (314, 375, 376).

Over 50% of the human population is infected with *Helicobacter pylori* in the gastric epithelium, but in the vast majority, it remains asymptomatic due to a highly adapted environment that maintains tolerance. Studies show that this tolerance is maintained by the β -defensins HBD2 and HBD3, which are upregulated during *H. pylori* infection and possess strong bactericidal activity (377, 378). Dysregulated HBD2-4 expression is linked to active *H. pylori* infection which has been associated with a variety of human diseases including peptic ulcer disease and gastric carcinomas (379, 380).

Respiratory tract diseases such as cystic fibrosis are also associated with dysregulated defensin activity. While healthy lung airway fluid is low in salt thus favoring the activity of defensins, studies show that the defective chloride channels in cystic fibrosis result in airway fluid that is very high in salt, which diminishes the activity of defensins and other antimicrobial peptides (83, 185, 186). As such, the respiratory epithelium in cystic fibrosis patients is progressively damaged due to recurrent infections with *P. aeruginosa* and inflammation. Other respiratory infections such as bacterial pneumonia are also associated with elevated levels of HBD2-4 in the respiratory tract and blood (138, 142, 187).

In the genitourinary tract, defensins are involved in host defense against bacteria, viruses and fungi. Indeed, numerous studies have been devoted to understanding the role of host immune defense factors in the protection against sexually transmitted diseases. In the female reproductive tract, secretion of cationic antimicrobial peptides, including HNP1-3, is linked to HIV-inhibitory activity (381). Furthermore, the cervico-vaginal fluid from healthy subjects, containing HNP1-3, inhibits HSV infection (382). Indeed, during bacterial vaginosis infections, vaginal fluids have reduced antimicrobial activity due to low levels of α - and β -defensins exposing patients to an increased risk for HIV and HSV infections (383). This observation helps explain why sexually transmitted diseases increase the likelihood of HIV-1 transmission. Recent studies show that the α -defensins HD5 and HD6 are secreted by vaginal epithelial cells in response to *Neisseria gonorrhoeae* infection (384). Interestingly, the secretion of HD5 and HD6

in response to *N. gonorrhoeae* was reported to promote HIV-1 infection, primarily during viral entry, due to the activation of TLR2 (385).

The skin performs an essential barrier function as it sustains a barrage of insults from the external environment, including microbial flora and external pathogens. It is, therefore, heavily invested with host defense peptides involved in antimicrobial immunity, wound healing and barrier function. In atopic dermatitis, skin is frequently infected with bacterial, viral and fungal pathogens due to the reduced levels of HBD2 and LL-37 expression (256). Conversely, psoriasis is characterized by increased levels of HBD2 and LL-37, which may contribute to disease pathogenesis by binding to self-DNA and directing autoimmune responses (85, 193).

In the oral and nasal mucosa, defensins have a critical protective function against the myriad of pathogenic organisms that come into contact with these epithelia. In Morbus Kostmann syndrome, a genetic form of periodontal disease, studies have shown a deficiency in the α -defensins HNP1-3 as well as LL-37 expression (386, 387). There are high levels of defensins in saliva, particularly HBD2 and HBD3, which inhibit HIV infection of the oral mucosa and helps explain why HIV does not appear to be transmitted through oral contact (388).

Although we have a great deal of understanding on role of defensins in host-pathogen interactions and immunity, little is known about the consequences of their dysregulation within the tumor microenvironment. Recent studies report that deregulated defensin expression can exacerbate an inflammatory environment by promoting deleterious inflammation. Studies on HBD1 expression in prostate and renal cell carcinomas have revealed that there is a cancer-specific loss of expression in the vast majority of these tumors (389, 390). This appears to be due to the presence of point mutations that dampen promoter activity. Indeed, when HBD1 is re-introduced to prostate and renal cancer cell lines, there is a corresponding decrease in proliferation and increase in apoptosis, suggesting that HBD1 is a potential tumor suppressor in these malignancies (391). Similar studies show that HBD2 expression is diminished in HPV-positive high-grade squamous intraepithelial lesions of cervical cancer (392). Furthermore, intratumoral expression of HNP1 in mouse models of breast and colon cancer resulted in

significant tumor growth inhibition due to the recruitment and activation of DCs and cytotoxic T cells (393).

Despite all the beneficial immunomodulatory effects of defensins, it is worth noting that the dysregulated expression of defensins can lead to perpetual inflammation which can be pro-tumorigenic. Human tumors overexpressing defensins have been found to possess increased populations of infiltrating monocytes, macrophages and DCs suggesting that the pro-inflammatory and pro-angiogenic functions of defensins may shape the development of these tumors. In mouse ovarian carcinoma, the expression of β -defensin 29, the murine homologue to HBD2, was found to chemoattract DCs through CCR6 and enhance tumor vascularization and growth through cooperation with VEGF-A (394). The α -defensins HNP1-3 are upregulated in the plasma and tissues of patients with gastric cancer and metastatic colorectal cancer, and have been proposed as potential biomarkers for prognostic assessment of these carcinomas (395, 396). In chronic myelomonocytic leukemia, which is characterized by the accumulation of heterogenous monocytes in the peripheral blood, the secretion of HNP1-3 by immature dysplastic granulocytes inhibits monocyte differentiation, suggesting that the α -defensins are directly responsible for the accumulation of leukemic cells (397). In oral cancers, HBD3 is widely overexpressed and has been associated with the trafficking of tumor associated macrophages by signaling through CCR2 (398-400).

1.3.3.5 Regulation of Defensin signaling and expression

The expression of α -defensins appears to be constitutive. The neutrophil peptides HNP1-4 are constitutively secreted by neutrophils and macrophages, while the enteric defensins HD5 and HD6 are constitutively secreted by the intestinal paneth cells. For this reason, α -defensins are thought to be involved in low level continuous immune surveillance of the epithelia on which they are secreted, in order to maintain homeostatic balance between these epithelia and the external environment.

In contrast, the expression of β -defensins is inducible at the transcriptional level, often involving a variety of mediators including TLR activation and pro-inflammatory cytokines. Indeed, numerous studies have been devoted to exploring defensin expression in mammalian systems (401). The first human β -defensin studied, HBD1, was found to be constitutively expressed by epithelial cells of the skin, oral mucosa, urinary and respiratory tracts (402). However, studies have shown that HBD1 expression can be upregulated in monocytes exposed to LPS and IFN- γ (401, 403).

Studies show that HBD2 is strongly induced in keratinocytes following LPS treatment, in a predominantly IL-1 dependent manner (326, 328). In fact, inhibition of the IL-1 receptor resulted in drastically reduced levels of HBD2 expression following peptidoglycan and LPS stimulation through TLR2 and TLR4 pathways, respectively (401). Human corneal epithelial cells also upregulate HBD2 in response to TLR2 activation (330), while intestinal epithelial cells upregulate HBD2 in response to TLR2 and TLR4 activation via NF- κ B and AP1-dependent pathways (329). Stimulation with TLR3 agonists such as poly(I:C) or double stranded RNA has been shown to induce HBD2 in uterine and airway epithelial cells (404, 405). Bacterial DNA or synthetic CpG oligonucleotides stimulate HBD2 expression in airway epithelial cells via TLR9 activation (406). In other studies, IL-17 is described as a potent inducer of HBD2 expression in airway epithelial cells (407). Additionally, HBD2 expression is induced by stimulation with TNF α , IL-1 β , PMA and *P. aeruginosa* (336). Genomic analysis of the HBD2 promoter has revealed the presence of putative binding sites for NF- κ B, AP1 and NF-IL6, all of which are transcription factors involved in the regulation of inflammatory processes (408, 409). Indeed, it has now been demonstrated that the TNF α , IL-1 β and IL-17-mediated induction of HBD2 requires binding of NF- κ B to proximal κ B-binding sites within the HBD2 promoter (328, 410). Furthermore, mutations in either the NF- κ B or AP1 binding sites severely reduce the TLR2 and TLR4 inducibility of the HBD2 promoter (329). Finally, in oral epithelial cells, microbial proteases have been shown to activate HBD2 expression through their binding to protease activated receptors (411).

On the other hand, the expression of HBD3 in keratinocytes is inducible by TGF α stimulation (412). However, it is notable that EGFR inhibitors are much more effective than TGF α inhibitors at blocking HBD3 upregulation, suggesting that other EGFR ligands can contribute to HBD3 upregulation. Indeed, other EGFR ligands; EGF, HB-EGF and amphiregulin, have been found to upregulate HBD3 in keratinocytes to varying degrees (401). HBD3 expression is also induced in response to IL-1 β , IFN- γ , PMA and *P. aeruginosa* (336). Unlike HBD2, the HBD3 promoter does not have any NF- κ B binding sites, but does have AP1, NF-IL6 and STAT3 consensus sequences. In keratinocytes, HBD3 expression is also strongly induced by IL-22 through the activation of STAT3 (335, 336). Histamine also induces HBD3 production in keratinocytes by activating STAT3 and AP1 transcription factors (413). Finally, prostaglandin D₂ induces HBD3 production in keratinocytes by activating AP1 family transcription factors (414).

HBD4 expression in keratinocytes is induced by stimulation with TNF α , IL-1 β and *P. aeruginosa*, and very strongly induced by PMA treatment through protein kinase C activation (336, 337).

1.4 SPECIFIC AIMS

1.4.1 To evaluate the significance of constitutive and inducible NF- κ B activation in regulating CCR7 expression in metastatic SCCHN.

We have previously reported a distinct chemokine expression pattern in SCCHN whereby CCR7 is upregulated in metastatic tumor cells. However, the signals that regulate CCR7 expression in these metastatic tumors have not been extensively studied. Several lines of evidence in the literature suggest that CCR7 is an NF- κ B target gene. Given the observed constitutive activation of NF- κ B complexes in SCCHN, we hypothesize that NF- κ B is a key transcription factor involved in inducing and maintaining CCR7 expression in metastatic SCCHN. To study this, we examined the CCR7 promoter region for potential NF- κ B binding sites, investigated their activity using luciferase assays, electromobility shift assays and chromatin immunoprecipitation assays. Furthermore, we tested the effects of NF- κ B activation and inhibition on CCR7 expression in metastatic SCCHN. The findings of this study are presented in Chapter 2.

1.4.2 To explore the significance of tumor-associated inflammatory signals in inducing CCR7 expression in primary SCCHN.

The mechanisms that are responsible for reprogramming malignant cells to express functional chemokine receptors are not well understood. However, it is increasingly clear that the tumor microenvironment contains crucial inflammatory factors that play a key role in transformation and the progression towards malignancy. In epithelial malignancies such as SCCHN, inflammatory antimicrobial peptides such as HBD3 are overexpressed and can contribute towards tumor aggressiveness by regulating tumor chemokine expression. Here, we hypothesize that

HBD3 overexpression in SCCHN regulates CCR7 expression through NF- κ B dependent mechanisms. We tested this by examining the effects of HBD3 treatment on primary SCCHN tumor cells. By using several specific inhibitors, we also investigated the dependence of the observed HBD3-induced CCR7 upregulation on NF- κ B activation. Moreover, *in vivo* studies explored the correlations between HBD3 secretion and CCR7 expression. The findings of this study are presented in Chapter 3.

1.4.3 To determine the significance of tumor-associated inflammatory signals in inducing antigen presenting cell maturation and CCR7 expression.

Literature reports reference the role of tumor-associated inflammatory signals in recruiting an immune cell infiltrate around a developing tumor. Indeed, HBDs have been reported to contribute to adaptive immune responses by recruiting and activating monocytes, dendritic cells and T cells to sites of inflammation. Given our knowledge on the CCR7-inducing properties of HBD3 on tumors, we hypothesized that HBD3 can enhance the maturation of DCs thereby acting as an adjuvant to the immune activation of these cells. Using flow cytometric analyses, we examined the effects of HBD3 exposure on DC maturation and CCR7 expression. We also tested the consequences of NF- κ B inhibition on this process. The findings of this study are presented in Chapter 4.

**REGULATION OF CHEMOKINE RECEPTOR 7 (CCR7) GENE EXPRESSION BY NF- κ B IN
SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK (SCCHN)**

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Format adapted for Dissertation

2.0 REGULATION OF CHEMOKINE RECEPTOR 7 (CCR7) EXPRESSION BY NF- κ B IN SCCHN

2.1 INTRODUCTION

Among the immune exploitive mechanisms used by tumors to facilitate their growth, survival and metastasis, is the ability to exploit the tumor microenvironment for physiologic signals that enhance tumor progression. Several lines of evidence now show that the expression of chemokines by tumors is one such manipulative mechanism (81, 123). Chemokines are small secreted pro-inflammatory proteins that mediate the selective recruitment and trafficking of leukocytes to sites of inflammation. They signal through seven-transmembrane G-protein-coupled chemokine receptors expressed by the migrating cell (78). CCR7 has two known ligands, CCL19 (MIP-3 β /ELC) and CCL21 (6Ckine/SLC) that are predominantly expressed in the lymphatic endothelium and the secondary lymphoid organs, and provide migratory cues for the homing of CCR7-positive cells to these tissues.

In SCCHN, we have previously reported the upregulation of CCR7 expression in metastatic tumors as compared to primary tumors (141). Given the distinct nodal metastasis pattern observed in this malignancy, it is likely that CCR7 is a key contributor to the organ-specific spread. Indeed, studies have shown that the expression of CCR7 correlates with metastatic potential and lymph node migration of oral SCC tumors (139-141). Furthermore, our previous work has shown that autocrine (and paracrine) CCR7 signaling provides pro-survival and invasive signals to SCCHN tumors (143, 144). However, despite our knowledge on the role of CCR7 in tumor progression, little is known about the nuclear regulatory factors responsible for the expression of CCR7 on malignant cells.

The inducible expression of chemokines and their receptors occurs in response to inflammatory cytokines, growth factors and/or pathogenic stimuli regularly present within the tumor microenvironment. These inflammatory stimuli are often potent activators of the NF- κ B family of transcription factors, thereby potentially linking chronic inflammation to cancer progression (149). The NF- κ B family consists of homo- and hetero-dimers of five structurally related Rel/NF- κ B proteins. Following activation by a variety of stimuli, the cytoplasmic inhibitory (I κ B) proteins are phosphorylated, ubiquitylated and subsequently degraded by the proteasome, which allows active NF- κ B to translocate into the nucleus and mediate transcription of target genes. In several malignancies, including SCCHN, NF- κ B is constitutively active and may facilitate tumor angiogenesis, growth and metastasis by regulating the genes involved in these processes. Indeed, the use of NF- κ B inhibitors has been shown to reduce tumor growth and metastasis using both *in vitro* and *in vivo* models (415, 416).

The cytokine-induced maturation of DCs results in upregulation of CCR7 expression, which is dependent on NF- κ B activation. In our studies, CCR7 is significantly upregulated in metastatic SCCHN (141) and furthermore, NF- κ B is constitutively active and known to contribute to the progression of these tumors (417, 418). Given our understanding of CCR7 regulation in DCs, we sought to determine whether NF- κ B was regulating the observed CCR7 expression in metastatic SCCHN. In addition, as we have recently reported, the autocrine and paracrine activation of CCR7 in metastatic SCCHN tumors (144), we explored the possibility that the observed NF- κ B activation was emanating from autocrine and/or paracrine sources. Recent evidence has linked NF- κ B activation to the expression of chemokine family genes, including the induction of CCL19 and CCL21 through non-canonical NF- κ B signaling pathways (166, 419). In breast cancer, NF- κ B was reported to promote metastasis by regulating the expression of CXCR4 (159), and in Hodgkin's lymphoma, the upregulation of CCR7 has been suggested to be an NF- κ B-mediated event (164, 165). Inducible and constitutively active NF- κ B, which has been demonstrated in more aggressive SCCHN phenotypes (420), could facilitate chemokine-mediated tumorigenesis and metastasis. For these reasons, we explore the role of NF- κ B in regulating basal and inducible CCR7 expression on metastatic SCCHN cells.

2.2 MATERIALS & METHODS

Cell lines

Human SCCHN cell lines PCI-6B, PCI-15B, PCI-37B were derived from the metastatic tumor site and characterized at the University of Pittsburgh (421). All cells were cultured in DMEM medium (Invitrogen Corp., Carlsbad, CA) containing 8% (v/v) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/ml penicillin G, and 100 units/ml Streptomycin (Invitrogen) and 4mM L-glutamine (Invitrogen, Carlsbad, CA). The cells were tested for mycoplasma on a regular basis to ensure that only mycoplasma-free cell lines were studied in our assays.

Antibodies & Reagents

Anti-human NF- κ B p65 (CT) and normal mouse IgG for the chromatin immunoprecipitation assays were purchased from Upstate (Temecula, CA). The antibodies used for EMSA supershift analysis were: p50, p65 (kind gifts from Dr. William H. Walker, University of Pittsburgh), p52 (447) and Rel-B (H-200) (both from Santa Cruz, CA), while the c-Rel antibody was obtained from Upstate (Temecula, CA). TNF α , CCL19 and CCL21 were purchased from R&D Systems (Minneapolis, MN). The PI3K inhibitor, LY294002 and Akt Inhibitor IV were purchased from Calbiochem (San Diego, CA).

Chromatin Immunoprecipitation (ChIP) Assay

Cells were serum-starved for 48 hours prior to stimulation. After appropriate treatment, the cells were fixed with formaldehyde (1% final concentration) (Sigma-Aldrich, Inc., St. Louis, MO) for 10 min. They were then quenched with Glycine (0.125M final concentration) (Sigma-Aldrich, Inc., St. Louis, MO) for 5 min. The cells were then washed twice with ice-cold PBS and scraped. After centrifugation, cells were lysed in SDS lysis buffer (Upstate, Temecula, CA) containing protease inhibitors. Chromatin was sheared by sonication for 5 x 10 seconds (at 25% of the maximum potency) to generate sheared DNA with an average length between 200 and 1000 base pairs. The immunoprecipitation of NF- κ B p65-bound

chromatin, washes and elution steps were performed using the Ez-ChIP™ kit (Upstate, Temecula, CA) and according to the instructions provided. Protein-DNA crosslinks were reversed at 65°C overnight. After RNase (10µg, 30 min at 37°C) and proteinase K (10µg, 2 hr at 45°C) (Sigma-Aldrich, Inc., St. Louis, MO) digestion, DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). About 1/10 of the purified DNA was used in each PCR using the following primers:

GAPDH: 5' TACTAGCGGTTTTACGGGCG 3' (s) and 5' TCGAACAGGAGGAGCAGAGAG-CGA 3' (as). IkB α : 5' GACGACCCCAATTCAAATCG 3' (s) and 5' TCAGGCTCGGGGAAT-TTCC 3' (as). CCR7 κ B0-1: 5' CTGAAAGAAGCCAGATGTGAAGGTCA 3' (s) and 5' AATATCACATGCCAGGC-CATGGGT 3' (as). CCR7 κ B1-2: 5' TTGGCCTAAACTACCCAGAA-GCCA 3' (s) and 5' AGAAAG-GTGACAGAGGGTGACAGT 3' (as). CCR7 κ B2-3: 5' ACCCA-TGGCCTGGCATGTGATATT 3' (s) and 5' TTAAGTTGTCGGAGAAGCCACCCT 3' (as).

Preparation of Nuclear Extracts

Metastatic SCCHN tumor cells were stimulated and nuclear extracts were prepared as follows: Cells were washed in ice-cold PBS, scraped, collected and resuspended in cell lysis buffer (10mM Hepes, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, Protease and Phosphatase Inhibitors). After allowing cells to swell on ice for 15 mins, 1% NP-40 was added and cells vortexed. After centrifugation, the nuclear pellet was collected and resuspended in nuclear lysis buffer (20mM Hepes, 400mM NaCl, 1mM EDTA, 1mM EGTA, Glycerol, 1mM DTT, 1mM PMSF, Protease and Phosphatase inhibitors). Samples were vortexed at 4°C for at least 1 hour. Nuclear supernatants were collected and stored at -80°C. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL).

Electromobility Shift Assay (EMSA)

The DNA binding activity of NF- κ B was examined using radiolabeled NF- κ B oligonucleotide probes in an electromobility shift assay. 6-10µg of nuclear extract was added to buffer (containing 20mM Hepes, 1mM EDTA, 10% glycerol, 5mM DTT, 1µg poly(dI-dC) and 2µg BSA) and incubated with 0.1pmol of

³²P-labelled oligonucleotide probe for 15 min at room temperature. The DNA binding activity of CREB was examined as a loading control. The probe sequences were as follows:

κB consensus: CGACACCCCTCGGGAATTTCCCACTGGGCC

CCR7 κB0: GTGGTTGCCAGGGGCTTTCGATGACAGGAG

CCR7 κB1: GGGAAGGATGGGGACACTTCTCTCACACAT

CCR7 κB2: AGGCAGCGCAGGGGGCTTTTTGAATGTAAA

CCR7 κB3: AGGCATTGAAGGGGCCCTGCATGAGTCAGG

CRE motif: GATCCGGCTGACGTCATCAAGCTAGATC

In the competition and antibody supershift analyses, the respective unlabeled oligonucleotides or antibodies were added to the reaction for 10 min before the addition of radiolabeled probe. All samples were run on 5% native gels and developed by autoradiography.

Plasmid Constructs & Transient Transfections

The CCR7 promoter constructs were amplified by PCR primers designed to encompass each κB region and the 100-200 flanking nucleotides. [Primers: κB0: 5' GGGGGTACCCTGAAAGAAGCCAGATGTG-AAGGTCA 3' (s) and 5' GGGAAGCTTCACAGAGTTATGCAACCATCACC-AC 3' (as). κB1: 5' GGGGGTACCCTTGGCCTAAACTACCCAGAAGCCA 3' (s) and 5' GGG-AAGCTTAATATCACATGCCAGGCCATGGGT 3' (as). κB2: 5'GGGGGTACCACCCATGGCCTGGCATGTGATATT 3' (s) and 5' GGGAAGCTTAGAAAGGTGACAGAGGGTGACAG-T 3' (as). κB3: 5'GGGGGTACCACTGTCA-CCCTCTGTCACCTTTCT 3' (s) and 5'GGGAAGCTTTTAAGTTGTCTGGAGAAGCCACCCT 3' (as)]

The constructs were then cloned upstream of the minimal promoter element in the pGL4.26 firefly luciferase vector (Promega, Madison, WI) using *KpnI* and *HindIII* restriction enzyme sites. Transient cotransfections along with a renilla luciferase control were performed using polyfect reagent (Qiagen). After 24 hours, cells were stimulated with relevant cytokine for another 24 hours and the luciferase activity measured. Where applicable, firefly luciferase activity was normalized to renilla luciferase.

Quantitative Real-time RT-PCR

RNA was isolated from metastatic SCCHN tumor cell lines using TRIzol Reagent (Invitrogen), and purified using RNA CleanUp (Qiagen, Valencia, CA), followed by DNase digestion (Ambion). Concentration and purity of RNA was determined by measuring absorbance at 260nm and 280nm. The reverse transcription of RNA was performed as described previously (422). Real-time PCR was then performed on the Applied Biosystems 7700 Sequence Detection Instrument, using Taqman Pre-developed assay reagent for human CCR7 (Applied Biosystems). Cycling parameters were: Initial denaturation at 95°C (12 min), 40 cycles of 95°C (15 sec) and 60°C (1 min). All experiments were performed in triplicate. Relative expression of the CCR7 target gene to endogenous control gene (β -glucuronidase (GUS)) was calculated using the Δ CT method: Relative Expression = $2^{-\Delta CT}$; where $\Delta CT = C_{T(CCR7)} - C_{T(GUS)}$ (141).

Human tissue samples & Immunohistochemistry (IHC)

Tumor NF- κ B levels were evaluated by immunohistochemical (IHC) staining of tumor and adjacent mucosal specimens arrayed in a previously described TMA as described in Chapter 3.2. Prior to incubation with anti-NF- κ B antibody (1:200) (Zymed, CA) for 60 minutes at room temperature, antigen retrieval was performed using Borg buffer (Biocare Medical, CA) in a Biocare Decloaking chamber and quenching and blocking were performed as in Chapter 3.2. Staining was developed by incubation with Mach 2 Rabbit Polymer (Biocare) for 30 minutes at room temperature followed by incubation with Substrate Chromagen. IHC scoring of NF- κ B cytoplasmic staining was done by a trained pathologist.

Statistical Analysis

Data are expressed as mean \pm standard error of at least three experiments. An unpaired t-test was used to calculate whether observed differences were statistically significant. For the IHC analysis, differences between paired tumor and adjacent mucosa levels of each protein were evaluated using the signed-rank test. Correlations between proteins were assessed using Spearman's nonparametric correlation coefficient. The threshold for significance was $p < 0.05$.

2.3 RESULTS

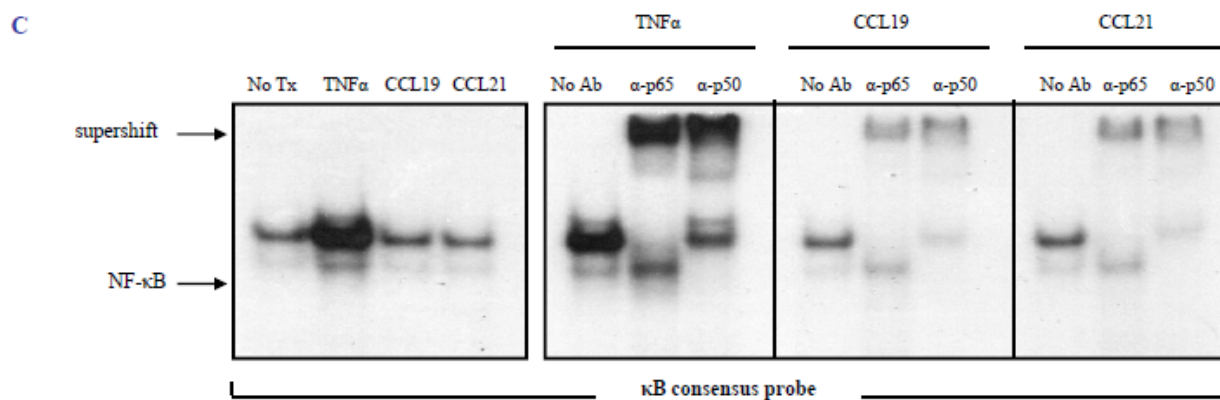
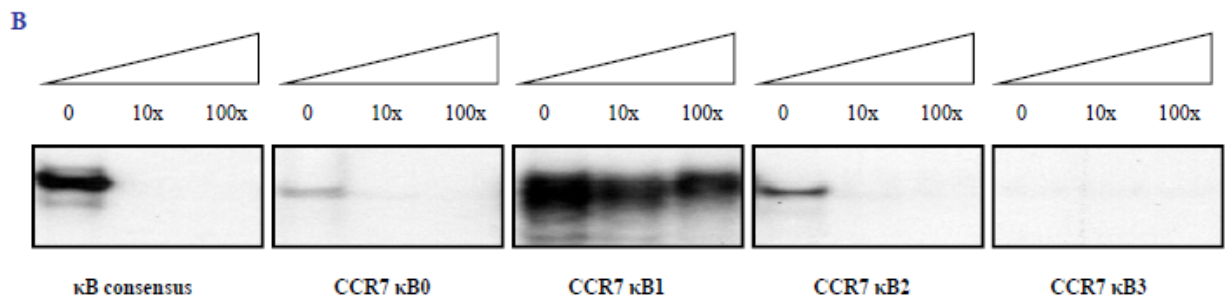
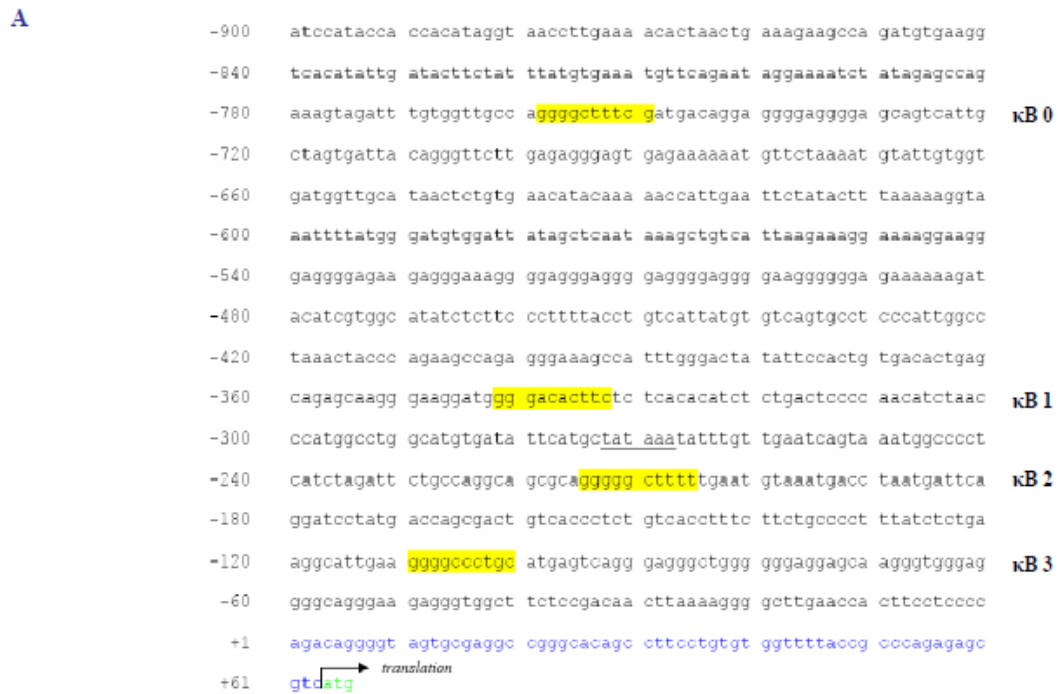
2.3.1 The CCR7 promoter contains functional κ B sites

Based on reports that CCR7 may be an NF- κ B target gene (164, 165), we examined the 1000bp upstream promoter for potential NF- κ B binding sites. At least four potential κ B sites were identified by virtue of their homology to the κ B consensus binding site sequence motif (Figure 2-1A). To determine whether these sites could bind to activated NF- κ B in nuclear extracts, oligonucleotide probes were constructed encompassing each CCR7 κ B site and the ten flanking nucleotides on either side. Nuclear extracts were prepared from TNF α stimulated cells, incubated with radiolabeled probe and the binding was analyzed by EMSA (Figure 2-1B). The κ B0, κ B1 and κ B2 probes were found to bind nuclear proteins from TNF α stimulated cells. The affinity of this binding was comparable to that observed on a consensus κ B probe. The binding observed for the CCR7 probes was specific as evidenced by the ability to compete away binding from each probe using increasing amounts of unlabelled κ B consensus probe. Protein binding to a specific probe containing a CRE motif was not competed away under these conditions (not shown).

To study whether the NF- κ B was the transcription factor responsible for the observed protein binding on the consensus κ B probe, we performed EMSA supershift analysis using antibodies to p50 and p65 (Figure 2-1C). The findings show that NF- κ B p50/p65 was the predominant heterodimer bound to the consensus κ B probe. Furthermore, there was no detectable increase in the binding of NF- κ B p50/p65 following stimulation with CCL19 or CCL21, suggesting that the previously reported autocrine and paracrine activation of CCR7 may not be responsible for activating NF- κ B in these cells.

Previous studies have suggested that different κ B sites can preferentially recruit and bind different NF- κ B subunits, potentially regulating selective gene activation. Indeed, the alternative NF- κ B complex p52/RelB was demonstrated to regulate activation of the CCR7 ligand genes (419). To examine which NF- κ B subunit complexes were bound to the CCR7 κ B motifs, we used antibodies to p50, p65, p52, RelB and cRel in a supershift EMSA (Figure 2-1D). Our results show that the p50/p65 heterodimer

was the predominant moiety bound to the CCR7 κ B sites. These findings suggest that the CCR7 promoter is at least partially influenced by the NF- κ B p50/p65 transcription factor.



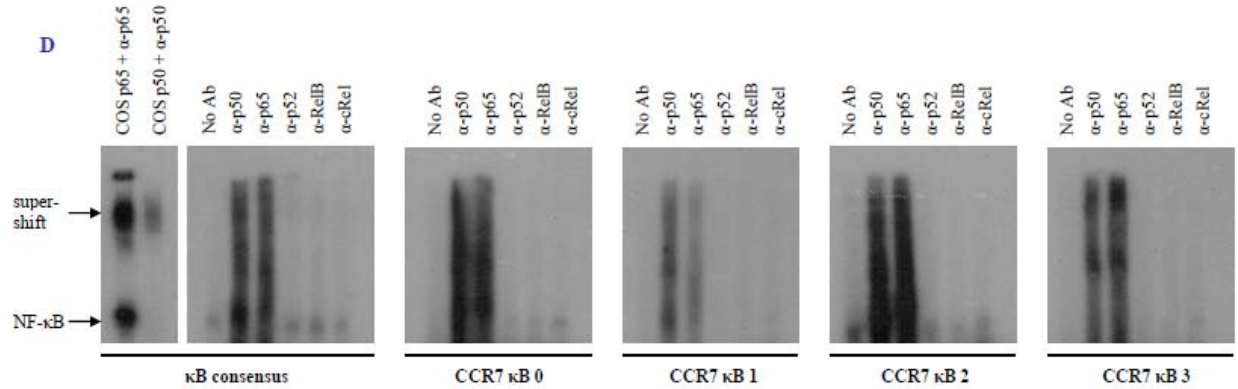


Figure 2-1: The CCR7 promoter contains NF- κ B binding sites. (A) Schematic representation of the CCR7 promoter, highlighting the four potential NF- κ B binding sites. (B) PCI-37B cells were stimulated with TNF α (20ng/ml, 10min) and the nuclear extracts incubated with 32 P radiolabeled CCR7 κ B probes and assayed by EMSA. Binding was competed using excess unlabeled κ B consensus probe. (C) PCI-37B cells were stimulated with TNF α (20ng/ml, 10min), CCL19 (100ng/ml, 10min) or CCL21 (100ng/ml, 10min). Nuclear extracts were prepared and incubated with radiolabeled κ B consensus probe in the presence of p50 or p65 supershift antibodies. (D) PCI-15B nuclear extracts obtained from untreated cells were incubated with each of the κ B probes and then subjected to supershift analysis using p50, p65, p52, RelB and cRel antibodies.

2.3.2 Activated NF- κ B regulates CCR7 promoter activity

To directly test the functional ability of each CCR7 κ B site to induce gene expression, we performed luciferase reporter assays. Reporter vectors consisting of each κ B site surrounded by the flanking 100-200bp nucleotides were inserted upstream of a minimum promoter-containing luciferase gene and transfected into a metastatic CCR7-positive SCCHN cell line. Reporter activity was measured in the presence or absence of TNF α , a potent NF- κ B inducing cytokine. Each κ B motif was capable of activating luciferase gene expression to varying degrees when stimulated with TNF α (Figure 2-2A). We also observed a high basal activation of the CCR7 promoter κ B3 region, raising the possibility that constitutively active NF- κ B in the SCCHN cells was responsible for elevating basal promoter activity. Nevertheless, TNF α stimulation was capable of inducing expression above this baseline (approximately 2 fold induction, $p < 0.05$).

We then tested the importance of NF- κ B in regulating the observed basal CCR7 activity by co-transfecting a luciferase construct containing the entire CCR7 promoter region (κ B0-3) along with a vector expressing the I κ B α AA super-repressor. The use of this super-repressor led to a 30-50% reduction in basal and inducible activity of the CCR7 promoter in PCI-15B and -37B cells, suggesting that NF- κ B is partially responsible for regulating basal and inducible CCR7 expression in these metastatic SCCHN cell lines (Figure 2-2B).

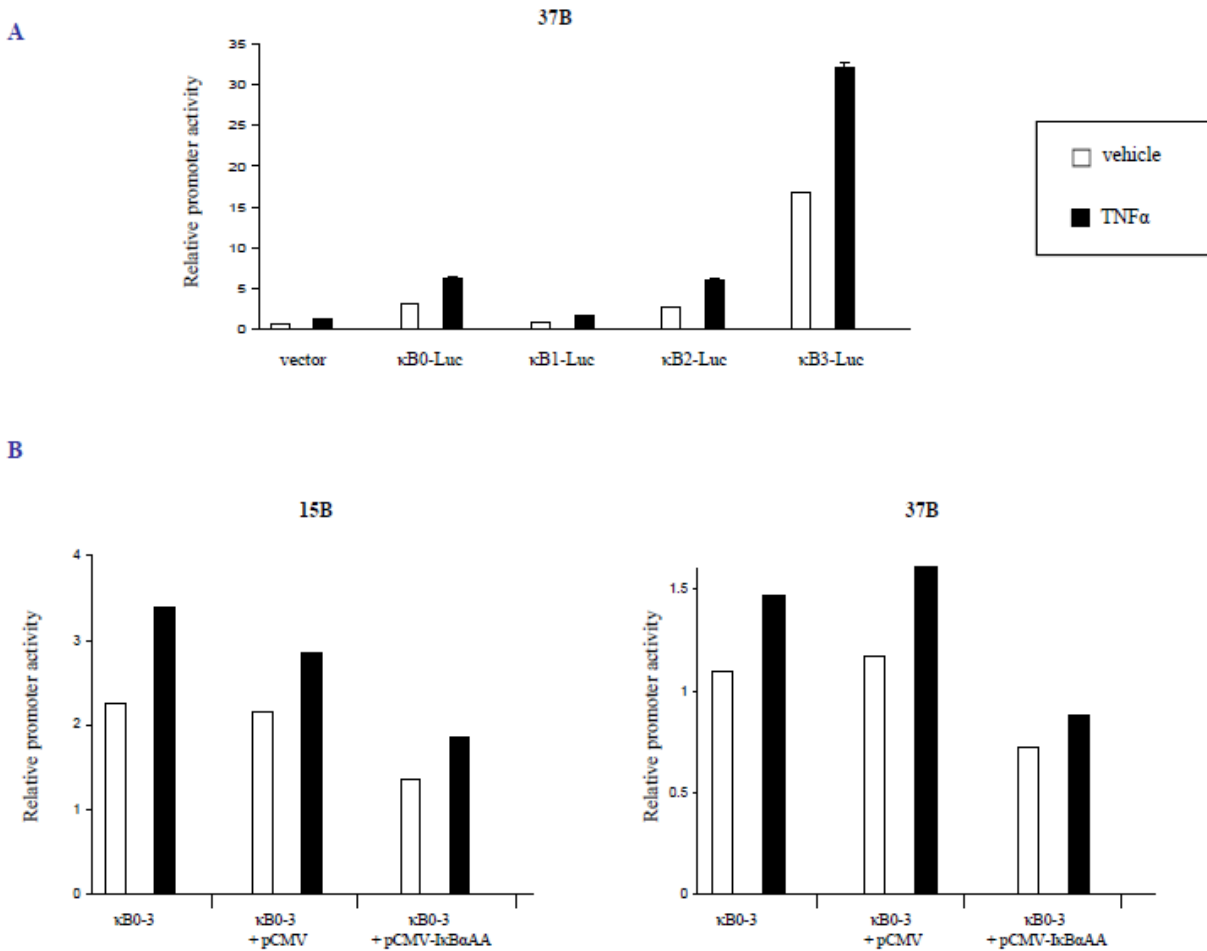
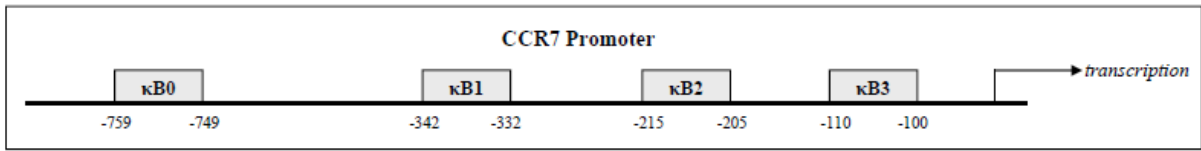


Figure 2-2: The CCR7 κ B sites are functionally important for inducible gene expression. (A) PCI-37B cells were transfected with each κ B luciferase construct. After 24hrs, the transfected cells were induced with TNF α (20ng/ml, 24hr), after which promoter activation was examined by assaying luciferase levels. Firefly luciferase activity was normalized to renilla luciferase to obtain relative promoter activity. Error bars show mean \pm standard error. (B) PCI-15B and -37B cells were co-transfected with the full length CCR7 κ B0-3 luciferase construct and an I κ B α AA superrepressor. After 24hr, transfected cells were induced with TNF α (20ng/ml, 24hr) and promoter activation assayed by measuring luciferase levels. Each experiment was performed at least twice with similar results.

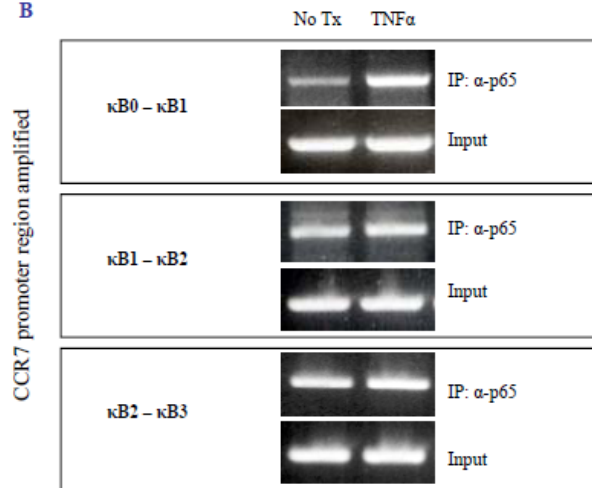
2.3.3 Activated NF- κ B is bound to the CCR7 promoter *in vivo*

To analyze whether activated NF- κ B is directly recruited and bound to the CCR7 promoter *in vivo*, ChIP assays were performed on metastatic SCCHN cells. A p65-specific mAb was used to immunoprecipitate genomic regions that were bound by NF- κ B. The results show that the κ B0- κ B1 region demonstrated inducible binding of NF- κ B p65 after TNF α stimulation, whereas κ B1- κ B2 and κ B2- κ B3 regions of the proximal promoter showed the greatest level of constitutive binding relative to input (Figure 2-3A, 2-3B) (See materials and methods – chapter 2.2 – for the primers used in ChIP analysis). Time course experiments show that NF- κ B was recruited to the CCR7 promoter within 30 min and remained bound to the promoter for up to 60 min after TNF α stimulation (Figure 2-3C). For comparison, the kinetics of p65 recruitment to the CCR7 promoter appear to resemble those of p65 recruitment to the well studied p65-dependent I κ B α promoter. These data confirm the *in vivo* recruitment of NF- κ B to the CCR7 promoter, and provide a direct mechanism for the modulation of CCR7 expression by constitutive and inducible NF- κ B activation. The observed constitutive NF- κ B binding suggests that NF- κ B may be contributing to the observed overexpression of CCR7 on these metastatic SCCHN cell lines.

A



B



C

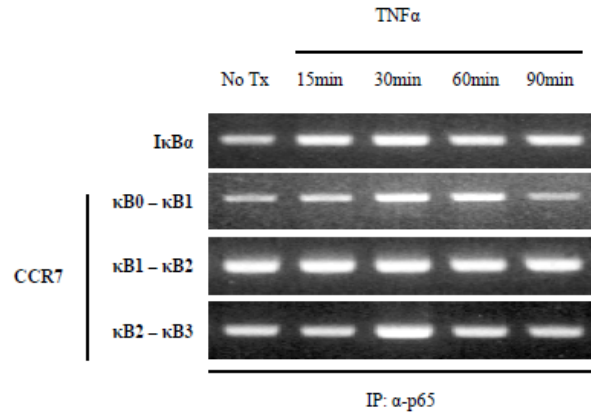


Figure 2-3: Activated NF- κ B is bound to the CCR7 promoter *in vivo*. (A) Schematic representation of the CCR7 promoter showing the position of each κ B site relative to the transcription start site. (B) ChIP assay on PCI-37B induced with TNF α (20ng/ml, 30min). Cell extracts were fixed, bound to a p65 antibody and the immunoprecipitated genomic regions were run in a PCR reaction using primers that encompassed overlapping sections of the CCR7 promoter. (C) PCI-6B cells were induced with TNF α as shown and the timeline of NF- κ B recruitment to the I κ B α and CCR7 promoter examined by ChIP assay.

2.3.4 Activation of NF- κ B induces CCR7 gene transcription

To evaluate whether activation and recruitment of NF- κ B to the CCR7 promoter was functionally related to CCR7 gene transcription, we transfected a vector expressing the I κ B α AA super-repressor into metastatic SCCHN cells and evaluated the expression of CCR7 mRNA using quantitative RT-PCR. The blockade of NF- κ B activation resulted in a 40-60% ($p < 0.05$) decrease in CCR7 mRNA expression (Figure 2-4A). We then investigated whether the activation of NF- κ B by a pro-inflammatory cytokine such as TNF- α could result in induction of CCR7 mRNA. The results show that TNF- α was able to induce CCR7 mRNA levels by up to 10-fold ($p < 0.05$) after 6 hours of stimulation (Figure 2-4B). The observed CCR7 mRNA induction subsided after 8 hours of stimulation possibly due to the induction of negative regulatory mechanisms on NF- κ B. Together, these data suggest that the basal and inducible activity of the CCR7 gene is dependent upon NF- κ B activation.

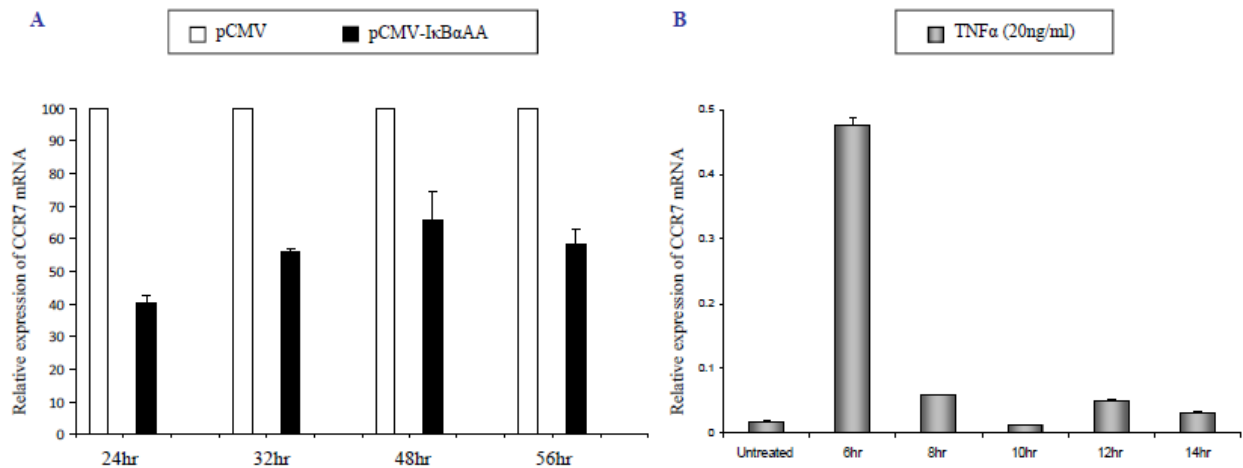


Figure 2-4: NF- κ B activation is required for CCR7 gene expression. (A) PCI-37B cells were transfected with either an empty pCMV plasmid or with one containing the I κ B α AA superrepressor. At various timepoints, CCR7 mRNA expression was examined by quantitative RT-PCR. (B) PCI-37B cells were treated with TNF α (20ng/ml) for different lengths of time as shown. CCR7 mRNA expression was assayed by quantitative RT-PCR. (* $p < 0.05$)

2.3.5 Constitutive NF-κB activation correlates with CCR7 expression *in vivo*

To evaluate the *in vivo* significance of constitutive NF-κB activation in SCCHN, we utilized immunohistochemical analysis on a tissue microarray. Firstly, we compared cytoplasmic NF-κB expression between tumor and adjacent mucosa (Figure 2-5A). Consistent with previous reports of constitutive NF-κB activation in SCCHN, we observed significantly higher NF-κB expression in the tumors as compared to adjacent mucosa ($p < 0.001$, $N = 13$). Interestingly, we observed a positive correlation when we compared cytoplasmic NF-κB activation to CCR7 expression (Figure 2-5B). These data confirmed the constitutive activation of NF-κB in SCCHN. Furthermore, the observed positive correlation between NF-κB and CCR7 expression provide further evidence that NF-κB may be a key transcription factor involved in the regulation of CCR7 expression.

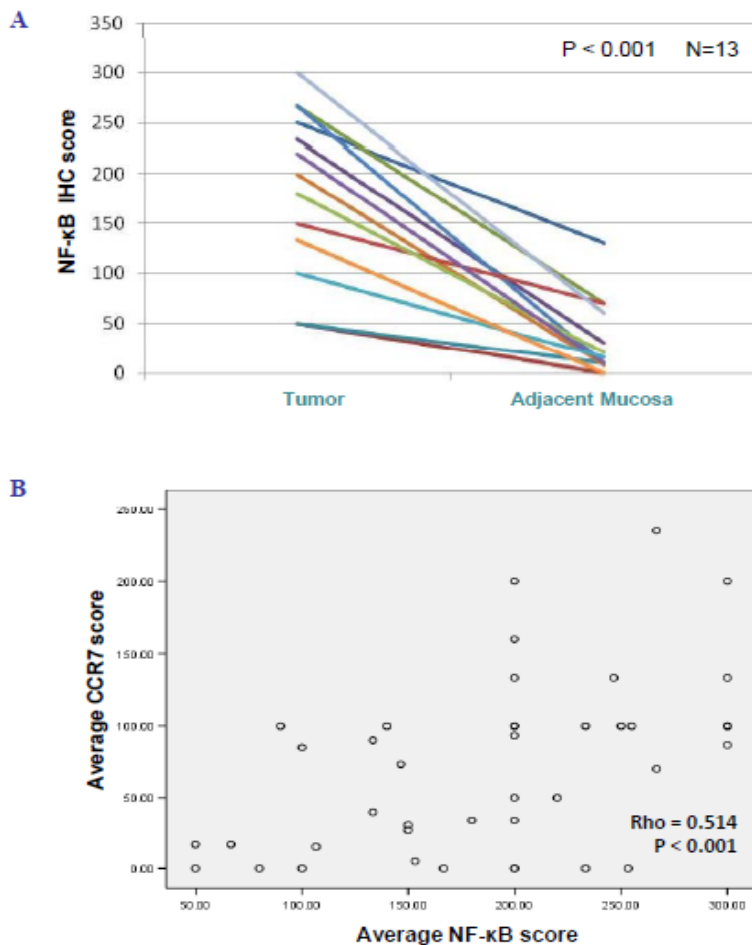


Figure 2-5: Constitutive NF-κB activation correlates with CCR7 expression *in vivo*. (A) Immunohistochemical scoring analysis of 13 paired specimens of a tissue microarray depicting NF-κB expression as scored by a pathologist. Comparisons are made between tumor tissue and adjacent mucosa. (B) Scatterplot of cytoplasmic NF-κB expression versus CCR7 expression in tumors. A positive Spearman's rank correlation coefficient (ρ) depicts positive correlation between the two variables.

2.3.6 Schematic of NF- κ B dependent CCR7 expression

Our proposed model for NF- κ B mediated CCR7 expression is outlined in Figure 2-6. Extracellular ligand-receptor interactions result in the activation of the I κ B kinase (IKK) complex, which in turn phosphorylates I κ B and targets it for proteasomal degradation. This allows for the release and subsequent activation of NF- κ B, which translocates into the nucleus and induces gene expression at the CCR7 locus. The resulting increase in CCR7 gene expression supports enhanced mRNA and surface receptor levels. The identification of this NF- κ B-dependent CCR7 pathway provides potential targets for clinical therapeutic intervention in metastatic SCCHN.

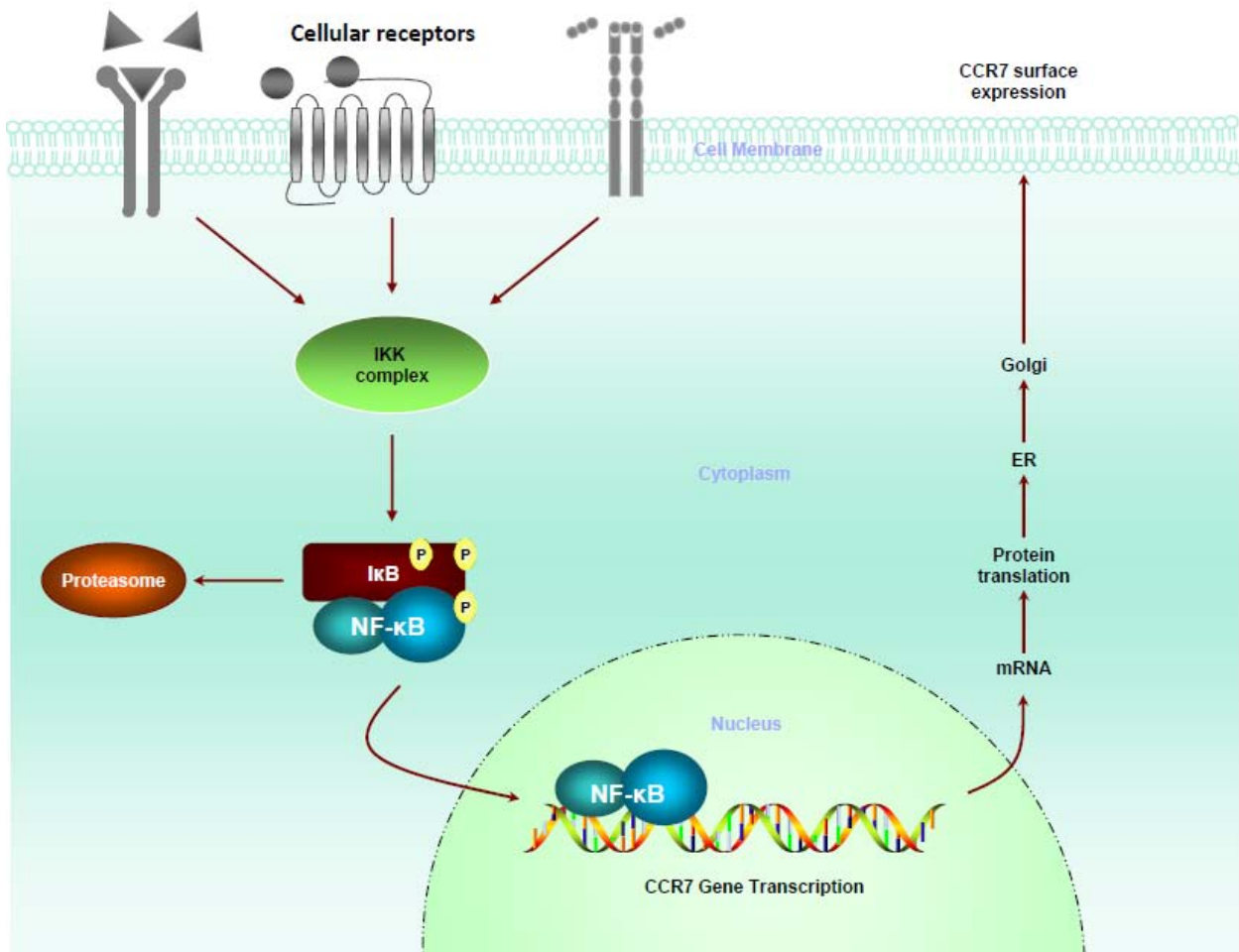


Figure 2-6: Proposed model of NF- κ B mediated CCR7 gene expression.

2.4 DISCUSSION

As previously mentioned, we and others have shown that CCR7 is highly expressed by metastatic lymph node-derived tumor cells. Indeed, in our studies, higher levels of CCR7 expression are correlated with a highly metastatic phenotype in SCCHN (141). As such, the expression of CCR7 is reported to be a poor prognostic factor in esophageal and squamous cell carcinomas. Recent studies have shown that CCR7 signaling can activate prosurvival pathways in tumors, DCs and T cells through PI3K/Akt activation, as well as pro-invasive characteristics in tumors (142, 143, 423). Furthermore, the expression of CCR7 in tumors was sufficient to induce lymph node metastasis in a B7 melanoma model suggesting that CCR7 was involved in facilitating the observed organ-specific metastases (109).

In our previous studies, we also observed that the CCR7-positive metastatic tumors secreted CCL19 and CCL21 which were responsible for maintaining a basal level of PI3K/Akt activation (144). This pathway has been suggested to result in the activation of NF- κ B, which is found basally active in many SCCHN cell lines. Some studies have suggested that CCR7 may be an NF- κ B target gene (165). Notably, a recent study showed that NF- κ B activation can regulate CCR7 expression and lymph node metastasis in breast cancer (424). There is also evidence to support the regulation of CCL19 and CCL21 by alternative NF- κ B complexes (419). As such, our observed autocrine secretion of ligands by CCR7-positive cells prompted us to investigate whether NF- κ B was a mediator between ligand involvement with the CCR7 receptor and downstream activation of the CCR7 gene and its ligands.

Our analysis identified four potential κ B elements on the CCR7 promoter whose activity was examined in EMSA and luciferase assays. Although two of these sites (κ B1 and κ B2) have been previously identified (165), they have not been extensively studied with respect to their specific contribution to CCR7 expression. To the best of our knowledge, the κ B0 and κ B3 sites are novel to our study and have not been reported elsewhere. Importantly, all four κ B sites appear to have significant NF- κ B binding and activity. It is also notable that κ B1 may be involved in some non-specific binding as evidenced by the inability of κ B consensus probe to compete away all the observed binding. Interestingly,

the p50/p65 heterodimer was the predominant NF- κ B moiety present in the cells, and bound to each CCR7 κ B site. Furthermore, our findings suggest that autocrine CCR7 signaling does not contribute to NF- κ B activation in these metastatic cells.

Using luciferase assays to test each κ B site separately, our results show significant basal activity at each site which was further inducible by TNF α . It is likely that constitutively active NF- κ B present in these cells was responsible for maintaining this basal activity. Remarkably despite little observable NF- κ B binding on the κ B3 site by EMSA analysis, the luciferase assay data show that the 200bp region encompassing the κ B3 site retained the highest promoter activity when compared to the other three sites. Moreover, the use of an I κ B α AA super-repressor only blocked approximately 30-50% of the basal promoter activity, suggesting that NF- κ B may not function solely and other transcription factors might be involved in the co-regulation of the CCR7 promoter. Evidence in the literature suggests that CCR7 expression can be co-regulated by both NF- κ B and AP1 transcription factors (425, 426). To be sure, subsequent examination of the CCR7 promoter revealed the presence of a consensus AP1 binding site within the CCR7 promoter κ B3 region (position -99 to -93). Further studies are required to determine whether AP1 transcription factors are indeed involved in the co-regulation of the CCR7 promoter.

The use of *in vivo* CHIP assays confirmed the direct recruitment of NF- κ B onto the CCR7 promoter. Our studies demonstrated constitutive NF- κ B binding on the CCR7 promoter which increased in the presence of inducible TNF α signaling. In addition, the data showed that the κ B0- κ B1 region appeared to be most responsible for inducible binding, although we cannot discount the involvement of the other κ B sites. As the results show, the recruitment of NF- κ B onto the CCR7 promoter appeared to follow the same mechanics as its recruitment to the I κ B promoter whereby binding was observed incrementally up to 30-60 minutes post stimulation and then dropped off, presumably due to negative regulatory mechanisms.

We also tested the effects of NF- κ B activation and inhibition on the expression of *CCR7* mRNA in metastatic SCCHN. The data show that induction of NF- κ B activity using TNF α resulted in increased levels of *CCR7* mRNA. Conversely, the inhibition of NF- κ B activity using an I κ B α AA super-repressor

resulted in decreased levels of *CCR7* mRNA. These findings offer relevance to the regulation of *CCR7* by NF- κ B by showing direct effects on *CCR7* mRNA levels.

Finally, an *in vivo* examination of NF- κ B activation confirmed increased expression in tumors as compared to adjacent mucosa. Moreover, a comparison of tumor NF- κ B and *CCR7* expression revealed a positive correlation between the two tumor markers further supporting our hypothesis that NF- κ B is a key regulator of *CCR7* expression. We present our model of NF- κ B-dependent *CCR7* expression in Figure 2-6. Despite the likely involvement of other co-transcription factors, the data presented here argues for a key role of NF- κ B in regulating *CCR7* expression. The therapeutic targeting of components along this signaling pathway should provide promise for clinical intervention in metastatic SCCHN.

HUMAN β -DEFENSIN 3 PROMOTES NF- κ B MEDIATED CCR7 EXPRESSION AND ANTI-APOPTOTIC SIGNALS IN SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

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Carcinogenesis, 2011; 32(2):168-74

Format adapted for Dissertation

3.0 HUMAN β -DEFENSIN 3 PROMOTES NF- κ B MEDIATED CCR7 EXPRESSION AND ANTI-APOPTOTIC SIGNALS IN SCCHN

3.1 INTRODUCTION

The β -defensins are highly expressed in cells and tissues that are involved in host defense such as the cutaneous and the mucosal surfaces, where they provide an innate first line of defense against invading pathogens. However, despite the advances in understanding the role of β -defensins in immunity, their function in oncogenesis of aerodigestive cancers, which are derived from HBD-producing epithelial cells (427-429) has not been well characterized. In oral squamous cancers such as SCCHN, HBD3 is frequently overexpressed and has been implicated in the pathogenesis of these malignancies (398, 430) but the mechanism(s) of such an effect is undetermined. In oral cancers, HBD3 expression has been correlated with the CCR2-mediated infiltration of tumor-associated macrophages, which are known to generate an inflammatory tumor microenvironment and promote tumor aggressiveness (399, 400).

We have previously reported that CCR7 is upregulated in metastatic SCCHN cells activating anti-apoptotic and invasive pathways, and contributing to cisplatin resistance (141, 143). However, the signals that can induce CCR7 expression in primary, non-metastatic tumors and promote tumor progression are largely unknown. Here, we examine the effects of hBD3 stimulation on non-metastatic SCCHN tumor cells. We hypothesized that hBD3 might induce CCR7 expression in these primary SCCHN tumor cells, providing migratory capability as well as pro-survival signals within the tumor microenvironment. We also investigated the role of NF- κ B as an inflammatory mediator of the putative hBD3-mediated CCR7 expression and pro-apoptotic effects. Our findings suggest that hBD3 expression can be exploited by developing tumors to enhance their growth, survival and evolution into a metastatic phenotype. In

particular, the hBD3-stimulated induction of CCR7 expression in primary tumors may contribute to the predictable pattern of regional lymph node metastases commonly observed in SCCHN.

3.2 MATERIALS AND METHODS

Cell lines

Human SCCHN cell lines PCI-6A, PCI-15A and PCI-37A were derived from the non-metastatic primary tumor site and characterized at the University of Pittsburgh (431). All cells were cultured in DMEM medium (Invitrogen Corp., Carlsbad, CA) containing 8% (v/v) heat-inactivated fetal bovine serum (Equitech-Bio, Ingram, TX), 100 units/ml penicillin G, and 100µg/ml streptomycin and 4mM L-glutamine (Invitrogen, Carlsbad, CA). The immortalized human keratinocyte cell line was a kind gift from Dr. Stephan Duensing (432). All cells were regularly tested for mycoplasma infection to ensure that only mycoplasma-free cell lines were studied in our assays.

Antibodies & Reagents

TNF- α , the CCR6 ligand, **Macrophage Inflammatory Protein-3 α** (MIP3 α /CCL20) and the CCR7 ligand, **Macrophage Inflammatory Protein-3 β** (MIP3 β /CCL19) were all purchased from R&D Systems (Minneapolis, MN). The human β -defensins HBD2, HBD3 and HBD4 were purchased from Peptides International (Louisville, KY). The PI3Kinase inhibitor LY294002 and the Akt/PKB inhibitor (1L6-Hydroxymethyl-chiro-inositol-2-(R)-2-O-methyl-3-O-octadecyl-*sn*-glycerocarbonate) were obtained from Calbiochem (San Diego, CA). BAY 11-7082, Wortmannin, Nocodazole, Cytochalasin D and Dimethyl Amiloride were all obtained from Sigma-Aldrich (St. Louis, MO). Peptide inhibitors were purchased from Imgenex (San Diego, CA): IKK- γ NEMO binding domain (NBD) inhibitor (DRQIKIWFQNRRMKWKKTALDWSWLQTE), MyD88 homodimerization inhibitor (DRQIKIWFQNRRMKWKKRDVLP-GT), and control peptide (DRQIKIWFQNRRMKWKK). The TRIF peptide inhibitor was purchased from InvivoGen (San Diego, CA): RQIKIWFQNRRMKWKKFCEEVQVPGRGELH, and control peptide: RQIKIWFQNRRMKWKK-SLHGRGDPMEAFII. Antibodies used were: fluorescein conjugated anti-human CCR7 (R&D Systems, Minneapolis, MN), rabbit anti-Akt, phospho-Akt (Ser 473) and pIkB α (Cell Signaling Tech., Beverly, MA), NF- κ B p65 and IkB α (Santa Cruz Biotech., CA) and γ -tubulin

(Abcam, Cambridge, MA). The HIV- κ B luciferase vector was a kind gift from Dr. Lawrence Kane (University of Pittsburgh, Department of Immunology), while the AP1-luciferase and Renilla-luciferase vectors were a kind gift from Dr. Pamela Hershberger (University of Pittsburgh Cancer Institute). The dual luciferase reporter assay kit was obtained from Promega (Madison, WI). Annexin V apoptosis detection kit was purchased from BD Biosciences. Fluorescein isothiocyanate (FITC) labeling kit was obtained from Pierce Biotechnology (Rockford, IL).

Flow Cytometry

Cells were cultured in serum-free media for 24-48hr prior to manipulation. Briefly, cells were treated with hBD3 (1 μ M, 24hr) or as indicated. Following treatment, cells were washed with sterile 1 x PBS buffer (Sigma-Aldrich, St. Louis, MO), collected by scraping and incubated with FITC-conjugated hCCR7 mAb (150503) (R&D Systems, Minneapolis, MN). Irrelevant isotype-matched FITC-IgG antibody (BD Biosciences, San Jose, CA) was used as a control. After incubation, cells were washed twice and fixed using 2% paraformaldehyde in PBS. Fluorescence was read using Beckman Coulter Epics XL cytometer, with the isotype control set to a mean fluorescence index x-mean of 5, and analyzed using Expo 32 software (Beckman Coulter, FL).

Transcription Factor ELISA

Serum starved cells were treated with appropriate stimulants and nuclear extracts prepared using the nuclear extraction kit provided by Active Motif (Carlsbad, CA). Following protein determination by BCA assay (Pierce Biotechnologies), 2-4 μ g of nuclear protein was loaded onto each well of a 96-well plate and assayed for NF- κ B family or AP1 family transcription factor activity using the respective TransAM transcription factor assay kits (Active Motif). This assay utilizes a plate-bound oligonucleotide probe which binds specifically to transcription factors contained in nuclear extracts. By using antibodies directed against each subunit, the transcription factor complex bound to the oligonucleotide is detected and quantified.

Chemotaxis Assay

Cell migration studies were performed as described previously (141). Briefly, 30 μ l aliquots of chemoattractant (CCL19 or CCL20 at 500ng/ml) were added in triplicate to the wells of a disposable 96-well chemotaxis chamber (ChemoTx Neuroprobe, Gaithersburg, MD) with an 8 μ m pore size filter and 5.7mm width/well. Cell suspensions (1×10^6 cells/ml) were placed on top of the filter. After 4hr incubation at 37°C, the filter was washed gently with media. To release adherent tumors cells, a 30 μ l aliquot of trypsin-EDTA was added to the filter (5min, 37°C) before centrifuging the plate (1400rpm, 5min). The filter was removed and the cells in each lower well were counted under a light microscope. The results are presented as chemotactic index, defined as the fold increase in cell migration in chemoattractant medium over migration in media alone.

Immuno-Blot Analysis

To prepare whole cell extracts, 50-70% confluent cells were serum-starved for 24-48hr. Cells were then treated with appropriate stimulation and then harvested in lysis buffer (10mM Tris-HCl pH 7.6, 50mM Na₄P₂O₇, 50mM NaF, 1mM NaV₃O₄, 1% Triton X-100 and protease inhibitors), vortexed at 4°C for at least 1hr and the supernatant containing whole cell extract was collected. To prepare nuclear extracts, cells were stimulated as appropriate, washed, collected and resuspended in cell lysis buffer (10mM Hepes, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT, 0.5mM PMSF, Protease and Phosphatase Inhibitors). After allowing cells to swell on ice for 15 mins, 1% NP-40 was added and cell suspension vortexed. After centrifugation, the nuclear pellet was resuspended in nuclear lysis buffer (20mM Hepes, 400mM NaCl, 1mM EDTA, 1mM EGTA, Glycerol, 1mM DTT, 1mM PMSF, Protease and Phosphatase inhibitors). Samples were vortexed at 4°C for at least 1 hour. After determining protein concentration using BCA Protein Assay kit (Pierce Biotechnologies), 50 μ g of whole cell or nuclear extract proteins were size-fractionated through a 4-12% SDS-PAGE gel, transferred to PVDF membrane and immunoblotted with the relevant antibodies.

siRNA Transfection

Cells were grown to 50% confluence and transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) with 200nM of either p65 (RelA) or non-targeting control siRNA (Ambion, Austin, TX). Transfected cells were incubated for 24-48hrs before analysis.

Luciferase Assay

The day before transfection, cells were plated so that they would be 50% confluent on the day of the transfection. Transient co-transfections of firefly luciferase (2 μ g) and a renilla luciferase (0.1 μ g) control were performed using Polyfect reagent (Qiagen, Valencia, CA). After 24 hours, cells were stimulated with relevant cytokine for another 24 hours. The cells were lysed in passive lysis buffer (provided in the dual luciferase reporter assay kit) and the firefly and renilla luciferase activity measured using an AutoLumat LB953 Luminometer (Berthold Technologies). Relative promoter activity was calculated as firefly luciferase activity / renilla luciferase activity.

Apoptosis Detection

Cells were pre-treated with hBD3 (0.25 μ M, 1hr) and then treated with apoptosis-inducing agent, cisplatin (40 μ M, 4hr). To examine whether hBD3-induced survival was dependent on Akt activation, cells were pre-treated with an Akt inhibitor (10 μ M, 3hr) prior to hBD3 and cisplatin treatment. Cellular apoptosis was assessed by Annexin V and 7-amino-actinomycin D (7AAD) staining using flow cytometry (where early apoptotic cells were Annexin V positive and 7AAD negative). Briefly, cells were harvested by gentle scraping and washed with 1 x PBS. The cells were then stained with 5 μ l Annexin V-FITC and 5 μ l 7AAD in 100 μ l binding buffer (10mM HEPES, 140 μ M NaCl, 5mM KCl, 1mM MgCl₂, 1.8mM CaCl₂ pH 7.4) for 15 min at room temperature in the dark. After staining, cells were analyzed within 1hr by flow cytometry.

Labeling & Detection of fluorescent HBD3

The labeling of HBD3 was performed using a FITC labeling kit from Pierce Biotechnology. Briefly, lyophilized HBD3 was resuspended at 1mg/ml in 0.05M borate buffer. The diluted HBD3 was then incubated with a pre-measured vial of FITC for 1hr at room temperature. Following resin purification to remove excess FITC, labeled HBD3-FITC was obtained and stored at 4°C until use. To analyze internalization of fluorescently labeled hBD3 peptide, tumor cells were collected by trypsinization, washed with 1 x PBS, resuspended in AIM-V medium and treated with 1-2µM hBD3-FITC for the indicated periods of time. At the conclusion of each treatment, 0.1% azide/PBS solution was added to the cells. The cells were then washed twice with 1 x PBS and fixed using 2% paraformaldehyde in PBS. Fluorescence was read using either: a Beckman Coulter Epics XL cytometer and analyzed using Expo 32 software (Beckman Coulter, FL), or using an ImageStream 100 imaging flow cytometer (AMNIS, WA). Alternatively, cells were grown on a cover slip and treated with 1µM hBD3-FITC for the indicated periods of time. The cells were then washed with 0.1% Azide/PBS solution and then with 1xPBS, fixed using 2% Paraformaldehyde/PBS, stained with PKH26 (membrane dye) and DAPI (nuclear dye) (Sigma-Aldrich, St. Louis, MO), and then examined by fluorescence microscopy using an Olympus Fluoview 1000 Confocal Microscope at 60X magnification.

NF-κB Immunofluorescence & Confocal Microscopy

Cells were grown on a cover slip and then treated with appropriate stimulation. The samples were then fixed in 2% paraformaldehyde/PBS for 15 min, followed by permeabilization in 0.1% Triton X-100 for another 15 min. After successive washes in 0.5% BSA/PBS, the samples were first blocked using 2% BSA/PBS for 1hr before being incubated overnight at 4°C in a 1:50 dilution of rabbit anti-NF-κB p65 antibody (Santa Cruz). Secondary goat anti-rabbit IgG-FITC antibody (Santa Cruz) was added at a 1:100 dilution for 2hrs at room temperature. Finally samples were stained for 1 min with nuclear DAPI stain (Sigma), washed and adhered to a slide using gelvatol solution. Confocal laser microscopy was performed using an Olympus Fluoview 1000 Confocal Microscope at 60X magnification.

Human tissue samples & Immunohistochemistry (IHC)

Tumor levels of HBD3 and CCR7 were evaluated by immunohistochemical (IHC) staining of tumor and adjacent mucosal specimens arrayed in a previously described tissue microarray (TMA) (433). For the studies presented here, the maximum number of evaluable tumor specimens was 47. Of these tumors, 13 had arrayed adjacent mucosal tissues available for analysis. A summary of patient and tumor characteristics is provided in Table 3-1. Tissue microarray quality assessment and morphologic confirmation of tumor or normal histology, one H&E-stained slide was evaluated for every ten tissue sections.

Arrayed tissues were IHC stained for HBD3 and CCR7, and tissue levels were evaluated semi-quantitatively. Prior to incubation with anti-CCR7 antibody (1:100) (cat#GTX7 1695; Gene Tex Inc., Irvine, California) for 30 minutes at room temperature, antigen retrieval was performed using Dako Citrate pH 6 buffer (cat#S1699; Carpinteria, California) in the Biocare Decloaking chamber. Endogenous peroxidases were quenched with 3% hydrogen peroxide (5 minutes at room temperature), and slides blocked with CAS block (cat#00-8120; Invitrogen, Carlsbad, California) for 10 minutes at room temperature. CCR7 staining was developed using Rabbit Envision polymer (cat# K4003; Dako) for 30 minutes at room temperature followed by incubation with Substrate Chromagen (cat# K3468; Dako) for 5 minutes at room temperature. Slides were counterstained with Harris Hematoxylin. The plasma membrane and cytoplasmic staining intensity (0 – 3) as well as percent of tumor to the nearest 5% were determined by a head and neck cancer pathologist (LW and RS). An IHC score was derived from the product of the intensity and percentage of tumor stained, and IHC scores for each core of a specimen were averaged.

Prior to incubation with anti-Defensin Beta 3 antibody (1:800) (Cat# NB200-17; Novus Biologicals, Littleton, Colorado) for 60 minutes at room temperature, antigen retrieval, quenching and blocking were performed as for the CCR7 staining. Staining was developed using Dual Envision+ secondary (cat#K4061; Dako) for 30 minutes at room temperature followed by incubation with Substrate Chromagen. IHC staining was evaluated and scored as described for CCR7.

Statistical Analysis

For the IHC analysis, differences between paired tumor and adjacent mucosa levels of each protein were evaluated using the signed-rank test. Correlations between proteins were assessed using Spearman's nonparametric correlation coefficient. In other experiments, data are expressed as mean \pm standard error of at least three repeats. An unpaired t-test was used to calculate whether observed differences were statistically significant. The threshold for significance was $p < 0.05$.

Table 3-1. Subject and Tumor Characteristics		
Characteristic	HNSCC Case	
	(N=47)	
Age (Years)		
Median (Range)	61 (24 - 80)	
Sex		
Male	33	70.2%
Female	14	29.8%
Ethnicity		
Caucasian	46	97.9%
African Amer.	1	2.1%
Smoking Status		
Never smoker	5	10.6%
Former smoker	20	42.6%
Active smoker	21	44.7%
Unknown	1	2.1%
Tumor Site		
Oral Cavity	20	42.6%
Oropharynx	8	17.0%
Hypopharynx	2	4.3%
Larynx	15	31.9%
Other	2	4.3%
Disease Stage		
I	3	6.4%
II	10	21.3%
III	5	10.6%
IV	25	53.2%
Unstaged [§]	4	8.5%
[§] Unstaged metastatic or recurrent HNSCC		

3.3 RESULTS

3.3.1 HBD3 is overexpressed in SCCHN and correlates with CCR7 expression *in vivo*

Previous studies have reported that HBD3 expression is deregulated in SCCHN (398). To confirm and extend these findings, we utilized immunohistochemistry on a tissue microarray to compare HBD3 expression in SCCHN tumor tissue versus adjacent normal mucosa (see Table 3-1 for subject and tumor characteristics). Indeed, increased levels of HBD3 staining were detected in tumor tissue compared to adjacent mucosa (Figure 3-1A). Furthermore, when 13 paired specimens were scored and compared, our data show that HBD3 expression was significantly increased in tumor tissue ($p < 0.001$) (Figure 3-1B). Interestingly, a comparison of HBD3 and CCR7 scoring intensities revealed a positive correlation between these markers (Figure 3-1C). Together, these *in vivo* data show that HBD3 is overexpressed in SCCHN and is significantly correlated with CCR7 expression.

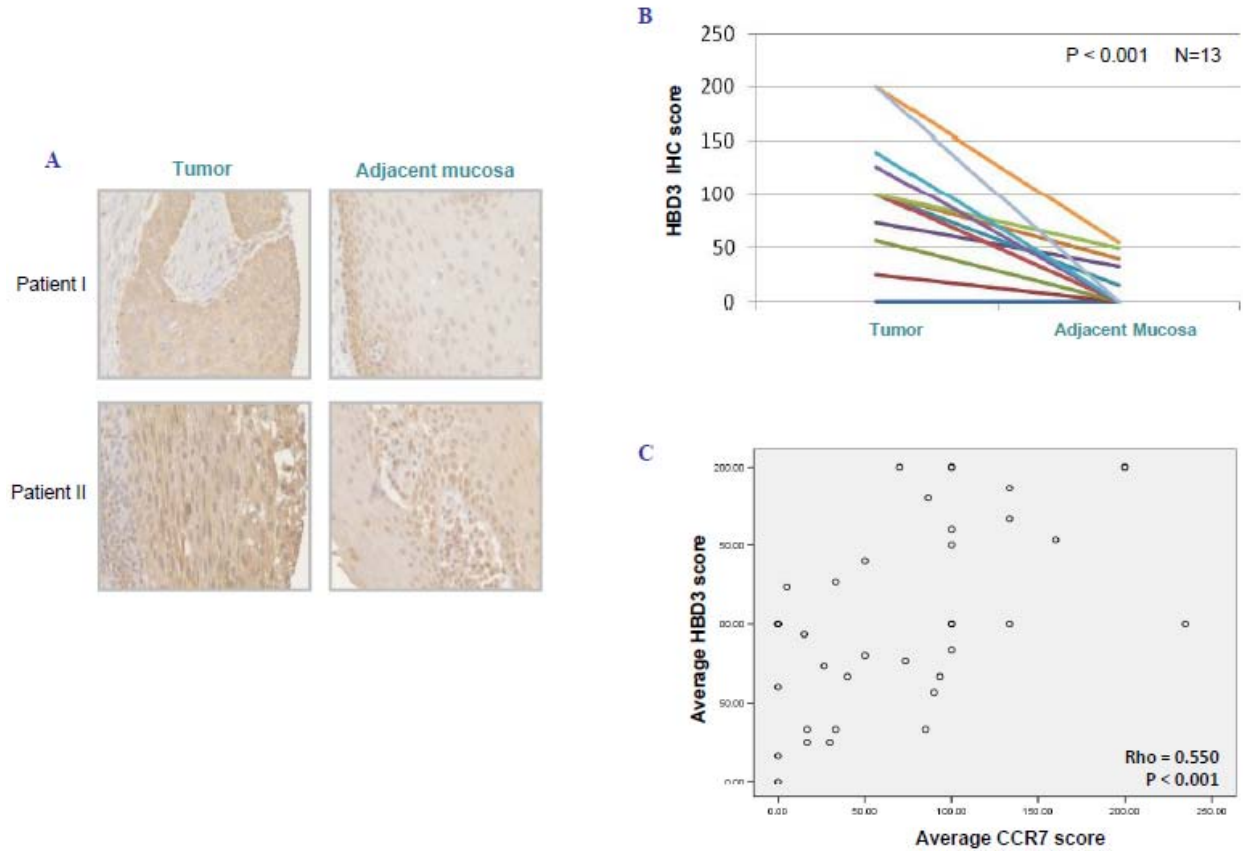


Figure 3-1: HBD3 is overexpressed by SCCHN tumors and correlates with CCR7 expression *in vivo*. (A) Representative immunohistochemical staining of HBD3 on two patient samples showing the staining on both tumor and adjacent mucosa. (B) Immunohistochemical scoring analysis of 13 paired specimens of a tissue microarray depicting HBD3 expression as scored independently by a pathologist. Comparisons are made between tumor tissue and adjacent mucosa. (C) Scatterplot of HBD3 expression versus CCR7 expression in tumors. A positive Spearman's rank correlation coefficient (ρ) depicts positive correlation between the two variables.

3.3.2 HBD3 upregulates CCR7 on primary, non-metastatic SCCHN tumor cells

We previously showed that CCR7 is upregulated in metastatic SCCHN cell lines (PCI-6B, -15B, and -37B) but not in the parental, nonmetastatic SCCHN cells lines derived from the primary head and neck tumor site (141). To determine whether HBD3 could stimulate CCR7 expression in nonmetastatic tumor cells, we treated the parental primary SCCHN tumor cells, PCI-6A, -15A and -37A, with HBD3 (1 μ M for 24hr at 37°C) and examined CCR7 expression by flow cytometry (Figure 3-2A). Under these conditions, we found that CCR7 surface expression was significantly upregulated in primary SCCHN tumor cells following HBD3 stimulation ($p < 0.05$). The induction of CCR7 expression showed a dose-response to HBD3 concentration and was detected at HBD3 concentrations $\geq 0.25\mu$ M (not shown), and CCR7 expression reached a plateau at approximately 1 μ M. Notably, the observed increase in CCR7 expression could not be attributed to a non-specific increase in cellular protein synthesis as the expression of HLA class I molecules did not increase under these conditions (Figure 3-2B). Furthermore, the use of heat-inactivated HBD3 or the use of other defensins HBD2 or HBD4 did not result in CCR7 upregulation (Figure 3-2C).

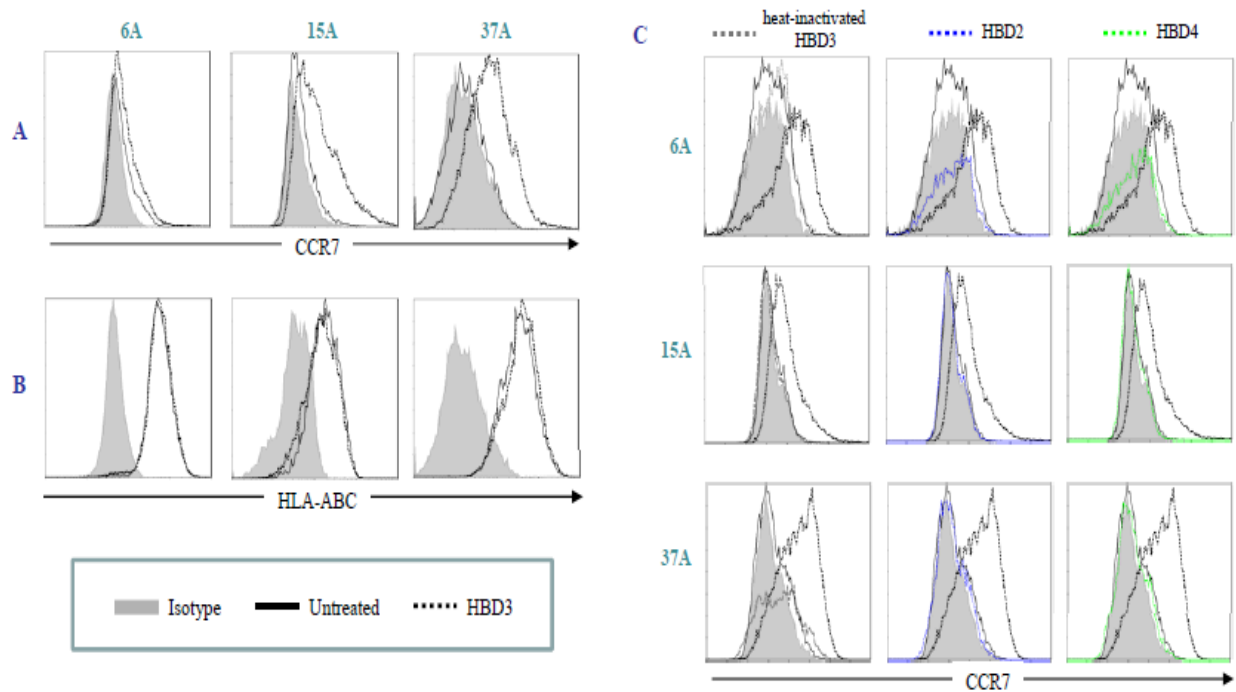


Figure 3-2: HBD3 stimulation activates CCR7 expression in primary tumor derived SCCHN tumor cells. PCI-6A, -15A, -37A were stimulated with HBD3 (1 μ M, 24hr) and assayed for (A) CCR7 expression or (B) HLA-class I molecules by flow cytometry. Results shown are representative of at least three experiments with similar results. (C) PCI-6A, -15A, -37A were stimulated with heat-inactivated HBD3, HBD2 or HBD4 (1 μ M, 24hr) and CCR7 expression compared that from to HBD3-induced cells.

3.3.3 HBD3-induced CCR7 promotes migration towards CCL19

To evaluate the functional significance of HBD3-induced CCR7 expression on primary SCCHN tumor cells, we assayed the migration of HBD3-treated tumor cells to the CCR7 ligand CCL19, using *in vitro* transwell chemotaxis chambers. As expected, untreated cells did not migrate towards CCL19 in the transwell assay. However, after HBD3 exposure (0.25 μ M, 24hr), we observed a significant ($p < 0.006$) increase in CCR7 and tumor cell migration toward CCL19 (Figure 3-3). Notably, since these primary SCCHN tumor cells express CCR6, they were able to migrate to the CCR6 ligand CCL20 in the absence of any stimulation. Furthermore, it appears that HBD3 stimulation had negligible effects on CCR6 expression since the CCL20-induced chemotactic activity was not significantly different between untreated and treated cells. More importantly, these data show that HBD3 stimulation of primary, non-metastatic SCCHN tumor cells results in the upregulation of a functional CCR7 receptor. Interestingly, we observed a two-fold increase in migratory response in both PCI-6A and -37A despite differing levels of CCR7 upregulation (Figure 3-2A), suggesting that even a small increase in CCR7 expression is sufficient to induce a functional response in these cells.

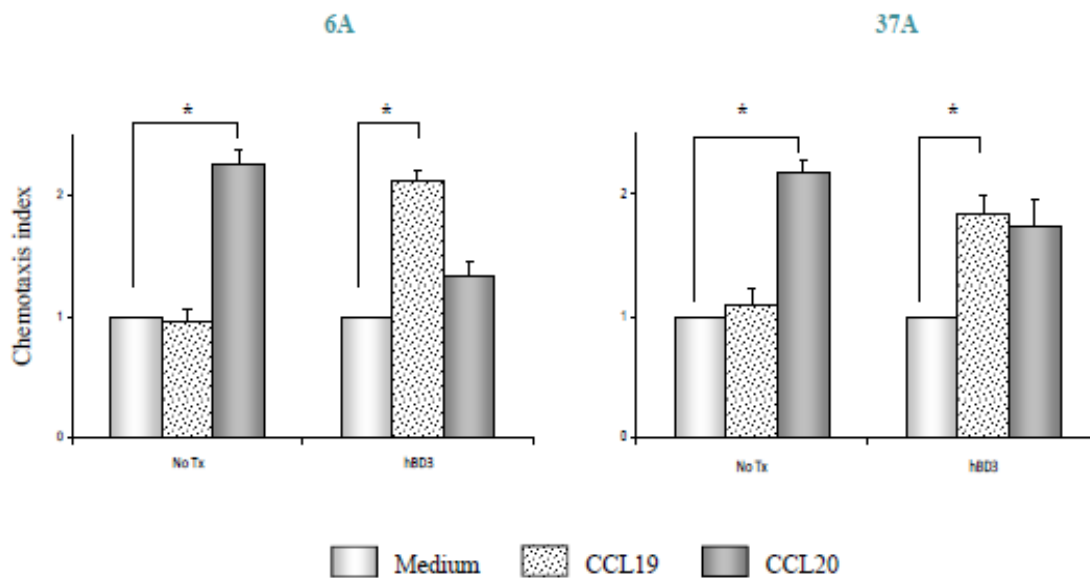


Figure 3-3: HBD3 induced CCR7 is functional. PCI-6A and -37A tumors were pre-treated with HBD3 (0.25 μ M, 24hr). The cells were then collected and added in triplicate to the upper chamber of a chemotaxis plate containing either medium, CCL19 (500ng/ml) or CCL20 (500ng/ml) in the lower wells. After 4 hours the number of cells migrated into the lower wells was counted. Results are expressed as chemotactic index (cells migrating toward chemokine / cells migrating toward medium). (* p <0.006)

3.3.4 HBD3 stimulation of CCR7 expression is NF- κ B dependent

Previous studies have suggested that CCR7 is an NF- κ B target gene (165) and we have observed that NF- κ B activation can regulate basal CCR7 expression in metastatic SCCHN tumor cells (434). To determine whether NF- κ B activation mediated HBD3-induced CCR7 upregulation, we blocked NF- κ B activation in PCI-6A, -15A and -37A tumor cells by using an IKK- γ (NEMO) inhibitor peptide (100 μ M, 18hr) (Figure 3-4A). The results showed a significant inhibition of CCR7 induction after HBD3 treatment, in the presence of the NF- κ B inhibitor. Similar results were observed when we used the I κ B α phosphorylation inhibitor BAY 11-7082 (10-100 μ M, 4hr), which was found to block CCR7 upregulation in a dose-dependent fashion ($p < 0.05$) (Figure 3-4B). To rule out the possibility of non-specific inhibition by the pharmacologic inhibitors, we also utilized siRNA to specifically knock down NF- κ B p65 expression and found that HBD3-mediated CCR7 expression was reduced (Figure 3-4C). The ability of all the aforementioned inhibitors to block NF- κ B activation was demonstrated by examining their ability to block NF- κ B p65 activation in TNF α -induced nuclear extracts. Additionally, the expression of HLA class I was not significantly affected by NF- κ B inhibition thereby ruling out non-specific effects of these inhibitors (Figure 3-4D). Taken together, these findings strongly indicate that HBD3 upregulates CCR7 expression in an NF- κ B-dependent manner.

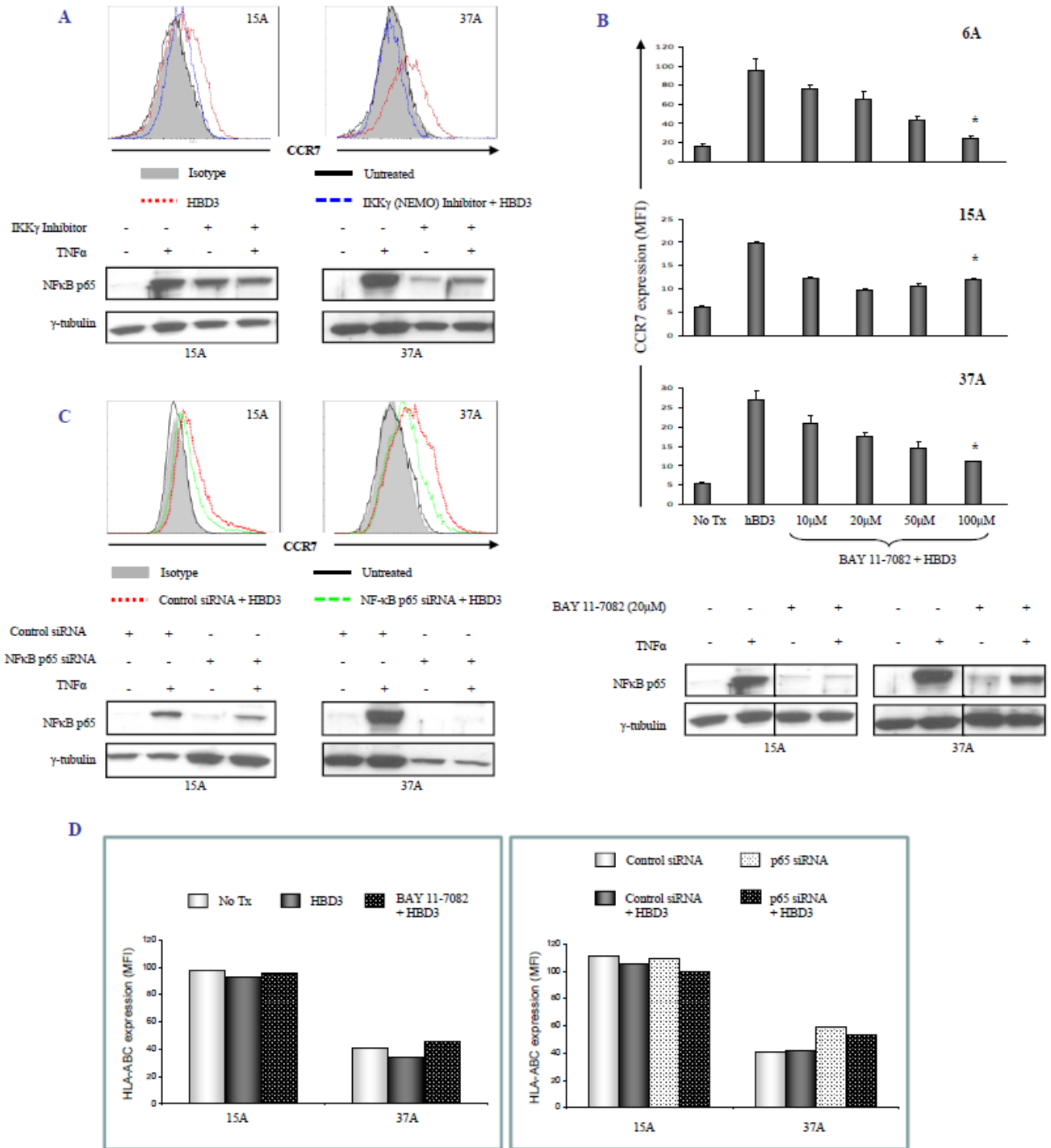


Figure 3-4: HBD3 induced CCR7 upregulation is dependent on NF-κB activation. PCI-6A, -15A and -37A tumors were pre-treated with: (A) an IKK-γ (NEMO) peptide inhibitor (100μM, 18hr) or (B) varying concentrations of BAY 11-7082 (4hr) or (C) NF-κB p65 siRNA (200nM, 24-48hr), and then stimulated with HBD3 (1μM, 24hr).

CCR7 expression was analyzed by flow cytometry. The mean fluorescence index \pm standard error is plotted on the histograms (* $p < 0.05$). Below each figure is an immunoblot of nuclear extracts showing the expression of NF- κ B p65 (or control γ -tubulin) when cells are stimulated with TNF- α (20ng/ml, 30min) in the presence or absence of the above inhibitors. All samples were run on the same gel. (D) Control experiments depicting HLA class I expression in PCI-15A and -37A as determined by flow cytometry following treatment using the aforementioned inhibitors.

3.3.5 HBD3 does not directly stimulate NF- κ B activation

Several specific NF- κ B inhibitors consistently blocked hBD3-mediated CCR7 upregulation, as shown in Figure 3-4. We therefore questioned whether HBD3 was responsible for directly stimulating NF- κ B activation in these cell lines. PCI-6A, -15A and -37A tumors were stimulated with TNF α (20ng/ml, 30min) or HBD3 (1 μ M, 30min) and the nuclear extracts were immunoblotted using NF- κ B p65 antibodies (Figure 3-5A). Furthermore, whole cell extracts from PCI-15A were immunoblotted using antibodies to pI κ B α and I κ B α (Figure 3-5B). The results show that there is no observable change in the nuclear accumulation of NF- κ B p65, nor is there any significant change in the phosphorylation of pI κ B α or degradation of I κ B α following HBD3 stimulation. Additionally, by utilizing an NF- κ B responsive luciferase vector to determine NF- κ B activation, our data demonstrate that HBD3 does not stimulate NF- κ B activation (Figure 3-5C). To further confirm and extend these findings, we used immunofluorescence and confocal microscopy to visualize the localization of NF- κ B p65 within the cellular environment (Figure 3-5D). In the absence of any treatment, constitutive expression of NF- κ B p65 is observed in the cytoplasm. Following stimulation with TNF α , a potent NF- κ B activator, we observed predominant localization of NF- κ B p65 in the nucleus. However, HBD3 stimulation did not induce any significant nuclear accumulation of NF- κ B p65. Finally, we used ELISA analysis to assay the induction of each NF- κ B family member. The results show that none of the NF- κ B transcription factors were induced in response to HBD3 treatment (Figure 3-5E). Together, these results indicate that HBD3 is not directly involved in stimulating NF- κ B activation.

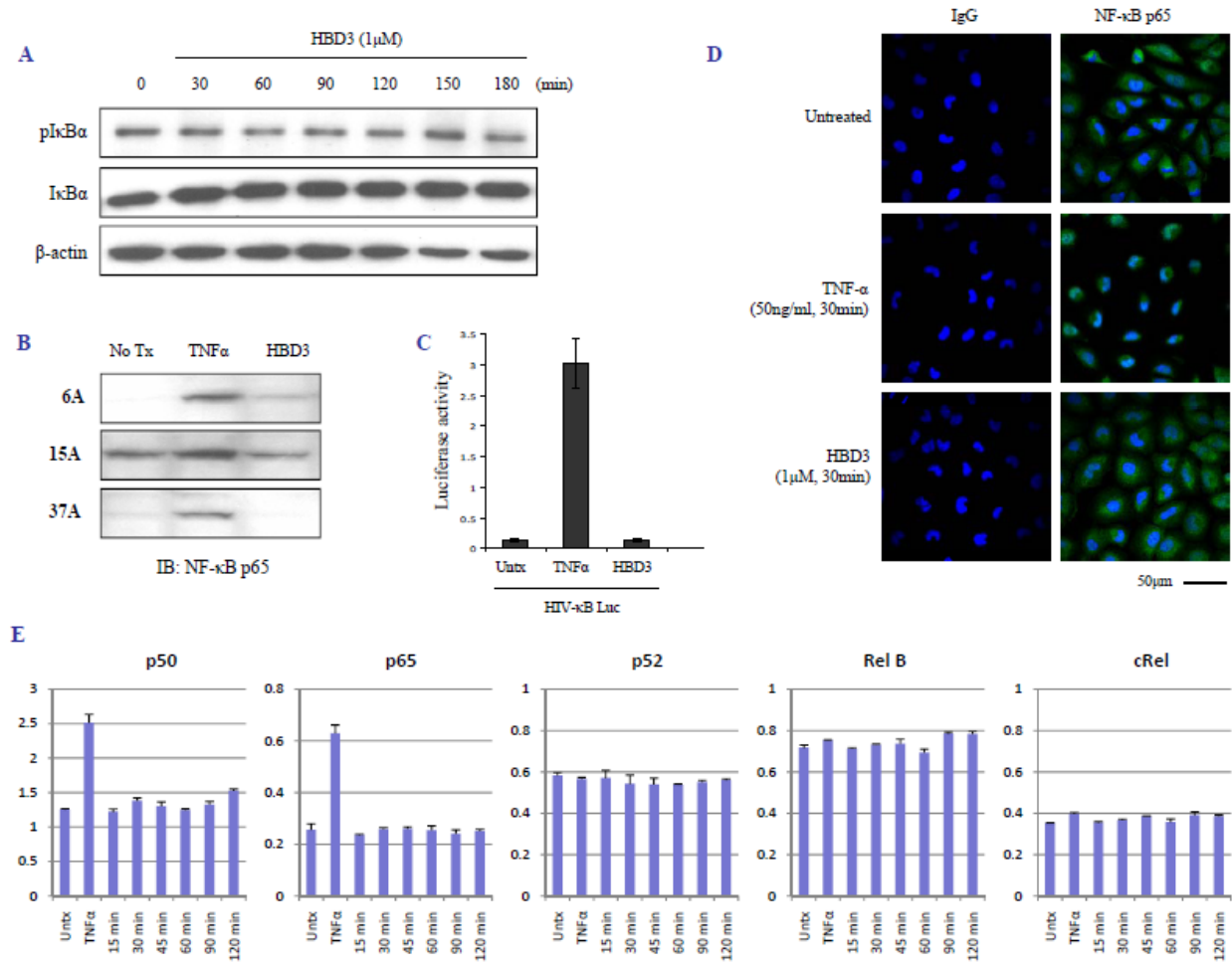


Figure 3-5: HBD3 does not stimulate NF- κ B activation. (A) PCI-37A cells were stimulated for varying periods of time with HBD3 (1 μ M). Whole cell extracts were then prepared and immunoblotted with antibodies to pI κ B α , I κ B α and β -actin. (B) PCI-37A cells were stimulated with TNF α (20ng/ml, 30min) or HBD3 (1 μ M, 30min). Nuclear extracts were prepared and immunoblotted for NF- κ B p65 expression. (C) An NF- κ B responsive HIV- κ B luciferase vector was transiently transfected into PCI-37A cells. After 24hr transfection, the cells were treated with either TNF α (20ng/ml) or HBD3 (1 μ M) for another 24hrs. Firefly luciferase activity was measured and normalized to renilla luciferase. (D) PCI-37A cells were treated with either TNF α (50ng/ml) or HBD3 (1 μ M) for 30 minutes. The cells were then fixed and stained using NF- κ B p65 antibodies or an isotype matched control. Cellular fluorescence was examined using confocal microscopy. Blue: Nuclear (DAPI) staining; Green: NF- κ B p65 staining. (E) PCI-37A cells were treated with either TNF α (20ng/ml, 30min) or HBD3 (1 μ M, for varying periods of time as indicated) after which nuclear extracts were prepared. 2 μ g of nuclear extracts were assayed by ELISA for expression of each of the NF- κ B family member proteins (p50, p65, p52, RelB and cRel). Results shown are averages of triplicate wells.

3.3.6 HBD3 stimulates activation of cFOS/AP1 transcription factors

Previously published reports have indicated that CCR7 transcriptional activity can be regulated through cooperation between NF- κ B and AP1 transcription factors (425, 426). Indeed, as discussed in chapter 2, we have established the presence of both NF- κ B and AP1 binding sites on the CCR7 promoter. Since we established that NF- κ B is not activated by HBD3 (Figure 3-5), we were curious to determine whether any of the AP1 family of transcription factors were induced by HBD3 stimulation. Using transcription factor ELISA analysis, we examined the activation of phospho-cJun, JunB, JunD, cFos, FosB and Fra-1 after HBD3 stimulation (Figure 3-6A). The results show that cFos demonstrated a gradual, time-dependent increase following HBD3 exposure. It is also notable that there was a modest increase JunD activation. These findings were confirmed in three different cell lines by immunoblot analysis (Figure 3-6B). Since cFos is reported to be activated through MAPK/ERK pathways (435, 436), we examined the ability of HBD3 to stimulate pERK activation using an immunoblot assay (Figure 3-6C). Indeed, HBD3 stimulation results in the activation and phosphorylation of ERK. Finally, we used a well established MAPK inhibitor (PD98059) to inhibit MAPK/ERK activity and examined the effect of this inhibition on cFos activation (Figure 3-6D). The data show that MAPK/ERK inhibition completely abrogates cFos activation by HBD3. In sum, these findings show that HBD3 induces cFos/AP1 transcription factor activation through MAPK/ERK-dependent signaling pathways.

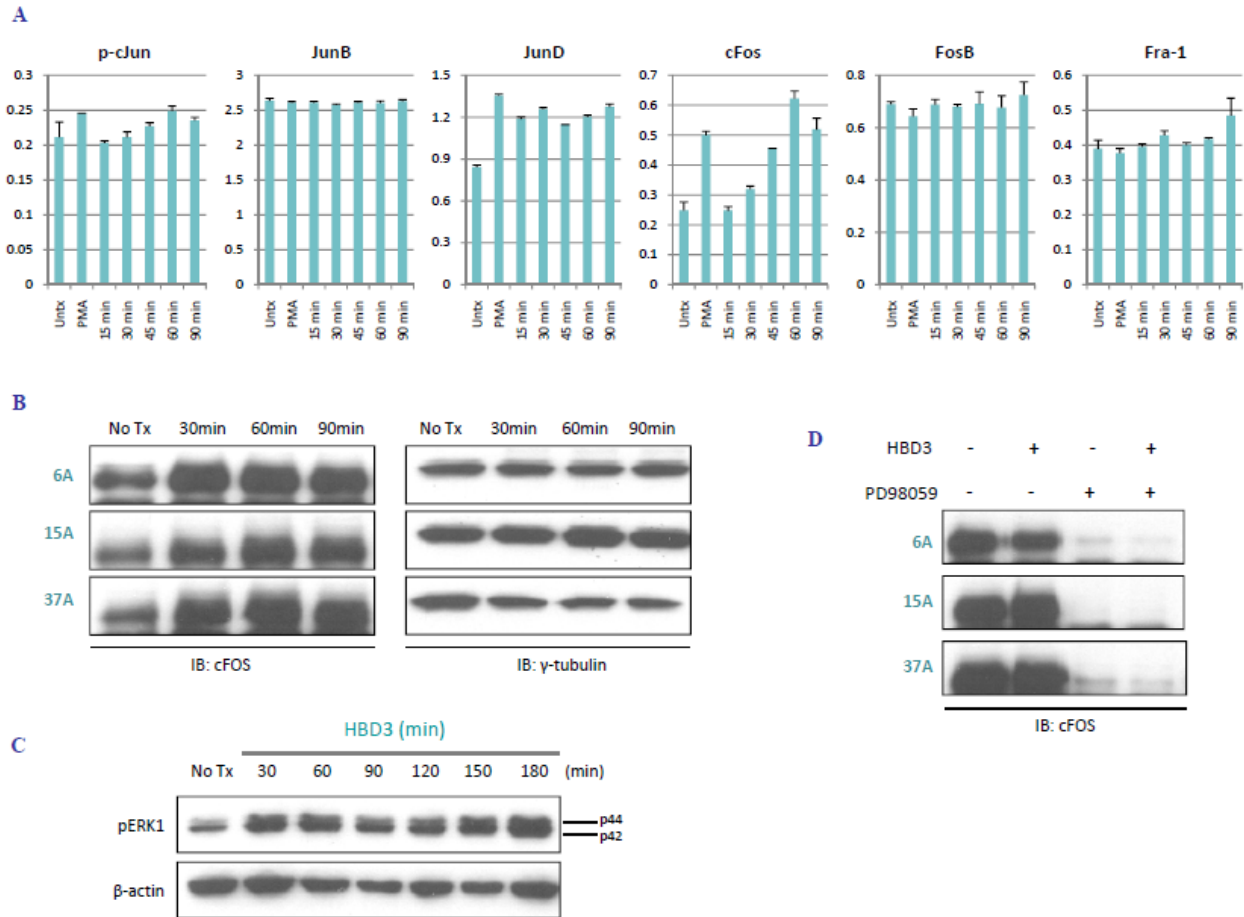


Figure 3-6: HBD3 stimulates activation of cFos/AP1 transcription factors. (A) PCI-6A cells were treated with either PMA (100ng/ml, 30min) or HBD3 (1 μ M, for varying periods of time) after which nuclear extracts were prepared. Using transcription factor ELISA analysis, 2 μ g of nuclear extracts were assayed for expression of each of the AP1 family member proteins (p-cJun, JunB, JunD, cFos, FosB, Fra-1). Results shown are averages of triplicate wells. Similar results were obtained using PCI-15A and PCI-37A cell lines. (B) PCI-6A, -15A and -37A cells were treated for varying periods of time with HBD3 (1 μ M). Nuclear extracts were prepared and immunoblotted for cFos and γ -tubulin expression. (C) PCI-15A cells were treated for varying periods of time with HBD3 (1 μ M). Whole cell extracts were prepared and immunoblotted for pERK and β -actin expression. (D) PCI-6A, -15A and -37A cells were pre-treated with the MAPK inhibitor PD98059 (100 μ M, 2hr) and then stimulated with HBD3 (1 μ M, 1hr) after which nuclear extracts were prepared and immunoblotted for cFos.

3.3.7 HBD3 activates PI3K/Akt pro-survival signaling pathways

We previously published that CCR7⁺ metastatic SCCHN cells can activate PI3K/Akt-mediated pro-survival and invasive pathways in response to CCL19 stimulation (143, 144). The activation of the Akt signaling pathway conferred a survival advantage to tumor cells. To determine whether HBD3 stimulation could also activate the PI3K/Akt pro-survival pathway, we stimulated tumor cells with HBD3 (1 μ M, 30min) and measured the expression of phosphorylated Akt (pAkt) in the presence or absence of the PI3K inhibitors, wortmannin (200nM, 2hr) (Figure 3-7A) or LY294002 (not shown). These experiments showed that pAkt expression was enhanced following HBD3 stimulation, and was blocked in the presence of wortmannin or LY294002. These inhibitors blocked both basal and HBD3-induced pAkt activation. Thus, our findings show that the activation of Akt by HBD3 is dependent on PI3K activation.

We then examined whether the HBD3-induced Akt activation enhanced the survival of tumor cells that were exposed to cisplatin, a commonly used pro-apoptotic chemotherapeutic agent for the treatment of SCCHN. PCI-6A and 37A tumor cells were pre-treated with HBD3 (0.25 μ M, 1hr) and then exposed to cisplatin (40 μ M, 4-6hr) (Figure 3-7B). Treatment of SCCHN cells with cisplatin alone induced apoptosis in 37% and 40% of tumor cells, respectively. However, pre-treatment of these SCCHN tumor cells with HBD3 led to a reduction in apoptotic cells to 7% and 3%, respectively. To investigate whether this HBD3-induced protection from cisplatin-induced apoptosis was mediated by the Akt survival pathway, we pre-treated the tumor cells with an Akt inhibitor (10 μ M, 2hr). In the presence of Akt inhibition, the anti-apoptotic effects of HBD3 were abrogated and cisplatin-induced apoptosis was found in 26% and 33% of SCCHN cells, respectively ($p < 0.04$). Notably, treatment with the Akt inhibitor alone did not significantly increase apoptosis of the tumor cells (not shown). Thus, HBD3 stimulation appears to contribute to anti-apoptotic signaling in SCCHN tumor cells, and this pro-survival effect was mediated mainly through an Akt-dependent pathway.

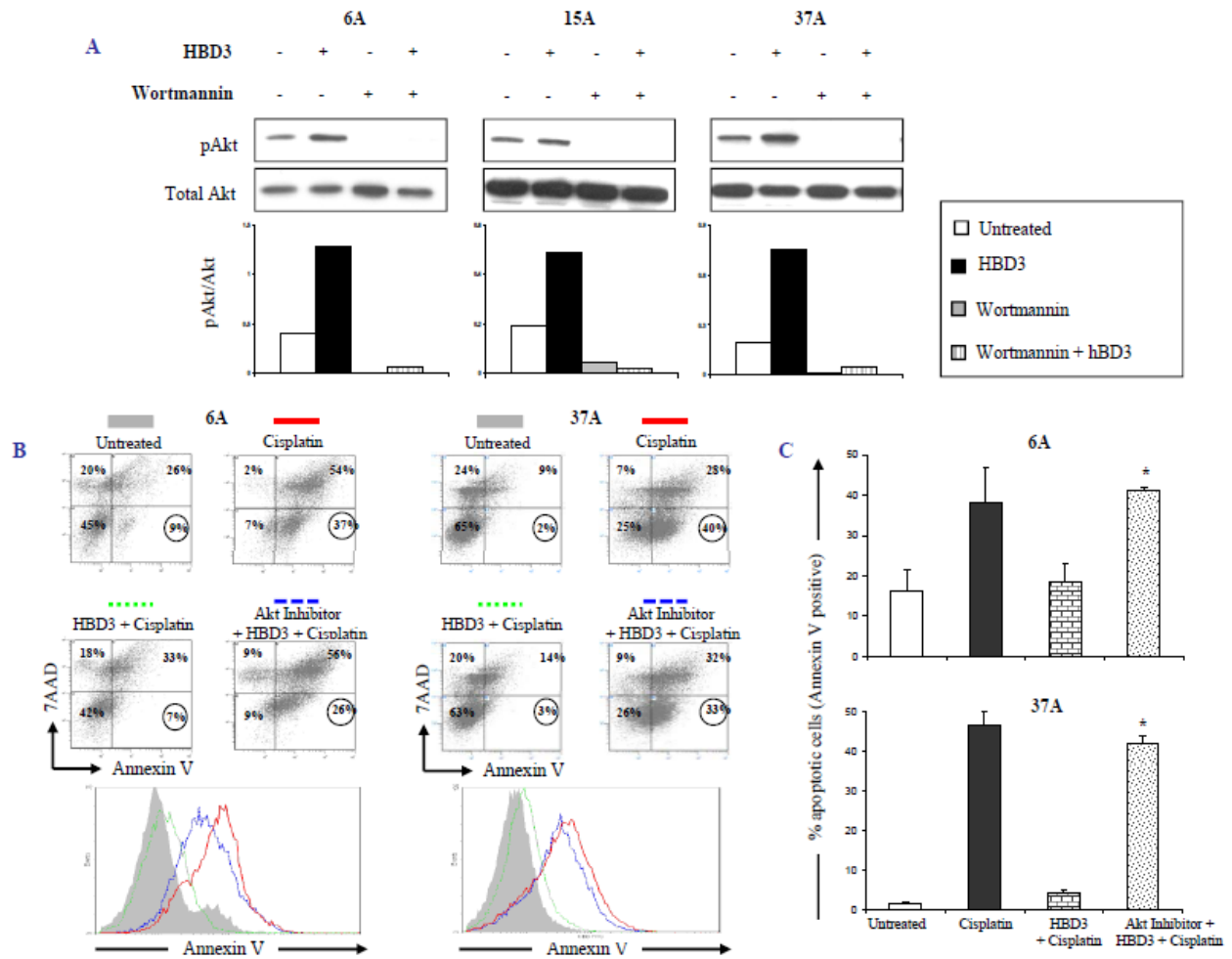


Figure 3-7: HBD3 stimulation activates PI3K/Akt pathways that enhance tumor survival. (A) PCI-6A, -15A and -37A tumors were left untreated or pre-treated with Wortmannin (200nM, 2hr) and then treated with HBD3 (1 μ M, 30min). Cellular extracts were prepared and blotted for pAkt (ser⁴⁷³) and total Akt. Relative expression of pAkt to Akt, as determined by densitometry, is shown below each blot. The experiment was repeated at least 3 separate times with similar results. (B) PCI-6A and -37A tumors were pre-treated with an Akt inhibitor (10 μ M, 2hr) and/or HBD3 (0.25 μ M, 1hr) followed by treatment with cisplatin (40 μ M, 4hr). Cellular apoptosis was assayed by Annexin-V and 7AAD staining using flow cytometry. Circles show early apoptotic (Annexin V positive, 7AAD negative) cells. Below the dot plots is a histogram portraying the Annexin V positive, 7AAD negative population under each treatment condition. (C) Mean \pm standard error of repeated experiments, showing Akt inhibition significantly ($*p < 0.05$) abrogates HBD3-mediated protection from apoptosis.

3.3.8 HBD3 does not utilize G-protein coupled receptor (GPCR) or Toll-like receptor (TLR) pathways to upregulate CCR7 in SCCHN tumors

To investigate the receptor(s) mediating HBD3-induced migratory and anti-apoptotic effects on SCCHN cells, we studied the role of GPCRs and TLRs, two classes of receptors previously associated with the binding of β -defensins and NF- κ B pathway activation (366, 369). Since PCI-6A, -15A and -37A tumor cells express both of these types of receptors (437, 438), we investigated whether these receptors were involved in mediating HBD3-induced CCR7 upregulation. SCCHN tumor cells were pre-treated with increasing concentrations of the $G\alpha_i$ inhibitor pertussis toxin (100-500ng/ml, 4hr) (Figure 3-8A), with a MyD88-peptide inhibitor (100 μ M, 18hr) (Figure 3-8B), or with a TRIF-peptide inhibitor (80-100 μ M, 18hr) (Figure 3-8C) and then were stimulated with HBD3 (1 μ M, 24hr) in the continued presence of each inhibitor. Interestingly, neither GPCR nor TLR blockade appeared to inhibit HBD3 stimulation of CCR7 upregulation. Of note, the GPCR blocking ability of pertussis toxin was confirmed in separate chemotaxis assays, where it was found to block the chemotactic migration of CCR7⁺ DCs towards CCL19 (Figure 3-8A). The MyD88 and TRIF inhibition by the peptides was confirmed by examining the blockade of DC maturation following LPS and Poly IC stimulation, respectively (Figure 3-8B, 3-8C). Together, these findings suggest that the observed HBD3 upregulation of CCR7 is not mediated through GPCRs or TLRs.

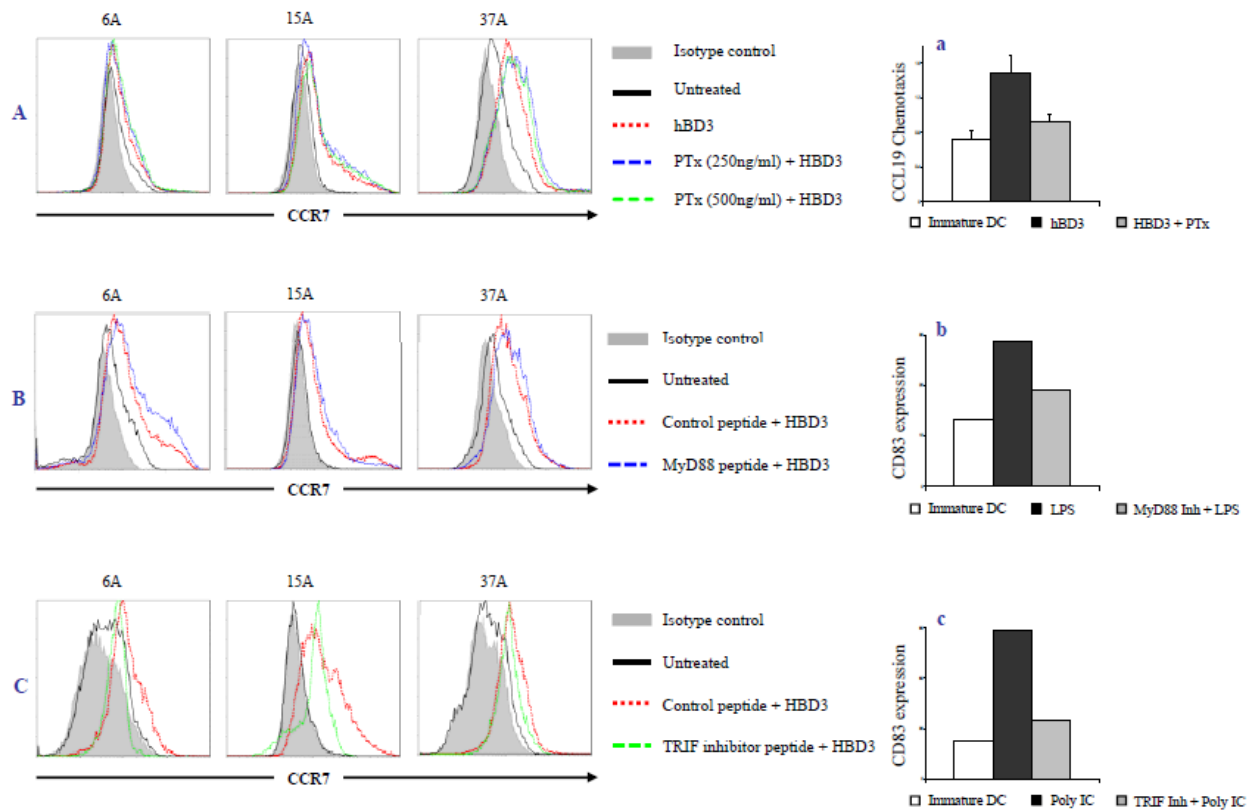


Figure 3-8: HBD3 stimulation of tumor cells is independent of GPCR and TLR signaling. PCI-6A, -15A and -37A tumors were pretreated with (A) increasing concentrations of Pertussis toxin (250-500ng/ml, 4hr) or (B) MyD88-inhibitor peptide (100 μ M, 24hr) or a control peptide (100 μ M, 24hr) or (C) TRIF-inhibitor peptide (80-100 μ M, 24hr) or a control peptide (80-100 μ M, 24hr). Cells were then stimulated with HBD3 (1 μ M, 24hr) in the continued presence of each inhibitor and CCR7 expression was assayed by flow cytometry. Control experiments: (a) Immature day 6 DC were stimulated with HBD3 (5 μ M, 24hr) and then left untreated or pretreated with 100ng/ml pertussis toxin for 1hr before being added to the upper chamber of a chemotaxis plate containing either medium or CCL19 (500ng/ml) in the lower wells. After 4 hours the number of cells migrated into the lower wells was counted. (b and c) Immature day 5 DC were pretreated with either MyD88 or TRIF inhibitory peptide respectively (80-100 μ M, 18hr) and then stimulated with HBD3 (5 μ M, 24hr). DC maturation was assayed by examining CD83 expression.

3.3.9 HBD3 is internalized through an endocytic mechanism

Several reports suggest that small cationic and anti-microbial peptides possess the ability to directly penetrate cellular membranes and potentially induce their effects through binding to intracellular receptors (293, 294, 439). To evaluate whether HBD3 was capable of penetrating the cellular membrane, we examined cellular fluorescence from cells incubated at 4°C or 37°C with 1-2µM fluorescein-labeled HBD3 for different periods of time (Figure 3-9A). The data show significantly increased HBD3 fluorescence at 37°C compared to 4°C at each time point ($p < 0.01$), suggesting that there is an active mechanism facilitating the internalization of HBD3 into tumor cells. Moreover, staining using fluorescein-labeled cetuximab antibody to EGFR which displays predominantly surface binding remained the same at 4°C and at 37°C (not shown). We further used imaging flow cytometry to examine the relative fluorescence from intracellular sources compared to that from the cell surface (Figure 3-9B). In our analysis, the intracellular fluorescence increased steadily over 4hrs, while the surface fluorescence remained constant. This strongly suggests that the observed increase in fluorescence at 37°C is due to the intracellular internalization and accumulation of HBD3 peptide. The internalized HBD3 was further visualized using confocal microscopy to determine its intracellular location. PCI-15A and -37A tumor cells were treated with HBD3-FITC and then observed by microscopy (Figure 3-9C). The results show detectable pockets of HBD3-FITC within the cytoplasm, which appear to congregate and accumulate in the perinuclear regions.

To determine the mechanism of the observed accumulation of intracellular HBD3, we inhibited endocytic mechanisms in the tumor cells using the microtubule polymerization inhibitor, nocodazole (10µg/ml, 1hr). Interestingly, the use of nocodazole significantly blocked, but did not completely abrogate intracellular uptake of HBD3-FITC (Figure 3-9D) into SCCHN cells ($p < 0.05$). The inhibition of pinocytosis or actin polymerization using dimethyl amiloride and cytochalasin D, respectively, did not significantly reduce HBD3 uptake or signaling (not shown). Thus, in tumors, HBD3 appears to accumulate intracellularly using active endocytic mechanisms.

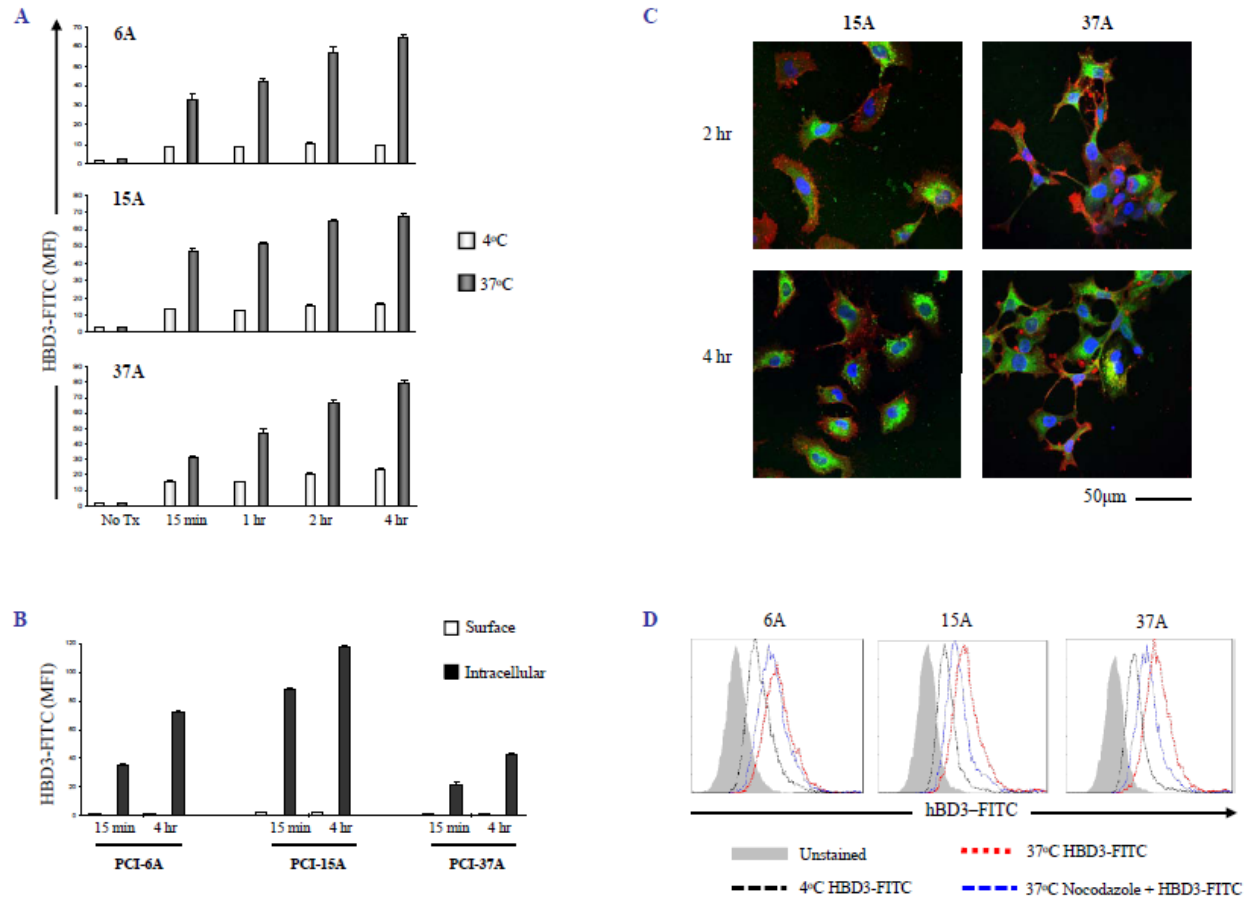


Figure 3-9: HBD3 accumulates intracellularly using an endocytic mechanism. (A) PCI-6A, -15A and -37A tumors were incubated with fluorescently labeled HBD3 (1-2 μ M) either at 4°C or 37°C for different lengths of time as indicated. Cellular fluorescence was examined by flow cytometry. (B) PCI-6A, -15A and -37A tumors were incubated with fluorescently labeled HBD3 (1-2 μ M, 37°C) for 15min or 4hr. Imaging flow cytometry was used to quantify the changes in surface and intracellular fluorescence at each time point. Results are presented as mean fluorescence index \pm standard error. (C) PCI-15A and -37A grown on cover slips were incubated with 1 μ M HBD3-FITC for 2hr or 4hr. Cellular fluorescence was observed by confocal laser scanning microscopy at 60X magnification. Blue: Nucleus (DAPI); Red: Cell membrane; Green: HBD3-FITC. (D) PCI-6A, -15A and -37A tumor cells were pre-treated with nocodazole (10 μ g/ml, 1hr, 37°C) prior to incubation with fluorescent-HBD3 (1-2 μ M, 1hr) at 4°C or 37°C. Cellular fluorescence was examined by flow cytometry.

3.4 DISCUSSION

Human β -defensins comprise a class of inflammatory molecules, whose primary role at epithelial surfaces appears to be the initiation of an early host immune response to clear invading microorganisms (440, 441). They are capable of bridging innate and adaptive immunity through their ability to attract antigen presenting cells to inflammatory sites of tissue injury and to provide ‘danger signals’ that facilitate the maturation of these immune cells. However, despite our understanding of the role of defensins in host-pathogen interactions and immunity, little is known about their functions within the context of an inflammatory tumor microenvironment. Particularly for squamous mucosal malignancies, which are a source of HBD secretion, even less is known about any potential pro-tumorigenic properties that may result from a dysregulation of inflammatory mediators such as defensins. In this chapter, we demonstrate that HBD3 induces CCR7 expression in primary SCCHN tumor cells in an NF- κ B dependent manner, and provides migratory and pro-survival signals to the developing tumors. Furthermore, the stimulation of tumors with HBD3 appears to confer a survival benefit to these tumors, mediated by phosphoinositide-3-kinase (PI3K)/Akt activation. Our findings suggest that HBD3 overexpression by epithelia can be exploited by developing tumors in autocrine and paracrine fashion to enhance their growth, survival and evolution into a metastatic phenotype. In particular, the HBD3-stimulated induction of CCR7 expression in primary tumors may contribute to the predictable pattern of regional lymph node metastases commonly observed in SCCHN.

Recent reports have begun to highlight the proinflammatory and pro-tumorigenic effects of HBD3. For instance, in mouse ovarian carcinoma, the expression of β -defensin 29, the murine homologue to human β -defensin 2, promotes DC recruitment through CCR6 and enhances tumor vascularization and growth through cooperation with VEGF-A (394). Human tumors overexpressing β -defensins have been found to possess increased populations of infiltrating monocytes, macrophages and DCs, suggesting that the pro-angiogenic and pro-tumorigenic functions of β -defensins may play a role in the development of these tumors. Indeed, the observed overexpression of HBD3 in oral squamous carcinoma has been

associated with the recruitment of tumor-associated macrophages (TAM), which enhance inflammation by secreting a wide array of tumor-promoting molecules, including cytokines, chemokines and growth factors (400, 442). Thus, by promoting TAM recruitment in oral SCC, HBD3 directs the generation of a prevailing inflammatory microenvironment which correlates with tumor aggressiveness (443, 444). Our own studies indicate that SCCHN cell lines secrete low levels of HBD3 into the culture medium. Interestingly, stimulation with proinflammatory cytokines such as IFN- γ can stimulate increased secretion of HBD3 in keratinocytes and PCI-37A (not shown). However, any direct effects of HBD3 on tumor growth and metastasis have not yet been elucidated.

In this study, we tested the ability of HBD3 to directly affect the survival and migratory ability of human SCCHN tumors. We previously identified a distinct pattern of chemokine expression in SCCHN tumors whereby CCR7 was upregulated in patient-matched metastatic tumors (141). The expression of CCR7 by metastatic SCCHN tumors may contribute to the frequent lymph node metastases that are reported in this and other malignancies. However, since CCR7 expression has generally been identified on tumors that are already present at the metastatic lymph node sites, the induction signals responsible for, and significance of, CCR7 expression on primary tumor cells emigrating from the mucosal tumor site, are still unclear. Our work here suggests that pro-inflammatory and cellular mechanisms cooperate to induce CCR7 expression and anti-apoptotic effects on the parental, non-metastatic SCCHN tumors.

Based on reports that β -defensins can signal through G-protein coupled receptors [specifically through CCR6 in T cells and DCs (366), and CCR2 in monocytes and macrophages (400)], and the subsequent upregulation of CCR7 that we observed in HBD3-treated DCs (see chapter 4), we tested the effects of exogenous HBD3 stimulation on the CCR7 expression of primary SCCHN cell lines. Using flow cytometry, we showed that HBD3 stimulation upregulates CCR7 expression. This effect was not due to an increase in cellular protein synthesis, since the expression of HLA-class I molecules did not change with HBD3 treatment. Furthermore, the CCR7 upregulation was specific to HBD3 as it was not observed when the tumor cells were treated with hBD2 or hBD4. Heat-denatured HBD3 also could not upregulate CCR7. Importantly, the upregulated CCR7 was functionally important in enhancing the chemotaxis of

these tumors towards CCL19. It is worth noting that these primary tumors do not lose their expression of CCR6 (as has been observed in metastatic SCCHN cell lines) and remain chemotactic to CCL20. This suggests that other cellular and immune mechanisms may be involved in the switch from primary tumor to metastatic lymph node. Nevertheless, HBD3 upregulation of CCR7 may be a contributing factor towards attaining a 'metastatic phenotype'. Our data show that HBD3 upregulates CCR7 expression and function, but the staining of tumor tissue shows little (if any) CCR7 expression at the primary tumor site, presumably because any CCR7 positive cells progressively migrate out of the primary tumor site into the metastatic lymph node sites.

We and others have shown that CCR7 is an NF- κ B target gene (see chapter 2) (165). We therefore questioned whether the activation of NF- κ B was necessary for the observed HBD3-mediated CCR7 upregulation. Consistent with this notion, blocking NF- κ B activation in the tumors significantly abolished the observed HBD3-induced CCR7 upregulation. The tumorigenic effects of deregulated NF- κ B activation in oral SCC have been well documented since this transcription factor has been shown to regulate various oncogenes, including CCR7 (445). Remarkably, although CCR7 upregulation is clearly dependent on NF- κ B activation, and is reduced following p65 knockdown, HBD3 does not appear to directly stimulate NF- κ B activation in these cells. Based on these data and literature reports suggesting that AP1 transcription factors also regulate CCR7 expression (425, 426), we hypothesized that HBD3 induces other transcription factors that, along with the constitutive NF- κ B activation already present in these tumors (446), can co-regulate CCR7 expression. Indeed, we show that HBD3 treatment induces cFos/AP1 activation. Further studies are required to establish whether NF- κ B/cFos cooperation is responsible for the observed HBD3-dependent CCR7 induction.

In previous reports, the expression of CCR7 in immune cells and tumors has been shown to provide a survival advantage to cells and is correlated with tumor aggressiveness (143, 144). This increased survival has been linked to the activation of the PI3K/Akt signaling pathway. In this study, we found that HBD3 stimulation activates the Akt signaling pathway in a PI3K-dependent manner. The activation and phosphorylation of Akt was biologically significant as it enhanced the survival of SCCHN

tumor cells in the presence of cisplatin. Moreover, since the cells were pre-treated with HBD3 for only 1hr prior to cisplatin treatment, we can attribute the observed pro-survival benefit directly to HBD3 stimulation of PI3K/Akt. However, it is also worth noting that continued HBD3 stimulation over a longer period which results in the upregulation of CCR7 would also contribute to increased survival through CCR7-mediated activation of PI3K/Akt signaling (143).

Finally, it was of interest to identify the potential receptors involved in the observed HBD3 effects on primary SCCHN cells. Interestingly, even though our primary SCCHN tumor cells express various GPCRs and TLRs (437, 438), the HBD3-mediated CCR7 upregulation was not blocked by the $G\alpha_i$ inhibitor pertussis toxin, or by TLR peptide inhibitors, both of which inhibit β -defensin effects on immune cells (366, 369). Interestingly, we report that HBD3 is taken up through endocytic mechanisms and accumulates in the peri-nuclear regions in tumor cells. It is also worth noting that although other hBDs (such as hBD2) do not have similar effects on tumor CCR7 expression as HBD3, it appears that they penetrate tumor cells in a similar manner to that described for HBD3 (not shown). Indeed, evidence in the literature suggests that the small cationic nature of these peptides allows them to associate with cellular membranes and translocate through the membrane using temperature and energy-dependent processes (447, 448). Our findings show that incubation at low temperatures or the inhibition of microtubule polymerization using nocodazole significantly reduced the observed internalization of HBD3 ($p < 0.05$). It is notable, however, that over an extended incubation period, HBD3 overcomes nocodazole inhibition and penetrates cells. This observation, along with the toxicity of nocodazole at higher concentrations made it difficult to directly assess the effects of nocodazole treatment on HBD3-induced CCR7 expression. Nevertheless, our data suggest that HBD3 signaling can occur through direct binding to intracellular receptors/targets as has been described for other antimicrobial peptides (293, 294). Indeed, HBD3 could be binding to yet unidentified receptors on tumors.

**HUMAN β -DEFENSIN 3 INDUCES MATURATION OF HUMAN DENDRITIC CELLS: AN
ANTIMICROBIAL PEPTIDE THAT ALSO FUNCTIONS AS AN ENDOGENOUS ADJUVANT**

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Format adapted for Dissertation

4.0 HUMAN β -DEFENSIN 3 PROMOTES NF- κ B MEDIATED CCR7 EXPRESSION IN DENDRITIC CELLS

4.1 INTRODUCTION

Human β -defensins (HBDs) and cathelicidins are small antimicrobial proteins produced by monocytes, macrophages and epithelial cells of the cutaneous and mucosal linings. The expression of HBDs is particularly high in inflamed skin and mucosa, where they are capable of killing intruding pathogens that breach surface barriers. Thus HBDs provide a primitive but critical function in host defense. However, their deregulation can contribute to tumorigenesis as discussed in chapter 3. Studies have shown that HBD expression is induced in the skin under conditions of inflammation or infection, and increased expression of HBDs has been directly associated with stimulation by various proinflammatory stimuli, including TNF- α , IL-1 β , and LPS (324). While the over-expression of HBD3 appears to be disadvantageous to a growing tumor, little is known about the effects of HBD3 on antigen presenting cells such as dendritic cells, which themselves can be a source of HBD3 secretion to tumors.

In addition to their antimicrobial functions, recent evidence suggests that HBDs may contribute to the induction of adaptive immune responses by recruiting and activating monocytes, dendritic cells and T cells to sites of inflammation (discussed in chapter 1.3.3.3). For instance, HBDs have been implicated in the chemotaxis of immature DCs and T cells through their interactions with chemokine receptor 6 (CCR6). Recent studies also highlight the role of HBD2 and HBD3 in the selective chemoattraction of macrophages and monocytes through CCR2 binding (367, 400). HBD3 has also been shown to induce the expression of various costimulatory molecules on monocytes and myeloid DCs through its interactions with toll like receptors (TLRs) 1 and 2 (369). Interestingly, antimicrobial peptides can mediate either

induction or inhibition of DC activation, depending on experimental conditions. In mice, mBD2 has been shown to promote the maturation of DC via TLR4 (449). The human cathelicidin LL-37 has also been shown to induce DC maturation *in vitro* (263). Conversely, cathelicidins have been shown to block TLR4 mediated DC activation and inhibit allergic contact sensitization in mice (450). Both HBDs and cathelicidins have been implicated in the pathogenesis of inflammatory skin diseases. In fact, the expression of HBD3 is increased in psoriatic skin (451).

Surprisingly little is known about the effects of HBDs on human DCs. HBD3 was initially isolated from psoriatic skin lesions and was reported to retain antimicrobial activity at physiologic salt concentrations (331). Since HBD3 is made primarily by keratinocytes, we sought to study its effects on skin-resident antigen presenting cells. We therefore examined the effects of HBD3 on monocyte-derived DC (mDC) as well as langerhans cells DC (LC-DC), particularly with regard to phenotypic maturation and CCR7 upregulation. As with mDC, Langerhans cells (LC) play an important role in protecting the skin against pathogens, and are remarkably plastic and responsive to environmental signals enabling them to functionally bridge innate and adaptive immune responses. Under inflammatory conditions, LC migrate rapidly out of the skin in a CCR7-dependent fashion and move toward the draining lymph node where they present antigen to T cells and initiate antigen-specific immune responses (452). The LC-DCs can be generated *in vitro* from human monocytes by culture in the presence of GM-CSF and TGF- β 1 (453-455). LC-DCs differ from classic monocyte derived DCs generated by culture with GM-CSF and IL-4 and uniquely express molecules typical of Langerhans cells, including langerin, CCR6, and E-cadherin.

In this study, we show that HBD3 induces phenotypic and functional maturation of immature human mDC and LC-DC. In particular, we highlight the HBD3-mediated upregulation of functional CCR7 expression in mDCs and LC-DCs. The observed maturation appeared to be mediated, at least in part, by NF- κ B activation. Furthermore, similar to our studies in tumors, HBD3 does not appear to utilize any of the known receptors in inducing CCR7 expression, suggesting it may be signaling through yet unidentified cellular receptors. Our data suggest that through its APC modulatory properties, HBD3 may be an important contributor to cutaneous immune regulation.

4.2 MATERIALS & METHODS

Antibodies and Reagents

The following cytokines and chemokines were purchased from R&D Systems (Minneapolis, MN): rhGM-CSF, rhIL-4, rhTGF- β 1, rhTNF- α , rhCCL19, and rhCCL21. The following antibodies used for flow cytometric analysis were purchased from BD Biosciences (San Jose, CA): mouse anti-human CD83-FITC (clone HB15e), mouse anti-human CD86-PE (clone 2331), mouse anti-human CCR6-PE (clone 11A9), rat anti-human CCR7-FITC (clone 3D12), mouse anti-human HLA-DR-FITC (clone L243). PE-conjugated mouse anti-human langerin (CD207) (clone DCGM4), E-Cadherin and CD1a antibodies were kind gifts from Dr. Laura Ferris (University of Pittsburgh). Synthetic HBD3 was purchased from Peptides International (Louisville, KY). The MyD88 peptide inhibitor was purchased from Imgenex (San Diego, CA) while the pertussis toxin was obtained from Sigma-Aldrich (St. Louis, MO). Adenoviral constructs were obtained from the vector core facility at the University of Pittsburgh.

Cell culture

Leukopaks from healthy donors were obtained from the Pittsburgh Central Blood Bank through an IRB-exempt protocol. Peripheral blood mononuclear cells (PBMC) were separated over a Ficoll density gradient by centrifugation at 1800 rpm for 20 minutes at room temperature. The PBMC-containing buffy coat layer was then washed 3 times in RPMI while centrifuging at 1000 rpm for 10 minutes. Whole PBMCs were plated at a density of approximately 10×10^6 cells/ml and the monocytes were allowed to adhere to plates for 2hr at 37°C after which the non-adherent PBMCs were washed away using pre-warmed RPMI. The monocytes were then cultured in serum-free AIM-V medium supplemented with rhGM-CSF (150 units/ml) + rhTGF- β 1 (160 units/ml) for LC-DCs; or rhGM-CSF (1000 units/ml) + rhIL-4 (1000 units/ml) for mDCs. Fresh cytokines were added on culture day 3. Cells were harvested and used on day 6-7 of culture.

DC treatment and maturation

Day 6 DCs were stimulated with HBD3 (5 μ M, 24hr) after which they were collected and assayed for surface marker expression. For the GPCR blocking experiments, day 6 DCs were pre-incubated for 1hr with pertussis toxin (100ng/ml) followed by HBD3 stimulation as above. For the MyD88 blocking and Adenoviral transduction experiments, day 5 DCs were treated overnight (16-18hr) with either MyD88 inhibitory peptide (100 μ M) or with 1000 PFU (plaque forming units)/cell of Adenovirus containing the IkB α super-repressor. Following the overnight treatment, the DCs were stimulated with HBD3 as above.

Flow cytometry

Briefly, cells were harvested by placing at 4 $^{\circ}$ C and resuspending in cold 1x PBS. The cells were then resuspended in 1% FBS/PBS buffer and stained for 1hr using the manufacturer's recommended concentration of antibody (or if no recommendations were given a concentration of 5 μ g/ml was used). Cells were then washed twice in wash buffer and fixed in 2%PFA/PBS prior to data acquisition. Flow cytometric analysis was performed using a Beckman Coulter Epics XL cytometer with an isotype control set to a fluorescence mean index of 5.

Chemotaxis assay

Cell migration studies were performed as described previously (141). Briefly, 30 μ l aliquots of chemoattractant (CCL19, CCL20 or CCL21 at 500ng/ml) were added in triplicate to the wells of a disposable 96-well chemotaxis chamber (ChemoTx Neuroprobe, Gaithersburg, MD) with a 5 μ m pore size filter and 5.7mm width/well. A 50 μ l cell suspension (1 \times 10⁶ cells/ml) was placed on top of the filter. After 4hr incubation at 37 $^{\circ}$ C, the filter was washed gently with media. The filter was removed and the cells in each lower well were counted under a light microscope using trypan blue staining. The results are presented as chemotactic index, defined as the fold increase in cell migration in chemoattractant medium over migration in media alone. All assays were done in triplicate.

4.3 RESULTS

4.3.1 HBD3 induces phenotypic maturation of mDC and LC-DC

DCs with features of LCs (LC-DCs) were generated from PBMC by culture with GM-CSF and TGF- β 1 using previously described methods (453, 454). The identity of these LC-DCs was confirmed by flow cytometric staining for the LC markers CD1a, E-cadherin, CCR6, and CD207 (Langerin). This was compared to mDC staining of the same markers (Figure 4-1A, 4-1B). The immature mDCs and LC-DCs also expressed HLA-DR, as well as the characteristic low to moderate levels of the costimulatory molecules CD83, CD86 and CCR7.

Maturation stimuli such as inflammatory cytokines induce the maturation and activation of DCs, including increased cell surface expression of costimulatory molecules. To evaluate the potential effect of HBD3 stimulation on the maturation of mDC and LC-DCs, day 6 immature DCs were stimulated with HBD3 (5 μ M, 24hr). The DCs were then stained with antibodies against HLA-DR, CD83, CD86 and CCR7, and surface expression was evaluated by flow cytometry (Figure 4-1C, 4-1D). Our findings show that HBD3 consistently induced increased surface expression of HLA-DR, CD83, CD86 and CCR7. By comparison these markers were similarly upregulated using a pro-inflammatory cytokine cocktail consisting of TNF α , IL-1 β , IL-6 and PGE₂ (not shown). Notably, HBD3 strongly upregulated the lymph node homing receptor, CCR7. Thus, these data show that HBD3 induces phenotypic changes in mDCs and LC-DCs consistent with DC activation.

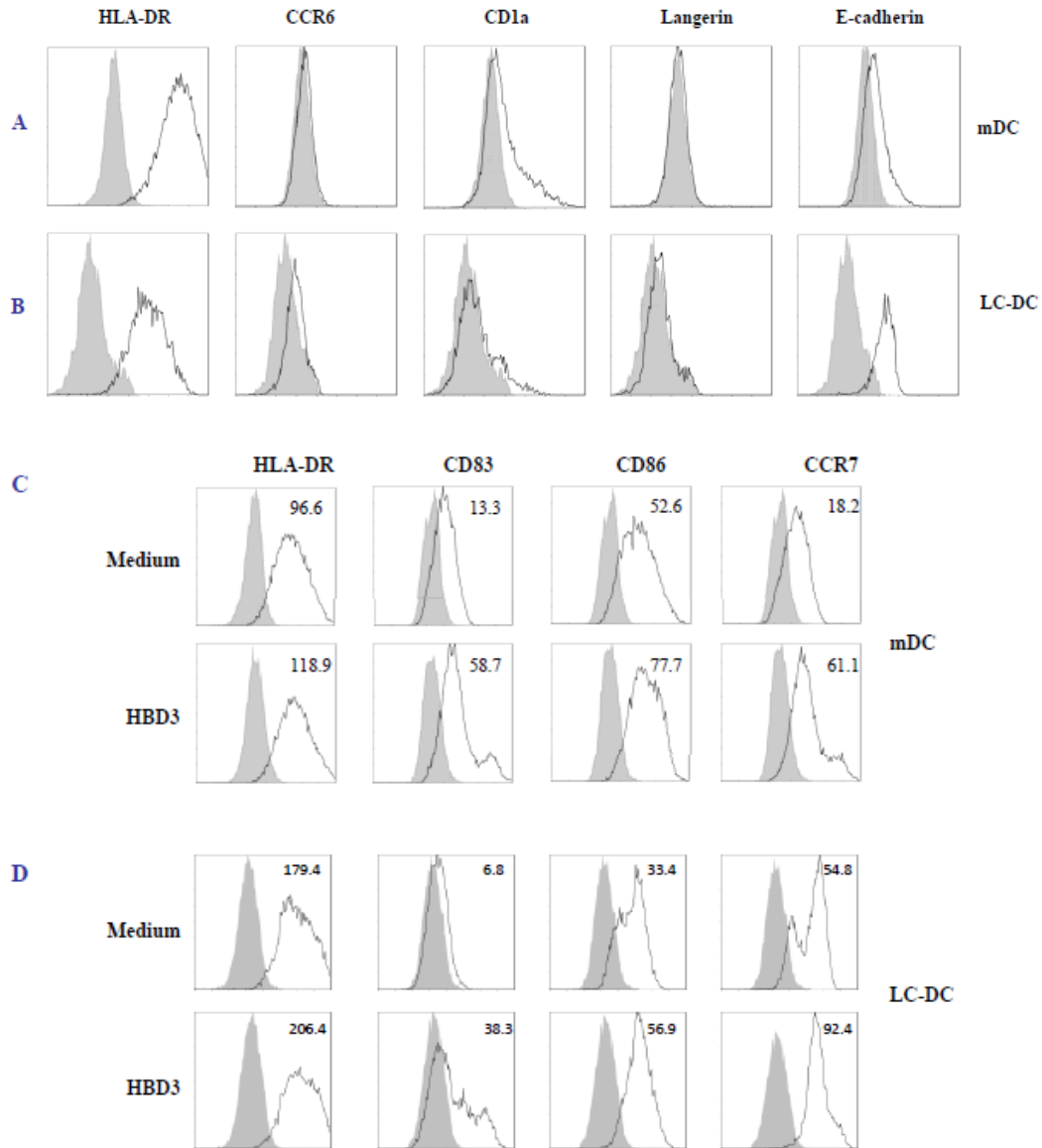


Figure 4-1: HBD3 stimulation activates phenotypic maturation of mDC and LCDC. Human monocytes were isolated from the blood of healthy donors by plastic adherence and cultured in serum-free AIM-V medium with either (A) rhGM-CSF + rhIL-4 (mDCs) or (B) with rhGM-CSF + rhTGF- β 1 (LC-DCs) for six days. Cell surface expression of HLA-DR, CCR6, CD1a, langerin and E-cadherin was assessed by flow cytometry. Additionally, day 6 immature (C) mDCs or (D) LC-DCs were cultured for 24 hours with medium alone or HBD3 (5 μ M). The surface expression of HLA-DR, CD83, CD86 and CCR7 were assessed by flow cytometry. Histograms show staining of cells with isotype-matched irrelevant antibody (gray shading) and antibody to indicated cell surface protein (black line). Values represent mean fluorescent intensity in treated cells. Results are representative of three separate experiments with cells generated from different donors.

4.3.2 HBD3-induced CCR7 promotes migration toward CCL19 and CCL21

To determine the functional significance of HBD3-induced CCR7 expression, we examined the ability of HBD3-treated DC to migrate toward the CCR7 ligands CCL19 and CCL21. Again, day 6 immature mDCs were stimulated with HBD3 (5 μ M, 24hr) or for comparison, a well-described pro-inflammatory cytokine cocktail (TNF α , IL-1 β , IL-6 and PGE₂) (456) known to induce mDC maturation. Chemotactic migration toward CCL19 and CCL21 was measured using an *in vitro* transwell system. Stimulation of mDCs with HBD3 enabled significant migration toward both CCR7 ligands across a transwell membrane (Figure 4-2). This effect appears to be CCR7-specific as the use of a CCR7 blocking antibody or pertussis toxin abolishes migration toward the ligands. Interestingly, the results show that unlike the DC phenotype obtained using pro-inflammatory cytokines whereby CCR6 expression is lost upon maturation, the mDCs that are matured with HBD3 still express CCR6 and remain chemotactic to CCL20. Nevertheless, these data suggest that HBD3 can promote CCR7 expression and the subsequent migration and lymph node localization of mDCs.

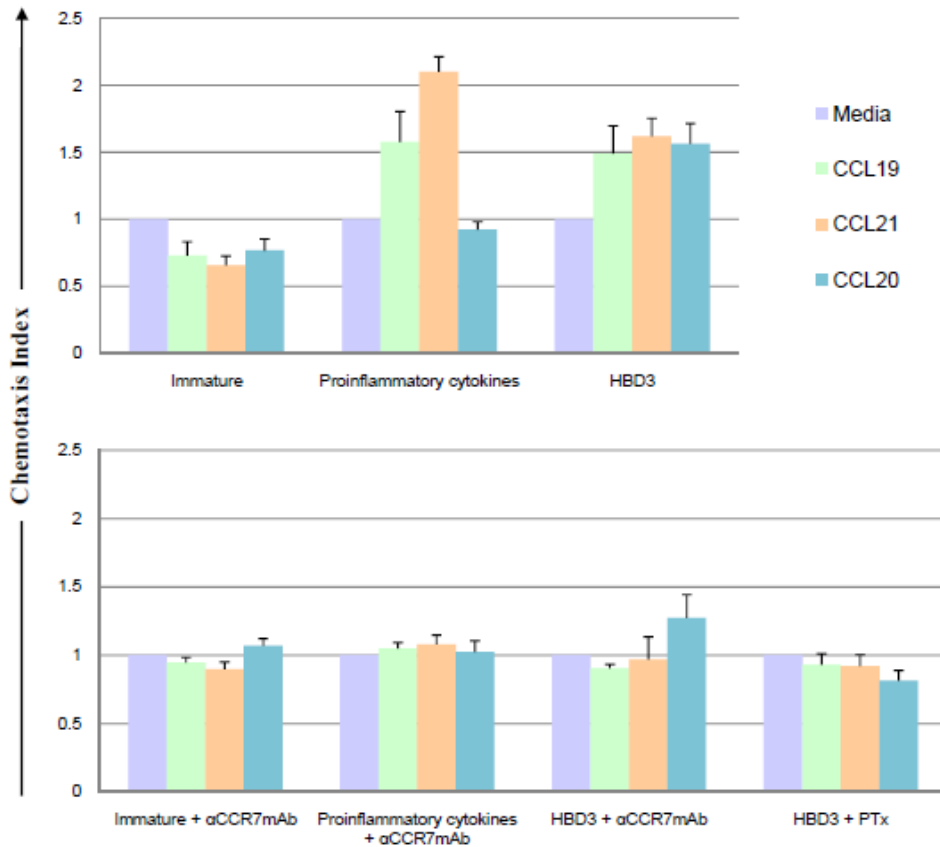


Figure 4-2: HBD3 induced CCR7 is functional. Day 6 mDCs were treated for 24 hours with medium alone, a well established pro-inflammatory cytokine cocktail ($\text{TNF}\alpha$, $\text{IL-1}\beta$, IL-6 , PGE_2) or HBD3 ($5\mu\text{M}$). The cells were then collected and added to the upper chamber of a chemotaxis plate containing medium, CCL19 (500ng/ml), CCL21 (500ng/ml) or CCL20 (500ng/ml) in the lower wells. Migration of cells toward the chemokine over a 4 hour time period was measured by counting the number of cells migrated into the lower wells. Results are expressed as chemotactic index (cells migrating toward chemokine / cells migrating toward medium). To show specificity of chemotactic response, following overnight exposure to maturation stimuli, mDCs were pre-treated for 1 hour with either anti-CCR7 mAb ($10\mu\text{g/ml}$) or Pertussis Toxin (100ng/ml). Results represent an average of triplicate wells.

4.3.3 HBD3 stimulation of CCR7 is NF- κ B dependent

The nuclear factor (NF)- κ B is an important transcription factor that is activated by various inflammatory stimuli and is associated with the maturation and survival of antigen presenting cells. To determine whether NF- κ B activation was required for HBD3-induced maturation and CCR7 upregulation, we transduced the LC-DCs with an adenovirus vector encoding the I κ B α AA dominant negative super-repressor, or with a control, blank adenovirus. NF- κ B blockade resulted in a significant inhibition of HBD3-induced CD86 and CCR7 upregulation in both mDCs and LC-DCs (Figure 4-3 and data not shown). These data suggest that HBD3 stimulates the maturation and CCR7 upregulation of DCs through an NF- κ B dependent pathway.

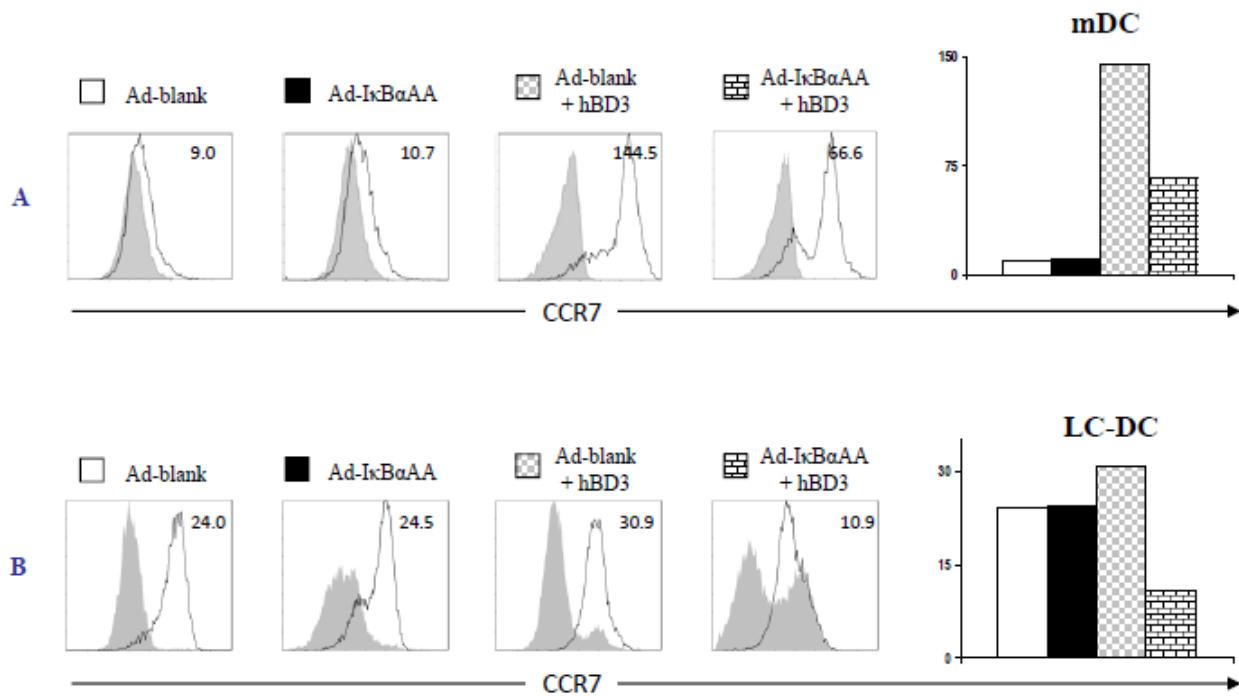
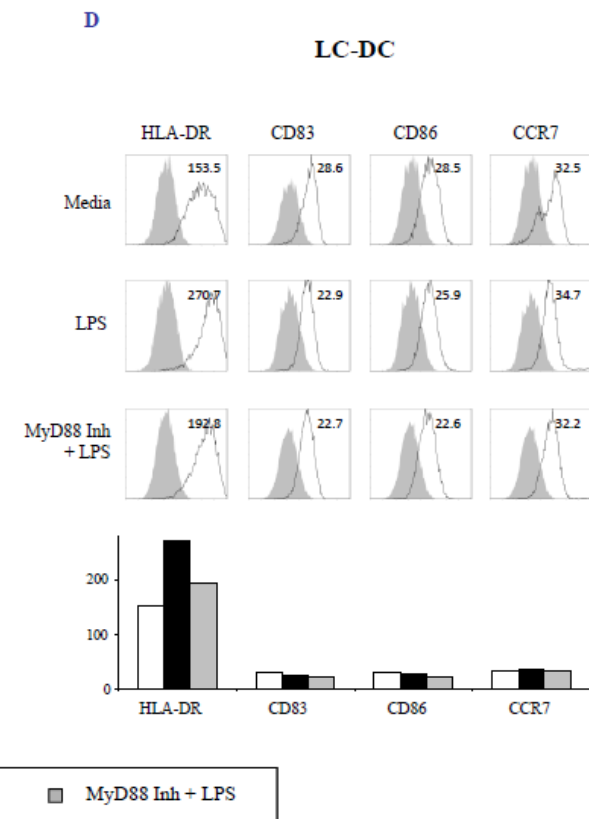
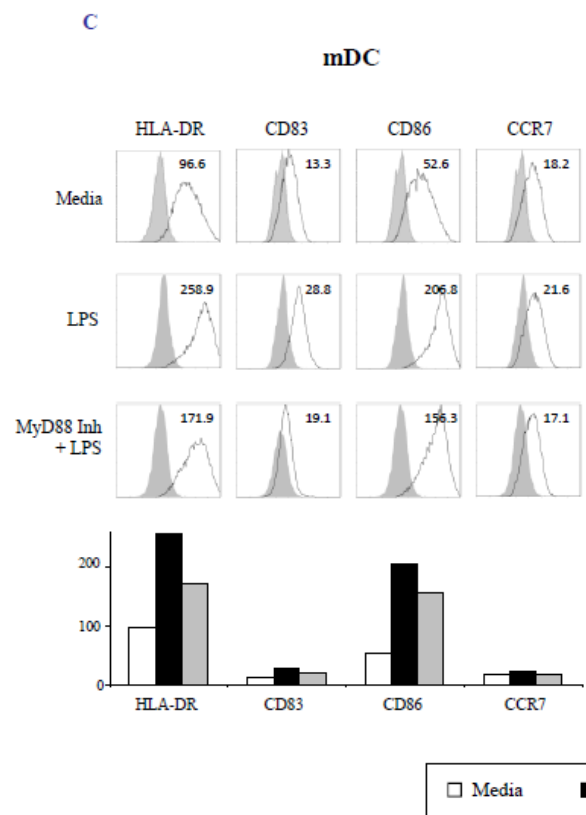
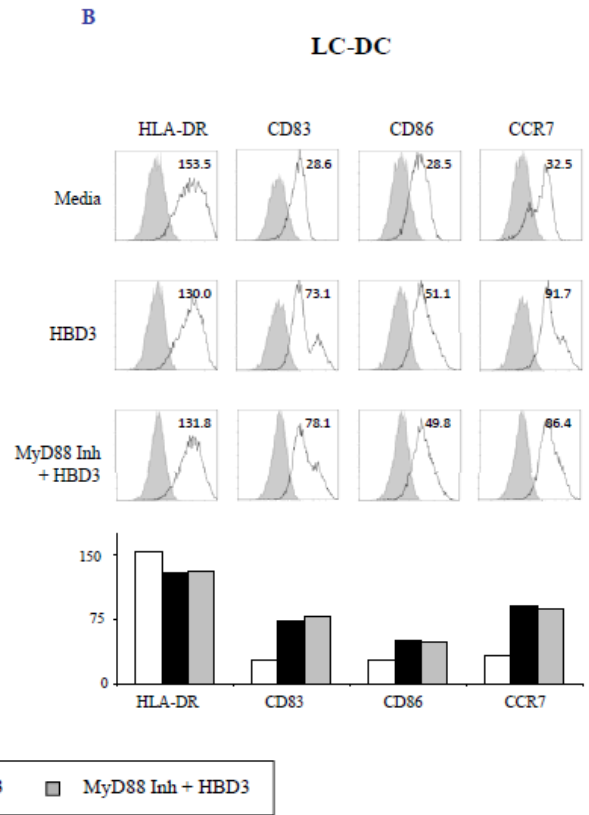
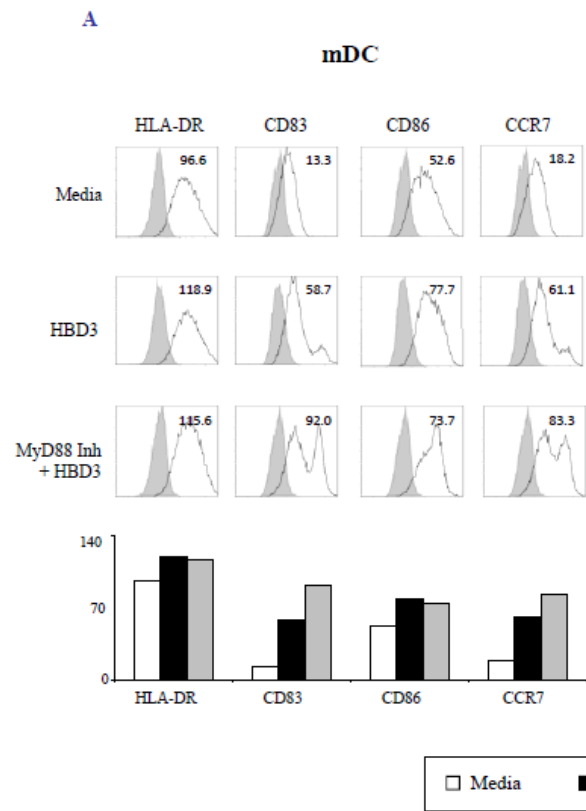


Figure 4-3: HBD3 induced CCR7 upregulation is dependent on NF- κ B activation. Day 5 mDCs (A) or LC-DCs (B) were transduced with either an empty adenoviral construct or an adenoviral construct containing the I κ BAA super-repressor over a 24hr time period. The DCs were then treated with HBD3 (5 μ M, 24hr). Expression of CCR7 was assayed by flow cytometric analyses. Values represent mean fluorescent intensity in treated cells.

4.3.4 HBD3 does not utilize G-protein coupled receptor (GPCR) or Toll-like receptor (TLR) pathways to upregulate CCR7 in mDCs and LCDCs

Recent studies have shown that HBD3 can signal through TLR1/2 heterodimers in a MyD88-dependent manner and through CCR2 which belongs to the GPCR family of receptors. To determine whether MyD88-dependent TLR signaling was required for the maturation of mDC and LC-DC by HBD3, we pretreated them with a MyD88 peptide inhibitor (100 μ M, 18hr) prior to HBD3 (5 μ M, 24hr) treatment. The inhibition of MyD88 signaling had no detectable effect on the upregulation of several phenotypic maturation markers in neither mDCs nor LC-DCs (Figure 4-4A, 4-4B). In separate experiments, we confirmed the MyD88 inhibitory activity of the peptide by observing the inhibition of LPS-induced DC maturation at similar concentrations (Figure 4-4C, 4-4D). To further explore the possible role of GPCRs in HBD3 mediated maturation, we pretreated mDC and LC-DC with pertussis toxin (100ng/ml, 1hr) and then treated with HBD3 (5 μ M, 24hr) after which phenotypic markers of maturation were examined using flow cytometry (Figure 4-4E, 4-4F). The data show that neither GPCRs nor TLRs were involved in the HBD3-mediated maturation and upregulation of CCR7 in DCs.



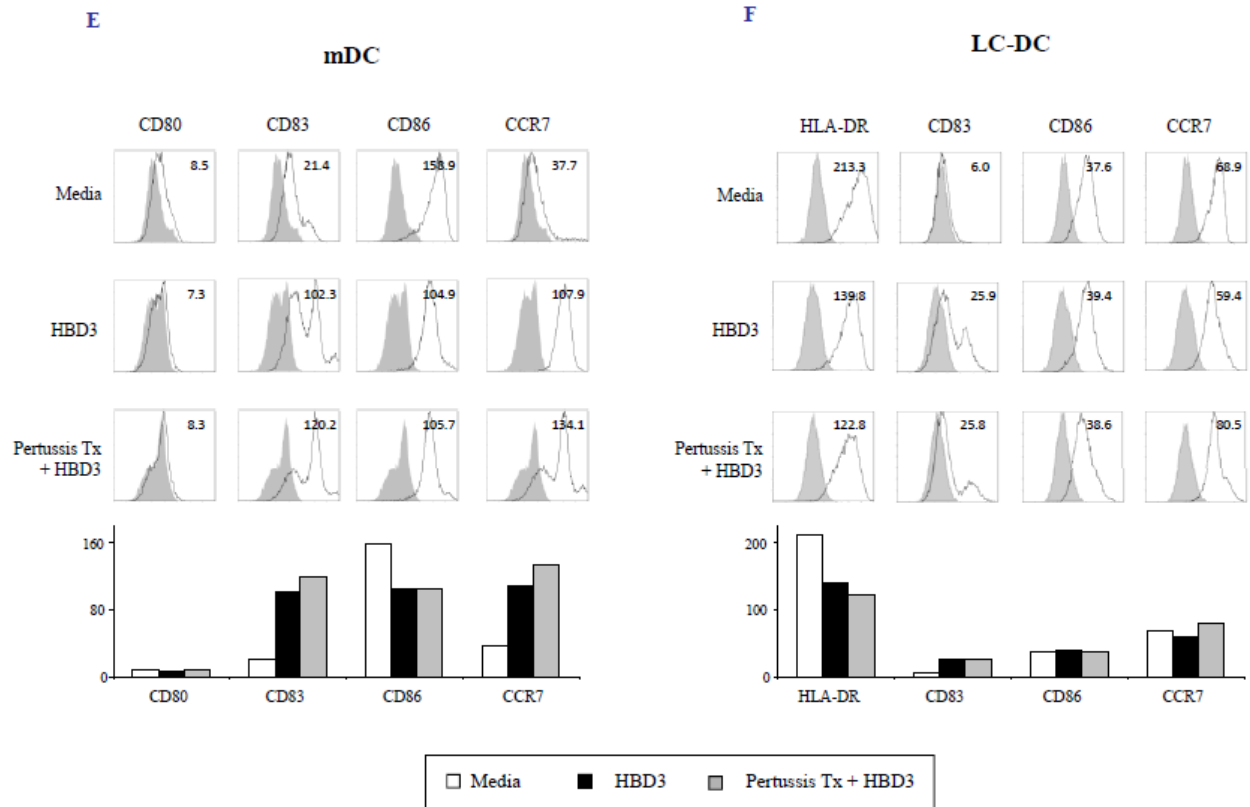


Figure 4-4: HBD3 stimulation of mDC and LCDC is independent of GPCR and TLR signaling. Day 5 mDCs and LC-DCs were pre-treated overnight with MyD88 inhibitory peptide (100 μ M). On day 6, the cells were stimulated with either (A and B) HBD3 (5 μ M, 24hr) or (C and D) LPS, and then assayed for cell surface expression of HLA-DR, CD83, CD86 and CCR7 by flow cytometry. (E and F) To assay GPCR involvement, day 6 mDCs and LC-DCs were pre-treated for 4hr with Pertussis Toxin and then stimulated with HBD3 (5 μ M, 24hr). Values represent mean fluorescent intensity in treated cells.

4.4 DISCUSSION

Antimicrobial peptides, including HBDs and cathelicidins, are made by keratinocytes under conditions of inflammation or infection. Their production is stimulated by exogenous microbial danger signals such as LPS or by endogenous mediators of inflammation such as TNF α and IL-1 β (324). Indeed, these inflammatory mediators can induce the activation of skin-resident DC directly by promoting their maturation, or indirectly by stimulating keratinocytes to produce HBDs which subsequently induce DC activation and maturation (see chapter 1.3.3.3). However, while the role of HBDs in innate immunity is well-established, their influence on adaptive immune responses is less clear. We addressed this issue by investigating HBD effects on dendritic cells, critical antigen presenting cells capable of priming antigen-specific T-cell responses and skewing adaptive immunity in response to environmental stimuli. Since defensins are made primarily in skin, we studied the effects of HBD3 on cultured DCs that most closely resemble those found in skin. Using well-established techniques, we generated and manipulated sufficient numbers of human mDCs and LC-DCs from peripheral blood precursors obtained from normal donors. The immature LC-DCs used in our studies express the Langerhans cell markers CD1a, langerin, and E-cadherin.

HBD3 has been shown to be chemotactic for macrophages, monocytes, DCs and T cells (see chapter 1.3.3.3). We now show HBD3 also has an important role in the stimulation of adaptive immune responses by inducing phenotypic and functional maturation of mDC and LC-DC *in vitro*. As part of the maturation process, HBD3-treated mDC upregulate expression of CCR7 and become responsive to the lymph node homing CCR7 ligands, CCL19 and CCL21. Together, our findings suggest that HBD3 may act as an endogenous danger signal that alerts the immune system to possible infection and mobilizes, activates, and polarizes dendritic cells to become effective activators of T cell responses. Interestingly, HBD3-mediated maturation of mDCs did not result in downregulation of CCR6 and the DCs remained responsive to CCL20 induced chemotaxis.

In previous studies using a mouse model, Biragyn *et al.* found that mBD2 induced phenotypic maturation and improved antigen presentation function in MLRs (449). These results were consistent with a mechanism whereby mBD2 induced DC maturation via TLR4. In contrast, cathelicidin peptides have been shown to block TLR4 mediated activation of DCs in a murine model (450). Interestingly, in studying the response of human monocytes to HBD3 maturation, recent studies showed that activation of TLR1/2 heterodimers was required for HBD3-induced maturation (369). Since the use of a MyD88 peptide inhibitor had no significant effects on the maturation of mDCs and LC-DCs by HBD3, our data suggest that neither mDCs nor LC-DCs use this pathway. Moreover, other studies have reported that HBD3 binds to CCR2 (367), CCR6 (366), and CXCR4 (inducing downregulation of this receptor and preventing its use by HIV as a co-receptor for T cell infection) (354). Using pertussis toxin as an overall inhibitor of G-protein coupled receptor signaling, our findings suggest that these receptors were not involved in mediating the observed HBD3 maturation of mDCs and LC-DCs. However, further work is needed to determine which receptors are utilized by HBD3 in binding to monocyte-derived DCs.

The role of inflammatory NF- κ B-inducing signals in promoting the maturation of various antigen presenting cells is well documented, since the NF- κ B transcription factor controls several genes involved in DC maturation (151). Our studies demonstrate that activated NF- κ B is required for HBD3-induced maturation in DCs. In the presence of an I κ B super-repressor, there is significant inhibition of CCR7 upregulation in both mDCs and LC-DCs. It is interesting to note however, that the inhibition was only partial, suggesting potential co-regulation with other transcription factors. This suggests that NF- κ B is required, but not sufficient, to fully activate the DC maturation pathways.

Together, these studies contribute to our understanding of the adjuvant role of HBD3 in the adaptive immune system through the activation and maturation of APC. Future studies will be directed at finding the receptor(s) important for the observed HBD3-mediated effects and on shedding greater light on the intracellular pathways activated and the transcriptional program induced by HBD3 in DCs.

5.0 SUMMARY & SIGNIFICANCE

In light of the variable and often heterogeneous pattern of chemokine expression that is observed on tumor cells, the mechanisms that are responsible for reprogramming malignant cells to express functional chemokine receptors are worthy of examination. Indeed, given the degree to which metastatic tumors differ both genotypically and phenotypically from their parental primary tumors, it is generally agreed upon that tumor environmental factors must play a key role in shaping the repertoire of receptor expression on the evolving neoplasm. Whether these receptor changes occur at the primary tumor site or at the secondary tumor site remains to be determined. What is now evident is that tumors can co-opt physiological chemokine-directed mechanisms of leukocyte migration to further their own survival, lymphatic invasion and metastasis into draining lymph nodes. This dissertation presents an examination of pro-inflammatory and pro-tumorigenic factors within the tumor microenvironment, and their subsequent signaling pathways, that enhance tumor development. The work presented here argues that the tumor environment provides crucial signals that are responsible for inducing and maintaining CCR7 expression in SCCHN both at the primary tumor and at the metastatic lymph node site.

The expression of CCR7 by metastatic (lymph node – derived) SCCHN tumor cells appears to be important for their invasive, migratory capacities as well as for survival and protection from apoptosis. For these reasons, we began with an examination of the regulation of CCR7 expression in metastatic tumor cells. As reported in chapter 2, CCR7 expression is dependent on NF- κ B signaling. We confirmed the presence of NF- κ B binding sites on the CCR7 promoter and examined their relevance using luciferase, EMSA and ChIP assays. Interestingly however, the inhibition of NF- κ B activity appears to confer a modest, albeit significant, decrease in CCR7 expression prompting us to hypothesize the involvement of other co-transcription factors in coregulating CCR7. Upon further analysis, we identified

an AP1 binding site at position -99 to -93 (TGAGTCA) on the CCR7 promoter. It is worth noting that since this AP1 site is directly adjacent to the κ B3 site at position -110 to -100, the AP1 site is included as part of the flanking nucleotides within the κ B3-Luc fragment depicted in Figure 2-2A. It is therefore highly likely that AP1 activity contributes to the basal (and inducible) activity observed on the κ B3-Luc region. No doubt further studies are required to clarify the specific role of AP1 and how it cooperates with NF- κ B in the regulation of CCR7. Nevertheless, reports in the literature support the notion that NF- κ B and AP1 transcription factors coregulate CCR7 expression (425, 426). This possible involvement of AP1 in the coregulation of CCR7 expression highlights the complexity of gene regulatory networks, and the subsequent need to elucidate these pathways for the generation of clinically relevant therapeutic targets.

The mechanism(s) leading to *de novo* induction of CCR7 expression on tumors are poorly understood. In general, since pro-inflammatory signals are involved in the induction of CCR7 on DCs, we hypothesized that similar tumor microenvironmental factors may promote CCR7 induction in SCCHN. Given the overexpression of HBD3 in oral malignancies, and the reported ability of HBD3 to phenotypically mature DCs, we hypothesized that HBD3 was involved in the induction of CCR7 expression on non-metastatic tumors. To our knowledge, ours is the first report to demonstrate the direct tumorigenic effects of HBD3 in human cancer. We show that HBD3 stimulation induces pro-inflammatory, NF- κ B-mediated signaling leading to functional CCR7 expression in SCCHN cells derived from primary, non-metastatic tumors. This gain in CCR7 expression appears to be important for facilitating the chemotactic migration of the tumor cells towards CCL19. In addition, consistent with our findings that CCR7 is an NF- κ B-dependent gene, the observed CCR7 upregulation occurs in an NF- κ B-dependent manner, although HBD3 is not directly responsible for activating NF- κ B. This is a fascinating observation. Not only does this finding confirm that NF- κ B is *required* for CCR7 expression, it alludes to the idea that NF- κ B activation is not solely *sufficient* for CCR7 expression suggesting the involvement of other co-transcription factors that are likely induced upon HBD3 stimulation. The data presented in chapter 3 confirms the activation of AP1 family transcription factors (cFos and JunD) upon HBD3 exposure of several SCCHN cell lines. These data, along with the findings from chapter 2, form a

compelling rationale for the hypothesis that the NF- κ B and AP1 family of transcription factors are involved in the coregulation of CCR7 expression. In order to comprehensively understand the regulation of CCR7, future studies should examine this cooperation between NF- κ B and AP1.

We further report that HBD3 stimulation provides pro-survival signals to primary SCCHN tumor cells through the activation of PI3K/Akt signaling, which may help explain the resistance of these tumors to standard chemotherapeutic strategies involving platinum reagents. Evidently, tumor survival and persistence depends on the sustained ability to evade immunologic and chemotherapeutic intervention. In this regard, it appears that HBD3 signaling provides a distinct advantage to tumors by activating pro-survival pathways.

The finding that HBD3 does not signal through previously identified GPCRs and TLRs but rather is endocytically internalized by tumors suggests that it is binding to as yet unidentified receptors. It is conceivable that HBD3 binds directly to intracellular targets thereby igniting a cascade of signaling pathways that result in the upregulation of CCR7 expression. An analysis of intracellular NOD-like receptors suggests that they are not involved in HBD3-mediated signaling (data not shown); although studies have shown that cationic antimicrobial peptides are capable of binding to a variety of intracellular targets. However, we also cannot exclude the possibility of receptor-mediated endocytosis of HBD3 involving a surface receptor. This remains a major unanswered question and future investigations into the receptor(s) involved in HBD3 stimulation of tumors could provide further insight into the pathways of CCR7 regulation.

Lastly, our studies on DCs provide some perspective on the role of HBD3 expression on immune cells. Epithelial cells, monocytes, macrophages and DCs themselves are a source of HBD3 secretion in the tumor microenvironment prompting us to examine the effects of HBD3 on CCR7 expression on antigen presenting cells, and whether there were any similarities to tumors. Studies using mDCs and LC-DCs show that HBD3 can induce their activation and maturation *in vitro*. The activated DCs upregulate CCR7 and become responsive to the lymph node homing chemokines CCL19 and CCL21. Furthermore, the observed HBD3-induced maturation of these DCs is not dependent on GPCRs or TLRs, two classes of

receptors previously associated with HBD binding. Nevertheless, HBD3 effects on DC maturation are dependent on NF- κ B activation. These results contribute to our understanding of the potential role of HBD3 in the pathogenesis of inflammatory skin diseases and demonstrate the adjuvant function of HBD3. Indeed, HBD3 appears to be a natural adjuvant capable of stimulating innate immunity and enhancing specific immune responses against antigens encountered by the skin. Our findings should be useful for the development of novel molecules that incorporate HBD3 and specific antigens to enhance the immunogenicity of DC-based immunotherapies and skin-targeted vaccines. However, the yin and yang that maintains the balance of beneficial HBD3 effects on immune cells, and destructive HBD3 effects on tumors is still not well understood and requires further investigation. Moreover, since our studies all utilized healthy donor-derived DCs, it is not clear to what extent similar HBD3 effects would be observed in cancer patient immune cells.

The studies presented in this dissertation advance our understanding of the inflammatory mechanisms of cancer metastasis in SCCHN. No doubt there are significant questions raised that still require further investigation in order to be fully addressed. Nonetheless, the identification of inflammatory HBD3-mediated pathways of CCR7 induction, and the described NF- κ B-dependence of CCR7 expression are novel findings that provide promising therapeutic avenues for future clinical strategies.

APPENDIX A: PUBLICATIONS

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Mburu YK, Walker WH, Ferris RL. Regulation of CCR7 gene expression by NF- κ B in Squamous Cell Carcinoma of the Head and Neck. Manuscript, 2011

Ferris LK, **Mburu YK**, Fluharty ER, Ferris RL, Falo LD. Human beta defensin 3 maturation of human langerhans cell-like dendritic cells: an antimicrobial peptide that also functions as an endogenous adjuvant. Manuscript, 2011

APPENDIX B: AUTHOR CONTRIBUTIONS

Ann Marie Egloff, Lin Wang and Raja R. Seethala were involved in the staining, analysis and presentation of the *in vivo* tumor microarray data presented in Figures 2-5 and 3-1. Koji Abe performed the chemotaxis assay presented in Figure 3-3 and the apoptosis experiments in Figure 3-7B and C. William H. Walker was involved in experimental design and analysis of the EMSA and luciferase experiments presented in Figures 2-1 and 2-2. Carter Van Waes, Saumendra N. Sarkar and Laura K. Ferris were involved in overall experimental design and analysis. Robert L. Ferris was involved in the conceptualization of the hypotheses, overall experimental design and analysis and co-writing of the manuscripts. I was involved in the conceptualization of the hypotheses and performed all the remaining experiments presented and co-wrote the manuscripts.

APPENDIX C: GLOSSARY & ABBREVIATIONS

APC: Antigen Presenting Cell

AP1: Activator Protein 1

COX-2: Cyclooxygenase 2

DC: Dendritic cells

EBV: Epstein-Barr Virus

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

ERK: Extracellular signal-regulated kinase

FPRL1: Formyl peptide receptor-like 1

Gamma-delta ($\gamma\delta$) T cells: A subset of T lymphocytes whose T cell receptor complex consists of a heterodimer of the γ - and δ -chains. They are 'innate-like' lymphocytes that circulate in tissues such as the skin and gastrointestinal tract without priming in lymphoid tissues.

HBD: Human β -defensin

HB-EGF: Heparin-binding epidermal growth factor

HCAP18: Human cationic anti-microbial protein 18

HD: Human defensin (epithelial-derived human α -defensin)

HIF-1 α : Hypoxia inducible factor 1 α

HIV: Human Immunodeficiency Virus

HPV: Human Papilloma Virus

HNP: Human neutrophil peptide (neutrophil-derived human α -defensin)

IL-1 β : Interleukin 1 β

IFN- γ : Interferon γ

I κ B: Inhibitor of κ B

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

MAPK: Mitogen activated protein kinase

NF- κ B: Nuclear factor κ B

NK cells: Natural killer cells

PMA: Phorbol 12-myristate 13-acetate

SCCHN: Squamous cell carcinoma of the head and neck

SCID: Severe combined immunodeficiency

STAT: Signal transducer and activator of transcription

TAM: Tumor associated macrophage

TGF α : Transforming growth factor α : Ligand to EGFR receptor

TNF α : Tumor necrosis factor α

TNM: A clinical staging system for grading SCCHN tumors: (T: refers to tumor size at the primary tumor site, N: refers to the status of cervical lymph nodes, and M: refers to the presence or absence of distant metastases)

TIL: Tumor infiltrating lymphocytes

TLR: Toll-like receptor

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