Immune-associated Epigenetic Regulation in the Tumor Microenvironment

by

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Dedication

He knows whom You let know

You have given me unconditionally loving, supportive, and inspiring family and friends, and cleared this path for me. What great opportunities have come in my life along the way! I will keep going.

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Each person has contributed something unique to me, none bigger or smaller.

My family has always unconditionally supported me and was always there to talk to me and inspire me every day. My extended family is also supportive, making me smile all the time.

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My friends I have met along the way, thank you for encouraging me when I needed it and allowing me to explore my hobbies and grow as a person.

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Abstract

The tumor consists of a microenvironment of many cells, including stromal and immune cells. The immune system not only can eliminate tumor cells but also can promote tumorigenesis. The tumor is constantly evolving with the pressure of the immune response and eventually creates an immunosuppressive environment and recruits immune cells that can promote its growth. This dissertation focuses on the tumor-immune crosstalk, both the effect of T cells on promoting tumor stemness as well as tumor intrinsic regulation of T cell trafficking. Both pathways are regulated epigenetically and can be reversed to suppress tumor growth.

The first project explores how Th22 cells can promote colon cancer stemness. We found that Th22 cells, through secreting IL-22, mediates cancer stemness through STAT3-mediated regulation of the epigenetic regulator DOT1L. Blocking IL-22 signaling leads to reduced *in vivo* tumor growth and cancer stemness through DOT1L-mediated gene regulation of stem cell genes. This mechanism predicts survival in colon cancer.

The second project is aimed at improving effector T cell trafficking into the tumor. The tumor creates a barrier to T cells by epigenetically repressing production of chemokines (CXCL9 and CXCL10), the key proteins that regulate trafficking of T cells into the tumor. The intrinsic repression in the tumor cells is mediated by the PRC2 complex and can be reversed by pharmacological drug leading to more chemokine production and T cell trafficking. Increased

effector T cells and chemokines and decreased PRC2 components predict better survival in colon cancer patients.

Finally, a recent project centers on trying to create a more powerful antitumor T cell by epigenetic manipulation. We focused on protein arginine deaminase (PAD)-mediated regulation of T cells. We found that PAD signaling can negatively affect T cells. Using an inhibitor of PAD4, F-Amidine, we observed that PAD inhibition increases T cell survival, proliferation, and effector cytokine expression in activated T cells.

Tumor and immune cells influence each other in the tumor microenvironment; some pathways, such as stemness and T cell trafficking, can be epigenetically regulated. Targeting these networks might be a strategy toward treating cancer patients.

Chapter 1

Introduction

1.1 Tumor immunology overview

The immune system is a highly complex system, which, in response to an insult, requires a beautifully orchestrated response of immune cells in a spatiotemporal arrangement. In response to inflammation such as a tumor response, the microenvironment is invaded with many cells such as macrophages, neutrophils, dendritic cells, T cells, and B cells. We now know that the immune system can eliminate tumor cells. However, the tumor can evolve and overcome antitumor immunity in three stages, known as immunoediting, where the tumor evolves but also shapes the immune system. Initially, the immune system may recognize and reject the highly immunogenic tumor (elimination stage), followed by an equilibrium phase where the immune system prevents tumor outgrowth but shapes tumor immunogenicity in the process, and finally, the tumor may escape detection and killing by tumor intrinsic mechanisms and/or by creating an immunosuppressive network that can promote tumorigenesis (Dunn et al., 2004, Zou, 2006, Zou, 2005, Schreiber et al., 2011). The influx of immunosuppressive or antitumor immune cells in a temporal manner can help the tumor evolve or impede its growth, respectively. In addition to the cancer cells in the microenvironment, there are other stromal cells like fibroblasts and endothelial cells that can also impact tumor growth. The crosstalk between all of these cells comprise a complex network that work together in tumor evolution

(Gajewski et al., 2013, Grivennikov et al., 2010). This thesis will discuss the dynamic interaction between tumor cells and immune cells and how different secreted proteins, like chemokines and cytokines, and also T cells themselves, are regulated epigenetically and how these pathways influence T cell trafficking, cancer stemness, and T cell immunity, respectively. These epigenetic pathways are reversible chemically and I will discuss how manipulating these pathways influence tumor immunity and tumor growth.

1.2 Chemokines in the tumor microenvironment

1.2a Introduction and history

In order for the immune cells to navigate to different areas of the body, such as the tumor site, from the blood, they respond to chemokines. The chemokines signal via chemokine receptors on the cell's surface. The cells then follow the chemotactic cytokines that form a gradient to control trafficking and migration of cells into specific sites. There are about 50 known chemokines and they can be classified into four groups according to their cysteine (C) residues: CXC, CC, C, and CX_3C, where X is a non-cysteine amino acid. With 20 known chemokine receptors (Allen et al., 2007), the chemokines are promiscuous and many chemokines bind to one receptor or one chemokine can bind to many receptors. In response to signals like cytokines, pathogens, or growth factors, many types of cells (including leukocytes, endothelial cells, fibroblasts, and epithelial cells) can secrete chemokines in order to specifically recruit cells with the corresponding receptor (Gupta et al., 1998, Scotton et al., 2001, Bartels et

al., 1996, Lukacs et al., 1995). Once the chemokine binds to the receptor, intracellular signals lead to directional migration (Rot and von Andrian, 2004).

Although the initial studies of chemokines were focused on innate immune cell recruitment in acute inflammation with the discovery of CXCL8 (Walz et al., 1987), the role of chemokines in various immune responses is now well appreciated. Chemokines are crucial for all immune cells and various diseases and immune processes (both homeostatic and inflammatory). The role of chemokines in immunity and host defense is reviewed elsewhere (Griffith et al., 2014, Charo and Ransohoff, 2006, Gerard and Rollins, 2001, Moser and Willimann, 2004, Rossi and Zlotnik, 2000). This chapter focuses on chemokines in the tumor microenvironment, especially T cell trafficking. T cells will create an inflammatory environment that can either promote or suppress the tumor based on the chemokines and cytokines produced in the microenvironment (review in (Grivennikov et al., 2010)). Initial observations of chemokine receptors were made in leukocytes but endothelial cells and epithelial cells can respond to and even secrete chemokines, greatly influencing the tumor response. Even though the best known function of chemokines is trafficking, they can have other functions and effects on non-immune cells, including angiogenesis, leukocyte granule/mediator release, metastasis, proliferation, and other pro-tumor effects, as discussed below. Since its crucial role in the tumor response, chemokine regulation as well as the functional role of chemokines on immune and non-immune cells (including trafficking and pro-tumor effects) and its relation to tumor biology will be discussed.

1.2b Chemokines and T cell trafficking

Many studies have shown that tumor-infiltrating effector T cells are associated with improved prognosis in multiple human cancers (Galon et al., 2006, Pages et al., 2005, Sato et al., 2005), whereas tumor-infiltrating regulatory T (Treg) cells are, for the most part, negatively associated with patient outcome (Curiel et al., 2004, Sato et al., 2005). One of the effector subtypes, T helper cell type 1 (Th1) cells, mediate a potent antitumor response by secreting cytokines and recruiting and activating cytotoxic T lymphocytes (CTL's) (Hung et al., 1998). Chemokine (C-X-C motif) ligand (CXCL)9 and CXCL10 are the major Th1-type chemokines, and mediate the trafficking of the main antitumor cells: Th1, effector $CD8^+$ T cells, and natural killer (NK) cells (as well as B cells, macrophages, and DC's) into tumor. CXCL9 and CXCL10 are induced in many cells by interferon (IFN) gamma (IFNγ) and T cells express the receptor, CXCR3. Particularly, increased levels of Th1 cells, CD8 T cells, and CXCL9/CXCL10 correlates with increased survival and less metastatic invasion in colon cancer (Galon et al., 2006, Pages et al., 2005, Ohtani, 2007, Mlecnik et al., 2010, Jiang et al., 2010). Remarkably, T cell and CXCL9/CXCL10 characterization in the tumor is a better predictor of patient survival than the standard colon cancer classification (Galon et al., 2006). In fact, in Hodgkin's lymphoma and Kaposi's sarcoma, Th2 attracting chemokines are expressed to weaken the Th1 mediated antitumor immunity (Skinnider and Mak, 2002, Sozzani et al., 1998).

In ovarian cancer, Th17 cells are present and positively correlate with effector T cells and predict patient outcome, promoting long term immunity (Kryczek et al., 2009, Kryczek et al., 2011, Wei et al., 2012, Zou and Restifo, 2010, Kryczek et al., 2007). They are polyfunctional, and through secreting IFNγ, stimulate chemokine CXCL9 and CXCL10 to recruit effector T cells

(Kryczek et al., 2009). Due to the chemokines' importance in mediating antitumor immunity, its regulation is tightly controlled, as discussed below.

Th22 cells, characterized as CD3+CD4+IL-22+ in humans, express CCR6 and migrate towards the tumor in a CCL20 dependent manner. Th22 cells and IL-22 expression is higher in colon cancer (Huang et al., 2015, Kryczek et al., 2014). IL-22 enhances colon cancer stemness via signal transducer and activator of transcription 3 (STAT3) and Disruptor of telomeric silencing 1-like (DOT1L) mediated H3K79 methylation (Kryczek et al., 2014). This pathway will be discussed in chapter 2. IL-22 can also increase colon cancer cell proliferation through another epigenetic complex, polycomb repressive complex 2 (PRC2) and STAT3 (Kryczek et al., 2016). Thus, targeting this pathway might be meaningful for colon cancer treatment.

Finally, regulatory T cells (Tregs), due to their function in maintaining immune tolerance to self-antigens can dampen T-cell immunity to tumor-associated antigens (TAAs). In ovarian cancer, Tregs suppress T cell antitumor immunity, leading to tumor growth and reduced survival (Zou, 2006). The macrophages in the tumor as well as tumor cells themselves secrete CCL22 ligand, which mediates the trafficking of Tregs into the tumor via CCR4 receptor on the Tregs. Other chemokines important for Treg recruitment in cancer include: CCR10 which migrate via CCL28 induced by tumor hypoxia (Facciabene et al., 2011) and CXCL9, CXCL10, and CXCL11 ligands in ovarian cancer to recruit $CXCR3^+$ Tregs (Redjimi et al., 2012).

Importantly, in cancer, Tregs preferentially migrate and are retained in the bone marrow. Once activated, Tregs express CXCR4, enabling them to migrate there via CXCL12 expression in the bone marrow (and tumor) and may explain why cancers often metastasis to the bone marrow in breast and prostate cancer (Zou et al., 2004, Zhao et al., 2012, Wei et al.,

2007). Our group has recently shown the existence of IL-8+FOXP3+CD4+ Treg cells in colon carcinoma and ulcerative colitis. These cells stimulate inflammatory cytokine production in the colon tissues and can also promote neutrophil trafficking through the chemokine IL-8 (Kryczek et al., 2016). All T cell subsets trafficking as well as other immune cell trafficking (B cells and antigen presenting cells) is summarized in Figure 1.1.

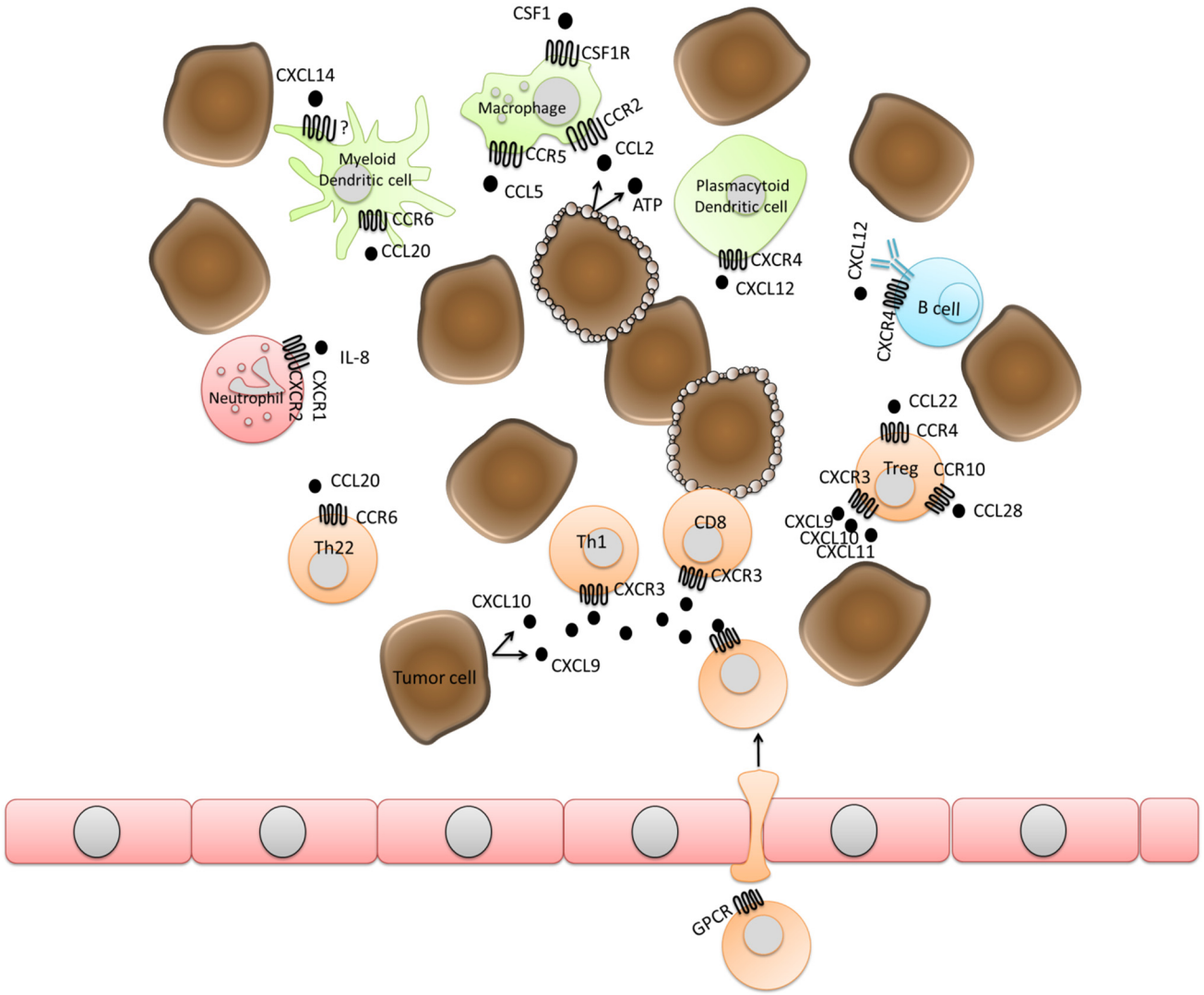

Figure 1.1: Immune effects of chemokines. Many immune cells are recruited to the tumor microenvironment through secreted chemokines in the environment. The immune cells possess specific chemokine receptors and once the chemokine binds to the receptor, intracellular signals lead to directional migration through the endothelial cells and into the microenvironment. For example, tumor cells can secrete CXCL9 and CXCL10 after stimulation with IFNγ and will recruit the main antitumor T cells into the environment, $CD8^{+}$ cytotoxic T cells and Th1 T cells. Dying tumor cells can also secrete chemokines like CCL2 and ATP, which can recruit myeloid cells. Adenosine triphosphate (ATP), cytotoxic T lymphocytes (CTL's), T helper cell type 1 (Th1), Treg (regulatory T cells).

1.2c Non-immune effects of chemokines

In solid malignancies, tumor development leads to an inflammatory network that will create a protumorigenic environment (reviewed in: (Mantovani et al., 2008, Grivennikov et al., 2010)). This inflammatory environment will recruit cells via chemokines in the tumor microenvironment as discussed above. The chemokine themselves can also have tumor promoting (or inhibiting) roles as discussed below. Some of these roles include promoting metastasis, invasion, proliferation, stemness, inflammation, and angiogenesis.

IL-8 (CXCL8)

In addition to IL-8's role in attracting neutrophils, IL-8 can influence endothelial cells and tumor cells. The receptor, CXCR1 and CXCR2 (Holmes et al., 1991, Murphy and Tiffany, 1991), is expressed on these cells suggesting a potential role in cancer progression. Melanoma cells produce IL-8 and the expression positively correlates with metastatic potential *in vivo* (Singh et al., 1994, Bar-Eli, 1999). They also express CXCR1 and CXCR2 and exhibit CXCR2 dependent IL-8 mediated invasion and migration (Gabellini et al., 2009). Interestingly, IL-8 signaling in prostate cancer cells contributes to hypoxia-induced apoptosis resistance (Maxwell et al., 2007). In terms of metastatic potential, IL-8 production in tumor cells can promote epithelial-mesenchymal transitions (and thus metastasis) in many ways, including promoting an inflammatory and angiogenic environment by attracting cells, inducing EMT in nearby epithelial cells, and maintaining a mesenchymal and invasive phenotype by autocrine IL-8 signaling (reviewed in (Palena et al., 2012, Fernando et al., 2011)). IL-8 signaling in endothelial cells regulates angiogenesis, through promoting endothelial cells survival and secretion of MMP-2 (Li et al.,

2003, Schwartz et al., 1998). To no surprise, lowering IL-8 levels in many cancers is associated with decreased angiogenesis, metastatic potential, and tumorigenecity in many cancers including prostate cancer, melanoma, bladder cancer, and pancreatic cancer (Inoue et al., 2000a, Huang et al., 2002, Inoue et al., 2000b, Mian et al., 2003, Shi et al., 1999).

There is also a link between IL-8, tumor EMT, and tumor stemness. IL-8 can induce stemness in many tumors including colorectal cancer, lung cancer, thyroid cancer, and pancreas cancer (Hwang et al., 2011, Liu et al., 2015, Visciano et al., 2015, Chen et al., 2014). However, IL-8 can promote immunogenicity of the tumor and potential antitumor response by translocating calreticulin to the outer surface of the plasma membrane, in response to mitoxantrone (Sukkurwala et al., 2014). Calreticulin exposure on the plasma membrane leads to immunogenic cell death to promote uptake by the DC and subsequent tumor-specific T cells (Obeid et al., 2007, Gardai et al., 2005). Thus, the effect of IL-8 can depend on the context (i.e. chemotherapy) (Ma et al., 2014).

CCL2

Even though CCL2 depletion is associated with tumor associated macrophage (TAM) function and is a therapeutic target, CCL2 depletion causes rapid and severe metastasis and angiogenesis. This suggests that CCL2 not only regulates monocyte recruitment and the inflammatory environment but also blood vessel formation (Bonapace et al., 2014, Saji et al., 2001, Goede et al., 1999). In addition to the tumor cells, endothelial cells express CCR2 and colon cancer-derived CCL2 leads to vascular permeability and consequently tumor extravasation and metastasis (Wolf et al., 2012). The metastatic cancer cells also proliferated

more. CCL2 can directly target the tumor cell and signaling promotes cancer cell survival and motility (Fang et al., 2012). The fibroblasts-derived CCL2 can also drive breast cancer stemness and stem cell renewal (Tsuyada et al., 2012).

CCL5

CCL5 can promote tumor growth through recruiting TAM's through CCR5 but also through promoting invasion, metastasis, and proliferation of cancer cells. In fact, in gastric cancer, high levels of CCL5 in patients were associated with more invasion, tumor size, higher tumor grade, and lymph node metastasis (Ding et al., 2016). In ovarian cancer, CCL5 expression from CD133+ ovarian cancer stem cells act on CD133- tumor cells and promote EMT and enhanced metastatic ability *in vitro* and *in vivo* (Long et al., 2015). One mechanism is through MMP-9 secretion. CCL5 induces MMP-9 secretion from the monocytes and cancer cells (Azenshtein et al., 2002). MMP-9, through degrading the matrix, allows for extravasation and is important for cancer invasion and tumorigenesis (Stamenkovic, 2000).

CXCL9 and CXCL10

In addition to CXCL10 being a potent chemoattractant for T cells, natural killer cells, B cells, macrophages, and DC's and thus inflammation in the tumor microenvironment, it is also an endogenous angiogenesis inhibitor and important for preventing neovascularization in a tumor (Belperio et al., 2000). CXCR3, the receptor for CXCL10, is expressed on endothelial cells, and as its signaling prevents proliferation, CXCR3 expression in endothelial cells could potentially explain the angiostatic activity (Romagnani et al., 2001). CXCL10 prevents both IL-8

and basic fibroblast growth factor-induced angiogenesis *in vivo* and *in vitro* (Strieter et al., 1995, Angiolillo et al., 1995). CXCL10, and possibly CXCL9, induces tumor necrosis in a mouse model of lymphoma (Sgadari et al., 1996, Sgadari et al., 1997). In terms of *in vivo* potency of angiostatic effects, a chimeric protein has been suggested to combine the functional moieties of CXCL10 and CXCL11 (called ITIP), which exerts more potent and prolonged antitumor efficacy and angiostatic effects in mouse models of multiple tumors. Both chemokines alone did not have a synergistic antitumor response, suggesting that a chimeric chemokine could be more effective in chemokine-based cancer therapy (Wang et al., 2010).

CXCL12/CXCR4

CXCR4 is generally expressed on many types of cancer cells and its signaling can affect the cancer cells in many ways. The chemokine for CXCR4, CXCL12 is expressed in many tissues and helps to retain the cancer cells in the primary site or at sites of metastasis. The signaling can enhance proliferation and survival of these cells in sites of minimal growth conditions (Balkwill, 2004). CXCR4 can also increase cancer cell invasion and metastatic abilities, as metastatic cells upregulate CXCR4 (Darash-Yahana et al., 2004, Kang et al., 2003, Helbig et al., 2003, Murakami et al., 2002). Indeed, anti-CXCR4 or CXCL12 antibodies prevented metastasis, reduced tumor weight, and prevented tumor extravasation (Muller et al., 2001, Rubin et al., 2003, Liang et al., 2005, Lapteva et al., 2005, Bertolini et al., 2002, Cardones et al., 2003). It is interesting to note that CXCR4 deficiency in colon cancer cells leads to lung metastasis but reduced proliferation of the cells upon arrival, suggesting that metastasis still occurs and the effect of CXCR4 is through a cell intrinsic proliferation mechanism (Zeelenberg et al., 2003).

In terms of cancer stemness, CXCR4 is a marker for cancer stem cells in ovarian cancer, non-small cell lung cancer, and colon cancer (Cioffi et al., 2015, Jung et al., 2013, Zhang et al., 2012). In colon cancer, these CD133+ CXCR4+ cancer stem cells had increased metastatic capacity and conferred worse survival in patients (Zhang et al., 2012). In non-small cell lung cancer, CXCR4 signaling maintained cancer stemness and these cells showed radiation resistance and tumorigenic potential (Jung et al., 2013). All this data suggest a diverse role for CXCR4 in tumor proliferation, metastasis, and stemness as CXCR4 antagonism leads to reduced tumor burden, metastasis, and resensitization to drugs.

All of these discussed mechanisms are summarized in Figure 1.2.

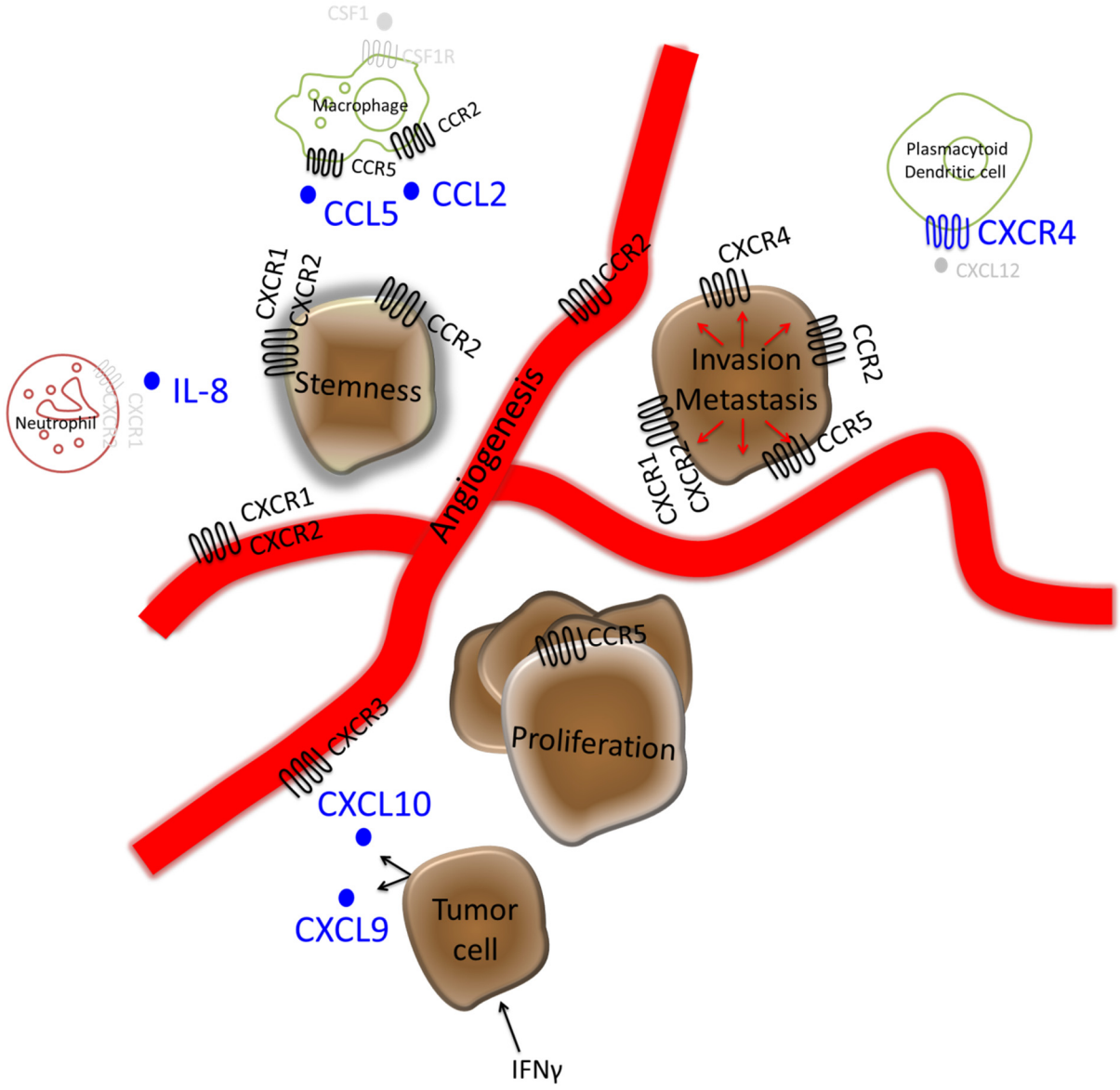

Figure 1.2: Non-immune effects of chemokines. Chemokine signaling is not only important for trafficking of cells but can also directly affect the cells. Signaling from IL-8, CCL2, CCL5, CXCR4, CXCL9, and CXCL10 in cells such as endothelial cells and tumor cells can influence tumor cell stemness, invasion/metastasis, proliferation and endothelial cell angiogenesis and survival.

1.2d Chemokine regulation

Environmental Cues:

Metabolism

In response to T cell activation and T cell receptor triggering, the kinase Akt plays a major role in controlling and increasing T cell metabolism. Akt controls T cell metabolism and survival as blocking PI3K, and thus Akt signaling, leads to less nutrient uptake, mainly amino acids and glucose (Cornish et al., 2006). However, Akt, at different threshold concentrations, can also control T cell migration through chemokine regulation. High levels of Akt, as in effector T cells, have low expression of CD62L, CCR7, and SIP1, chemokines important for lymph node trafficking. Conversely, T cells that retain the ability to traffic to the lymph nodes do not fully activate Akt and thus have higher levels of the lymph node homing chemokines (Waugh et al., 2009, Sinclair et al., 2008, Finlay et al., 2009). CD62L down regulation is mediated by Akt driven inhibition of forkhead box O (FOXO) proteins. FOXO transcription factors directly control and drive the expression of the transcription factor Krüppel-like factor 2 (KLF2), which regulates CD62L expression (Carlson et al., 2006, Bai et al., 2007). Therefore, AKT-driven downregulation of FOXO1 down regulates KLF2 and thus CD62L (Waugh et al., 2009). In conclusion, Akt and other metabolism signaling can be involved in chemokine regulation and thus T cell function. Chemokines themselves can also lead to downstream metabolic signaling. CCL5 signaling increases glucose uptake and intracellular ATP levels to support chemotaxis energy demands (Chan et al., 2012).

Metabolism in cancer cells has been studied quite extensively (Hsu and Sabatini, 2008); the cancer cells rely mainly of glycolysis with lactic acid production as a byproduct. This lactate

can signal in endothelial cells by inducing phosphorylation and degradation of the Inhibitor of κB (IκBα), and thus increasing NF-κB. NF-κB can then induce IL-8 production, resulting in angiogenesis in breast and colon cancer (Vegran et al., 2011).

Hypoxia and the immune milieu

In general, chemokines expression is regulated by many factors including cytokines, hypoxia, and the cell type and stage of differentiation. The versatile chemokine receptor, CXCR4, is regulated by many factors in the tumor microenvironment. As discussed above, CXCR4 mediated signaling in cancer cells leads to many protumor effects, thus its signaling must be tightly regulated. In many cells, including cancer cells, its expression is upregulated by TNF-α, VEGF, NF-κB, and hypoxia (Kulbe et al., 2005, Bachelder et al., 2002, Kryczek et al., 2005, Helbig et al., 2003, Schioppa et al., 2003, Staller et al., 2003). In vascular endothelial cells, VEGF increases CXCR4 expression; hypoxia in the tumor microenvironment increases both VEGF and CXCL12 expression, creating a synergistic angiogenic loop (Kryczek et al., 2005). Macrophages and TAM's are also regulated by hypoxia as hypoxia induces CXCR4 expression (Schioppa et al., 2003). The CXCL12 receptor, CXCR4, is induced in macrophages in hypoxic regions and may thus regulate trafficking in hypoxia rich tumor regions (Ceradini et al., 2004). In addition, hypoxia can retain the cells in hypoxic regions as hypoxia can inhibit CCR2 expression and thus CCL2 mediated macrophage trafficking (Bosco et al., 2004). Not surprisingly, as hypoxic regions exists in many tumors, TAM's generally accumulate in these avascular regions and can modulate the tumor response (Murdoch et al., 2004). As TAM accumulation and infiltration has been

suggested in promoting an angiogenic switch in cancer, understanding their mechanism of trafficking will help in inhibiting cancer progression.

Environmental oxygen tension in cells is regulated by the transcription factor, hypoxia-inducible factor (HIF). Other chemokines like CCL2 also has HIF-1 binding sites in the promoter and CCL2 can be induced in human astrocytes by hypoxia (Mojsilovic-Petrovic et al., 2007). Therefore, chemokines can be induced by hypoxia, with HIF-1 being a major chemokine regulator of vital tumor regulating chemokines.

Oncogenic regulation:

β-catenin

Antibody-mediated cancer immunotherapy works remarkably in a subset of patients, presumably due to the increased T cell infiltration in those patients (Ji et al., 2012, Harlin et al., 2009). A recent paper sought to further dissect the mechanism of variable T cell infiltration in tumors. Spranger and colleagues (Spranger et al., 2015) found that melanoma tumors with little to no T cell infiltration had activated β-catenin signaling. Using a genetically engineered mouse melanoma model with activated β-catenin, they found that activated β-catenin led to lower levels of CCL4, a chemokine essential for Batf3-lineage DC's ($CD103^+$ DC's) migration. Thus, when β-catenin was activated, these DC's were not being recruited or activated in the tumor leading to little or no T cell infiltration in the tumor and no priming against tumor-associated antigens. Consequently, therapeutic efficacy of anti-CTLA-4 and anti-PD-L1 monoclonal antibodies was not observed when β-catenin was activated, but could partially be restored with addition of intratumor DC's. The study suggests chemokine control through an intrinsic

mechanism in the tumor cells can influence DC migration, which will affect T cell migration and ultimately cancer immunotherapy efficacy.

Epigenetic repression

NF-κB transactivation is important for chemokine expression including IL-8 and CCL2 in ovarian cancer (Singha et al., 2014, Xu et al., 1999). Epigenetic modifier, histone deacetylase (HDAC) 1, generally associated with gene repression, can physically bind to the NF-κB subunit Rel homology domain of p65 which will lead to gene suppression. NF-κB sites exists in the IL-8 promoter region. Using TNFa treatment to activate NF-κB and an inhibitor of HDAC, an increase in IL-8 is observed suggesting that NF-κB signaling leads to IL-8 suppression via HDAC (Ashburner et al., 2001, Mayo et al., 2003).

As CXCL9 and CXCL10 mediate trafficking of clinically relevant antitumor T cells, their expression is repressed in ovarian and colon cancer. IFNγ-induced CXCL9 and CXCL10 are repressed by epigenetic complexes, PRC2 and DNA methyltransferase 1 (DNMT1) in ovarian cancer and PCR2 in colon cancer. Removing this inhibition by pharmacological drugs or RNA-interference leads to significantly higher chemokine production and increases effector T cell migration. This increase in migration *in vivo* leads to slower tumor progression. In addition, the PRC2 complex components are negatively associated with T cell infiltration and confer worse patient outcome in ovarian and colon cancer (Peng et al., 2015, Nagarsheth et al., 2016). Thus, epigenetic silencing of chemokines is a novel immune-evasion mechanism of tumors and is a new therapeutic strategy. This project will be discussed in chapter 3.

Mutations

While mutations exist for chemokines in the context of HIV, there is very little evidence of chemokines mutations in cancer. One report identifies a point mutation in CXCR4 (G574A) in one melanoma and one colon cancer cell line. The mutation still allows for CXCL12 mediated signaling but delayed tumor growth *in vivo*. Interestingly, treatment of the tumor with a CXCR4 inhibitor, AMD3100, leads to increased tumor growth, compared to wild-type cells (Ierano et al., 2009).

All of the discussed mechanisms of chemokine regulation are summarized in Figure 1.3.

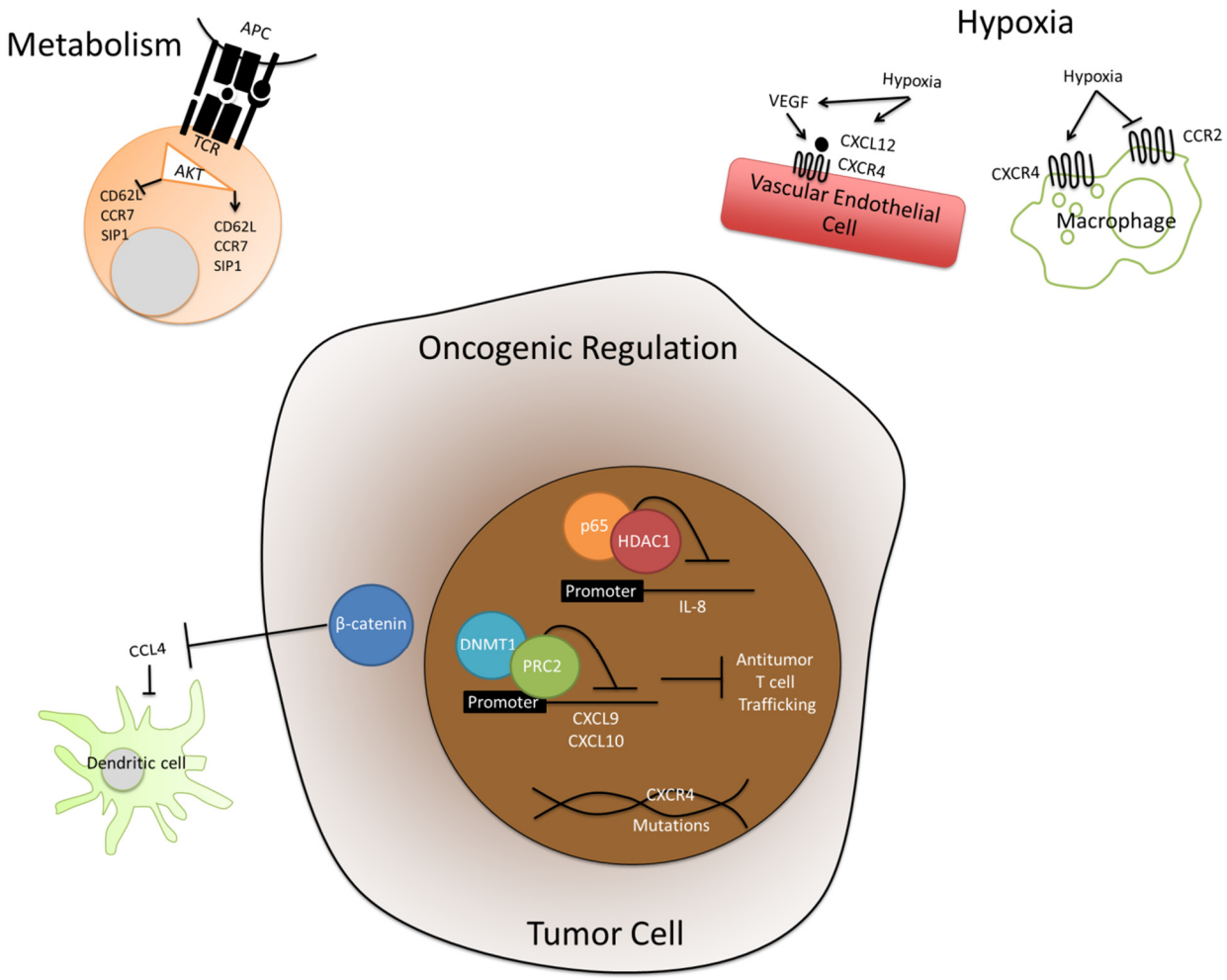

Figure 1.3: Chemokine regulation. Chemokines can be regulated by many mechanisms, including intrinsically through oncogenic regulation and extrinsically through environmental factors. Chemokines in the tumor cells can be regulated by epigenetic factors such as PRC2 and HDAC1 as well as DNA mutations. In addition, signaling through NF-κB (induced by many cytokines) and β-catenin in the tumor cell can also directly regulate chemokines such as IL-8 and CCL4. Externally, hypoxic environment can induce CXCR4 and CXCL12 expression in endothelial cells as well as macrophages. Hypoxia can also retain macrophages in hypoxic regions as hypoxia can inhibit CCR2 expression. Finally, activation induced metabolic signaling (such as Akt) in T cells can regulate T cell migration in the lymph nodes through chemokines important for lymph node trafficking. Antigen presenting cell (APC), T cell receptor (TCR), SMAD interacting protein 1 (SIP1), Histone deactetylase 1 (HDAC1), DNA methyl transferase 1 (DNMT1), Polycomb repressive complex 2 (PRC2)

1.2e Chemokines and immunotherapy

Cancers do not grow in isolation but in a microenvironment of stromal, inflammatory, and immune cells. Adaptive immunity plays a crucial role in tumor immunosurveillance (Dunn et al., 2004, Koebel et al., 2007, Vesely et al., 2011). Since effector T cells, especially CD8 and Th1 T cells, confer better patient survival in most tumors, a critical question still remains as to what controls "inflamed" tumors with effector T cell infiltration versus tumor with little infiltration. What defines the immune cell infiltration in the tumor microenvironment? As tumors can secrete chemokines, they can dictate the tumor immune response and infiltration. We propose different ways of regulation, such as oncogenic regulation within the tumor: metabolism, epigenetics, signaling through β-catenin, and genetic mutations can all regulate chemokine production and thus the immune infiltration. There are also many environmental factors and cues that can influence the inflammatory environment, including metabolic products, hypoxia, as well as the preexisting immune subsets in the microenvironment. Thus, epigenetic therapy, oncogenic therapy, and even chemokine blockade could be useful in regulating chemokines.

For cancer immunotherapy, the role of chemokines in relation to immune cell trafficking and infiltration will be an important parameter. Existing cancer immunotherapy can be divided into three types: "active immunization", "nonspecific immunomodulation", and adoptive cell transfer (Rosenberg et al., 2008). Active immunization includes using immunizing agents such as whole cells, peptides, and proteins to vaccinate the patient against their own tumor. This has not been successful except for sporadic and rare solid tumors, which could partially be due to the immunosuppressive microenvironment (Rosenberg et al., 2008, Rosenberg et al., 2005). To

address this microenvironment, nonspecific immunomodulation therapy has been developed and includes antibody-mediated therapy designed to block the suppressive immune mechanisms, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and B7 homolog 1 (B7-H1) receptors and ligands. Antibodies to CTLA-4 have shown remarkable clinical regression for melanoma and renal cancer (Leach et al., 1996, Hodi et al., 2010, Robert et al., 2011). Antibodies to PD-1 (Dong et al., 2002, Topalian et al., 2012) and its ligand (B7-H1) (Dong et al., 2002, Curiel et al., 2003) are also FDA-approved for certain cancers (Zou et al., 2016). Adoptive cell transfer has also gained acceptance (June, 2007). In particular, effector T cells: cytotoxic $CD8^+$ T cells (CTL's) and CD4+ T helper cell type 1 (Th1) cells, are transferred into patients. To make the T cells more persistent and effective in the tumor, patient-derived genetically modified T cells, or chimeric antigen receptor modified T cells (CAR cells), have been created (Eshhar et al., 1993, Gross et al., 1989). The CAR cells are then reinfused back into the patient and have been successfully used for treating hematopoietic malignancies in phase I clinical trials (Laport et al., 2003, Rapoport et al., 2005).

Many of the above mentioned chemokine targeting strategies can dramatically improve cancer immunotherapy, especially with antibody blockade. As shown above, epigenetic regulation of chemokine production can improve the antitumor immune response by increasing antitumor effector T cells in the tumor. However, combining epigenetic repression with PD-L1 therapy can dramatically improve the therapeutic efficacy. As chemokines are increased, adoptive T cell transfer is also improved (Peng et al., 2015). Another example is the gut microbiota. The microbiota may also play an important role as manipulating one microbe (*Bifidobacterium*) alone can completely alter and improve the immune response but addition of

anti-PD-L1 will further improve the response (Sivan et al., 2015). Finally, manipulating β-catenin activation in the tumor can lead to a better tumor response with the addition of Batf3-lineage DC's and the response is even greater with anti-PD-L1 and CTLA-4 (Spranger et al., 2015). Thus, combining the above-mentioned strategies with current immunotherapy has potential to increase therapeutic efficacy and serve as a new avenue for therapy.

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Chapter 2

IL-22$^+$ CD4$^+$ T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methlytransferase DOT1L[1]

2.1 Abstract

Little is known about how the immune system impacts human colorectal cancer invasiveness and stemness. Here we detected interleukin-22 (IL-22) in patient colorectal cancer tissues that was produced predominantly by CD4$^+$ T cells. In a mouse model, migration of these cells into the colon cancer microenvironment required the chemokine receptor CCR6 and its ligand CCL20. IL-22 acted on cancer cells to promote activation of the transcription factor STAT3 and expression of the histone 3 lysine 79 (H3K79) methytransferase DOT1L. The DOT1L complex induced the core stem cell genes NANOG, SOX2, and Pou5F1, resulting in increased cancer stemness and tumorigenic potential. Furthermore, high DOT1L expression and H3K79me2 in colorectal cancer tissues was a predictor of poor patient survival. Thus, IL-22$^+$ cells promote colon cancer stemness via regulation of stemness genes that negatively affects patient outcome. Efforts to target this network might be a strategy in treating colorectal cancer patients.

[1]Excerpts taken from Kryczek I, Lin Y, Nagarsheth N et al. IL-22(+)CD4(+) T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase DOT1L. *Immunity*. 40(5):772-84. PMID: 24816405.

2.2 Introduction

The interaction between tumor cells and immune elements might directly promote tumor development and progression (Ben-Neriah and Karin, 2011, Coussens et al., 2013), and/or result in immunoediting of the tumor that molds the cancer into either a dormant state (Dunn et al., 2002, Matsushita et al., 2012) or fosters tumor immune evasion (Pardoll, 2012, Zou, 2005). The role of tumor-infiltrating $CD8^+$ T cells and regulatory T (Treg) cells (Curiel et al., 2004, Galon et al., 2006) has been extensively studied in human cancers. Interleukin-22^+ (IL-22^+) immune cells are identified in humans and include both IL-22^+CD4^+ T (Th22) cells (Duhen et al., 2009, Trifari et al., 2009) and IL-22 expressing innate leukocytes (ILC22) (Cella et al., 2009, Spits and Cupedo, 2012), but the role of IL-22^+ immune cells is poorly defined in the human cancer microenvironment.

IL-22-producing immune cells could have a role in molding cancer, particularly colon cancer. The cytokine IL-22 has been shown to protect intestinal epithelial cells from bacterial infection and inflammation damage in mice (Aujla et al., 2008, Basu et al., 2012, Hanash et al., 2012, Pickert et al., 2009, Sonnenberg et al., 2012, Sonnenberg et al., 2010, Zheng et al., 2008) and supports thymic repair (Dudakov et al., 2012). Recent mouse studies have revealed that IL-22^+ cells stimulate tumor cell proliferation in a bacteria-induced colon cancer model (Kirchberger et al., 2013), and IL-22 binding protein (IL-22BP) reduces chemical carcinogen-induced colon-cancer development (Huber et al., 2012). Interestingly, IL22 polymorphisms might be associated with an increased risk of colon carcinoma development (Thompson et al., 2010). These data suggest a potential link between IL-22^+ cells and colorectal cancer development and progression in humans. However, the nature and clinical relevance of IL-22^+

cells is poorly defined in patients with colorectal cancer. It is not known whether and how IL-22$^+$ cells impact human colon cancer.

It has been demonstrated that cancer-initiating cells or cancer stem cells play an important role in shaping the invasive cancer phenotype by contributing to tumor initiation, metastasis/ relapse, and therapeutic resistance (Brabletz et al., 2005, Dean et al., 2005, Pardal et al., 2003, Reya et al., 2001, Vermeulen et al., 2012). The key issue in cancer stem cell biology is understanding the mechanisms that control cancer cell self-renewal and expansion. Recent evidence suggests some degree of external control from the microenvironment that defines the stem cell niche (Bendall et al., 2007, Cui et al., 2013, Scadden, 2006). Given that the protective role of IL-22 in epithelial cells (Aujla et al., 2008, Basu et al., 2012, Dudakov et al., 2012, Hanash et al., 2012, Pickert et al., 2009, Zheng et al., 2008) and its effects on bacteria (Huber et al., 2012) and chemical carcinogen (Kirchberger et al., 2013) induced cancer in mice, we hypothesized that colon cancer-infiltrating IL-22$^+$ immune cells contribute to cancer stem cell renewal and expansion, reshape the tumor invasive phenotype, and affect colon cancer patient outcomes. In this work, we focused on the interaction between IL-22$^+$ immune cells and cancer (stem) cells. We demonstrated that IL-22$^+$CD4$^+$ T cells promote colorectal cancer stemness via STAT3 transcription factor activation and induction of the methyltransferase disrupter of telomeric silencing 1-like (DOT1L) and that this is relevant for outcome in patients with colon cancer.

2.3 Materials and Methods

Human Subjects

Patients diagnosed with colon carcinomas were recruited in the study. All usage of human subjects in this study was approved by the local Institutional Review Board. One hundred fifty one formalin-fixed, paraffin-embedded tumor tissue blocks were obtained during surgery. These patients underwent resection of the primary tumor at the Second Department of General Surgery in Medical University of Lublin between 2007 and 2008. The follow-up period was an average 2.8 years. Additional 177 patients with colon cancer were evaluated from the National Center for Biotechnology Information Gene Expression Omnibus database (GSE17536) (Smith et al., 2010). Thirty-six fresh cancer tissues were collected from patients with colon cancer newly diagnosed at the University of Michigan and the University of Florida. Primary colon cancer cells, immune cell subsets and all the in vitro and in vivo functional assays were performed with single cells from fresh colon cancer tissues and peripheral blood.

Cell Isolation and FACS Analysis

Single cell suspensions were prepared from fresh colon cancer tissues as previously described (Curiel et al., 2004, Curiel et al., 2003, Kryczek et al., 2006, Zou et al., 2001). Immune cells and tumor cells were enriched with paramagnetic beads (StemCell Technology). Lin$^-$CD45$^-$EpCAM$^+$ cells primary colon cancer cells and CD4$^+$CD45$^+$ T cells were sorted from stained single cell suspensions with a high speed cell sorter (FACSaria, Becton Dickinson Immunocytometry Systems). Cell purity was >98% as confirmed by flow cytometry (LSR II, BD). Cytokine profile was determined with intracellular staining and analyzed by LSRII (BD).

Cell Culture and Sphere Formation

Three primary colon cancer cell lines (1, 2, 3) were established from fresh colon cancer tissues. DLD1 and HT29 cell lines (ATCC) were used in the experiments. Colon cancer cells were treated with recombinant IL-22 (R&D systems) and/or colon cancer infiltrating $CD4^+$ T cells for different time points. The DOT1L inhibitor EPZ004777 (10 mM) and relevant antibodies were added in conventional or sphere culture (Kryczek et al., 2012). Tumor cell sphere formation and gene expression were examined (Kryczek et al., 2012).

Lentiviral Transduction

Several lentiviral vectors were used to transduce colon cancer cells and establish stable cell lines. The lentiviral transduction efficiency was confirmed by GFP which was coexpressed by the lentiviral vector. The knockdown efficiency was assessed by immunoblotting. The vectors included pGIPZ lentiviral vector encoding gene-specific shRNAs for STAT3, DOT1L, or scrambled shRNA (Puromycin resistant); lentiviral vectors encoding an active form of Notch (the transmembrane and intracellular domains, comprising residues 1,704–2,531) Notch-IC cDNA (Notch IC) (Wang et al., 2011, Yamamoto et al., 2001), or constitutively active STAT3 domain (STAT3C) (EF.STAT3C.Ubc.GFP) (Bromberg et al., 1999, Li and Shaw, 2006). Human DOT1L cDNA was cloned into the entry vector pENTR223.1 (Thermo Scientific Open Biosystem, OHS5894-202503093) and inserted into the destination and expressing vector pDEST26 by GatewayRecombination Cloning Technique (Invitrogen). Human DOT1L expression vector (pDEST26-hDOT1L) was verified by restriction enzyme digestion and DNA sequencing.

Cytokine Detection

The amount of cytokines protein was detected either by ELISA (R&D) or flow cytometry analyzer (FACS) as described previously (Curiel et al., 2004, Kryczek et al., 2011). All samples were acquired with LSR II (BD) and analyzed with DIVA software.

Real-Time RT-PCR

The mRNA was quantified by real-time RT-PCR. SYBR Green Master Mix was used to detect fluorescence. Relative expression was calculated according to the Ct value with normalization to GAPDH.

Immunoblot

Immunoblotting was performed with specific antibodies against human STAT3 (9132, Cell Signaling), phosphorylated STAT3 (9138, Cell Signaling), Oct3/4 (sc- 5279, Santa Cruz biotechnology), Nanog (ab21624, Abcam), Sox2 (MAB4343, Millipore), H3K79me2 (ab3594, Abcam), H3K9me2 (ab1220, Abcam), H3K9me3 (ab8898, Abcam), H4K20me3 (07-463, Millipore), H3K27me3 (07-449, Millipore), H3K36me3 (ab9050, Abcam), acetyl-Histone H3 (06-599, Millipore), Histone H3 (9715, Cell Signaling), b-Actin (A5441, Sigma), Dot1L (ab72454, Abcam), and Cleaved Notch1 (NICD, ab52301, Abcam). Signals were detected by ECL reagents (GE Healthcare).

In Vitro and In Vivo Migration Assays

In vitro migration assay was performed in a Transwell system with a polycarbonate membrane of 6.5 mm diameter with a 3 mm pore size as described (Curiel et al., 2004, Curiel et al., 2003).

Purified T cell subsets were added to the upper chamber and CCL-20 (5 ng/ml, R&D) was added to the lower chamber. After 4 hr incubation at 37C, the phenotype and number of T cells in the upper and lower chambers was determined by FACS. In vivo migration assay was performed in female NOD/Shi-scid/IL-2Rγnull (NSG) mice (6–8 weeks old, Jackson Laboratory) (Curiel et al., 2004, Curiel et al., 2003, Kryczek et al., 2012). Subcutaneous DLD-1 (10^6) tumor was established in NSG model. Human T cell subsets (5 X 10^6) were treated with anti-CCR6 and isotype antibody and were intravenously transferred into these NSG mice. After 48 hr, human T cells were analyzed in the tumors by FACS.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed according to the protocol with exceptions stated below (Upstate, Millipore; http://www.millipore.com/techpublications /tech1/mcproto407). Crosslinking was performed with 1% formaldehyde or 1% paraformaldehyde for 10 min. To enhance cell lysis, we ran the lysate through a 27 g needle three times and flash froze it in 80C. Sonication was then performed with the Misonix 4000 water bath sonication unit at 15% amplitude for 10 min. Protein and DNA complex was precipitated with specific antibodies against H3K79me2 (abcam, ab3594), STAT3 (Santa Cruz, SC-482), p300 (Santa Cruz, SC-585), and immunoglobulin G control (Millipore). DNA was then purified using a DNA Purification Kit (QIAGEN). ChIP-enriched chromatin was used for real-time PCR with SYBR Green Master Mix, normalizing to input.

Immunohistochemistry (IHC)

Immunohistochemical staining on colon cancer tissue sections was performed on a DAKO Autostainer (DAKO,) with DAKO LSAB^{+} and diaminobenzadine (DAB) as the chromogen. Serial sections of deparaffinized tissue sections were labeled with rabbit polyclonal antibodies against human KMT4/Dot1L, H3K79me2 and CCL20 (AbCam), or mouse anti-human VCAM1 antibody (6G9, Abcam). H3K79me2 and DOT1L were localized in the nuclei and were scored with the H-score method (Pirker et al., 2012). The H score is a method of assessing the extent of nuclear immunoreactivity. The H score takes into account the percentage of positive cells (0%–100%) in each intensity category (0–3+) and computes a final score, on a continuous scale between 0 and 300. The score is obtained by the formula: (3 X percentage of strongly staining nuclei) + (2 X percentage of moderately staining nuclei) + (1 X percentage of weakly staining nuclei), giving a range from 0 to 300 (Pirker et al., 2012). Any discrepancies were resolved by subsequent consultation with a diagnostic pathologist. The tissues were divided into high and low H3K79me2 and DOT1L expression based on the median value of H3K79me2 and DOT1L expression per tissue section.

In Vivo Tumor Formation

Single cells were prepared from fresh colon cancer tissues. These colon cancer environmental cells contained all the primary cellular components in the colon cancer environment including CD3^{+} T cells within CD45^{+} immune cell population and lin^{-} CD33^{-}CD45^{-} FSC^{hi} SSC^{hi} primary colon cancer cells. These cells or colon cancer cells (10^{2}- 5 X 10^{6}) were treated with anti-IL-22 or IL-22, and were subcutaneously injected into dorsal tissues of NSG mice (6–8 weeks old, Jackson Laboratory) (Curiel et al., 2004, Curiel et al., 2003, Kryczek et al., 2012). Tumor size was

measured two times weekly with calipers fitted with a Vernier scale. Tumor volume was calculated based on three perpendicular measurements (Curiel et al., 2004, Curiel et al., 2003). Tumor incidence was monitored.

Statistical Analysis

Wilcoxon rank-sum tests were used to compare two independent groups; for paired groups, Wilcoxon signed rank tests were used for comparison. Correlation coefficients (Spearman correlation, denoted by r, for ordinal data and Pearson correlation, denoted by r, for continuous data), together with a p value (null hypothesis is that r is in fact zero), were computed to measure the degree of association between biomarkers. Log-rank test was used to compare time to tumor initiation between two groups. Overall patient survival was defined from date of diagnosis to disease related death. Data was censored at the last follow-up for patients who were disease-free or alive at the time of analysis. Survival functions were estimated by Kaplan-Meier methods. Cox's proportional hazards regression was performed to model survival (all classified as low and high based on the median value), after adjusting for age, grade and stage. The adequacy of the Cox regression model was assessed with graphical and numerical methods. All analyses were done with SAS 9.3 software. $p < 0.05$ was considered significant.

2.4 Results

2.4a IL-22 in the tumor environment promotes colon cancer stemness

As IL-22 protects intestinal stem cells from immune-mediated tissue damage in mice (Hanash et al., 2012), we hypothesized that IL-22^{+} cells might support cancer stemness in patients with colon cancer. High amounts of IL-22 mRNA were detected in primary colon cancer tissues compared to peripheral blood and colon tissue adjacent to the cancer (Figure 2.1A). Next we examined the potential effects of endogenous IL-22 on primary tumor formation in a female NOD Shi-scid IL-2Rγnull (NSG) immune-deficient mouse model (Cui et al., 2013, Curiel et al., 2004, Kryczek et al., 2012, Kryczek et al., 2011). To this end, single cell suspensions were made from fresh human colon cancer tissues. These cells contained all the primary cellular components in the colon cancer environment including CD3^{+} T cells within the CD45^{+} immune cell population, and lin^{-}CD34^{-}CD45^{-}FSC^{hi} SSC^{hi} primary colon cancer cells. We equally divided this primary colon cancer tissue into two groups and injected the cells into NSG mice with a one-time treatment of either anti-human IL- 22 monoclonal antibody (mAb) or isotype mAb. Anti-human IL-22 mAb dramatically reduced primary tumor volume (Figure 2.1B) and delayed tumor development (Figure 2.1C) and increased mouse survival (Figure 2.1D).

To confirm the tumorigenic potential of endogenous IL-22, we injected different concentrations of a colorectal adenocarcinoma cancer cell line, DLD-1 cells, into NSG mice to determine a nontumorigenic concentration. We found that 10^5 DLD-1 cells failed to form a tumor in the NSG mouse. However, exogenous IL-22 administration enabled tumor formation with 10^5 DLD-1 cells as shown by increased tumor volume (Figure 2.1E), accelerated tumor development (Figure 2.1F), and decreased mouse survival (Figure 2.1G). Thus, IL-22 might enhance tumorigenesis by altering cancer stem cell properties.

In support of this notion, IL-22 promoted tumor sphere formation in a dose-dependent manner (Figures 2.11H and I) and increased aldehyde dehydrogenase (ALDH1) activity in DLD-1, HT29, and two primary colon cancer cell lines (Figure 2.1J). ALDH1 is an operative marker of human cancer stem cells (Carpentino et al., 2009, Kryczek et al., 2012). Furthermore, IL-22 enhanced mRNA (Figure 2.1K) and protein (Figures 2.1L and M) expression of multiple core stem cell genes including NANOG, SOX2, and POU5F1 (OCT3/4). Altogether, IL-22 stimulates expression of genes associated with core cancer stemness and promotes colon tumorigenicity.

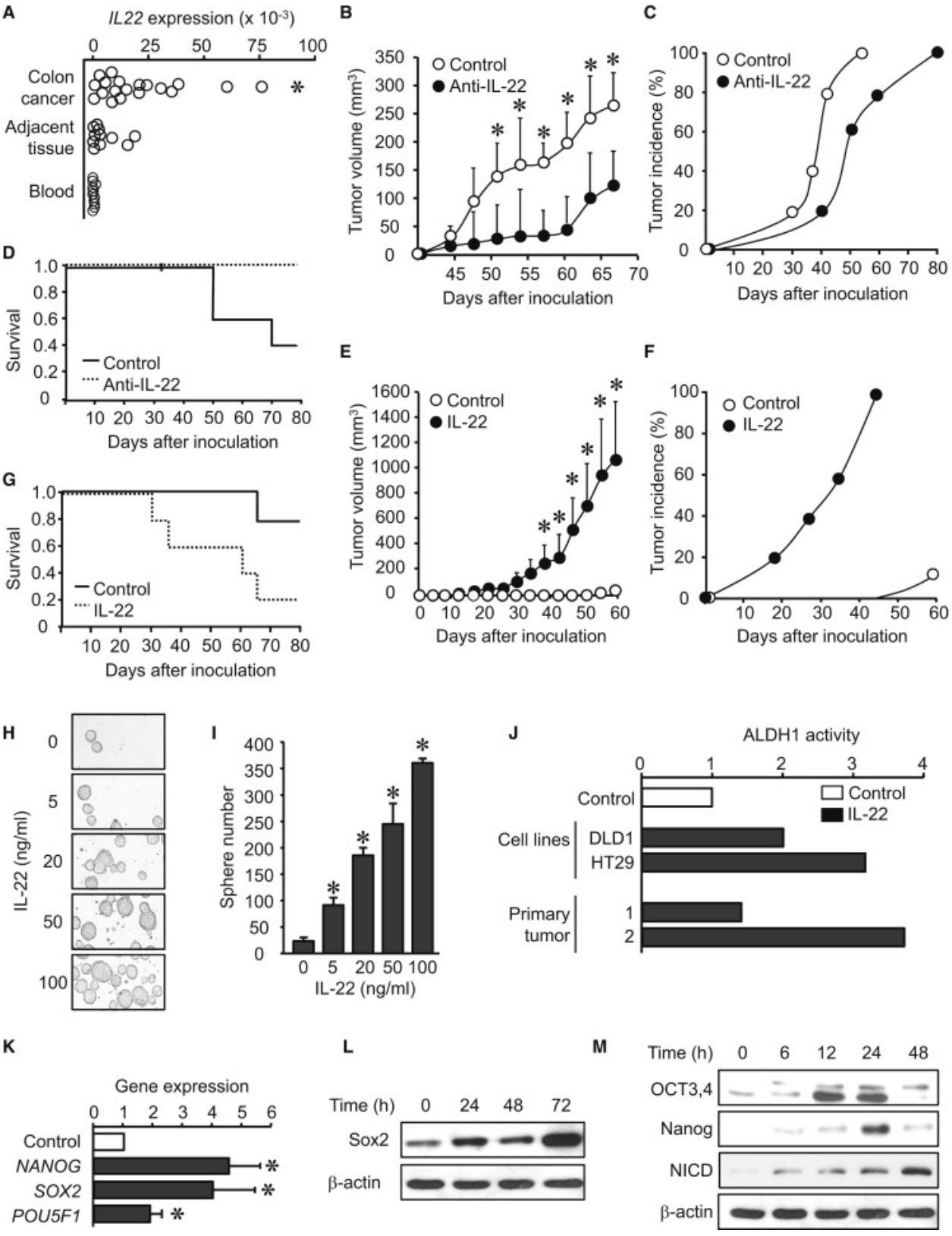

Figure 2.1 IL-22 in the tumor microenvironment promotes colon cancer stemness

(A) IL22 mRNA was detected by real-time PCR in colon cancer tissues, adjacent tissues, and peripheral blood. *$p < 0.05$ compared to blood and adjacent tissues, 20 colon cancer patients. (B–D) Single cells isolated from colon cancer tissue were mixed with anti-IL-22 antibody or control mAb, and then subcutaneously injected to NSG mice. Tumor growth (B, *$p < 0.05$, $n = 5$ per group), incidence (C, $p = 0.037$, $n = 5$ per group), and animal survival (D, $p = 0.013$, $n = 5$ per group) are shown. (E–G) DLD-1 colon cancer cells (10^5) were preincubated with IL-22 (20 ng/ml) for 1 hr, and then subcutaneously injected to NSG mice. Tumor growth (E, *$p < 0.05$, $n = 5$ per group, incidence), incidence (F, $p = 0.003$, $n = 5$ per group), and animal survival (G, $p = 0.013$, $n = 5$ per group) are shown. (H and I) DLD-1 colon cancer cells were cultured with IL-22. Sphere assay was performed with 2,000 cells. Representative image of spheres (H) and the mean numbers of spheres (I) are shown. *$p < 0.05$, $n = 5$. (J) Colon cancer cell lines (DLD-1 and HT29) and two primary colon cancer cells (1, 2) were cultured with IL-22 for 24 hr. ALDH1 activity was determined by FACS based on aldefluor fluorescence. Cells treated with DEAB inhibitor were negative controls. Results are expressed as fold changes of aldefluor fluorescence. ($p < 0.05$ for all, $n = 3$ repeats per group). (K–M) DLD-1 colon cancer cells were cultured with IL-22 (20 ng/ml). The mRNA of stem cell core gene was detected by real-time PCR (K) and proteins were detected by immunoblotting (L, M). (*$p < 0.05$, $n = 5$).

2.4b IL-22$^+$ cells are recruited into the tumor and promote cancer stemness via IL-22

Given that IL-22 promotes colon cancer stemness, we examined the cellular source of IL-22 and the phenotype of IL-22$^+$ cells in the human colon cancer environment. Real-time PCR revealed that IL-22 was expressed by CD45$^+$ immune cells in the colon cancer environment (Figure 2.2A). To further define the phenotype of the IL-22$^+$ cells, we sorted colon cancer associated CD45$^+$ cells into four populations: lineage negative cells (lin$^-$), CD33$^-$ CD3$^-$CD56$^+$ cells (with natural killer [NK] or potentially ILC), CD33$^+$ myeloid cells, and CD3$^+$ T cells. We found that IL- 22 mRNA expression was confined to CD3$^+$ T cells (Figure 2.2B). Furthermore, sorted colon cancer-associated CD45$^+$CD3$^+$ T cells, but not colon-associated CD45$^+$CD3$^-$ cells or blood CD45$^+$CD3$^-$cells, spontaneously released IL-22 (Figures 2.2C and D). We analyzed the cytokine profile of IL-22$^+$ cells in the colon cancer. We found that IL-22$^+$ cells were also CD3$^+$CD8$^-$CD4$^+$ and expressed the transcription factor RORγ. Of the CD3$^+$CD8-CD4$^+$IL-22$^+$ cells, 30% expressed IL-17, and whereas IL-4, IL-2, and interferon-γ (IFNγ) expression was detected in less than5% of cells (Figure 2.2E). Thus, IL-22 is predominantly expressed by CD4$^+$ T cells in the colorectal cancer microenvironment.

We next examined how peripheral blood IL-22$^+$CD4$^+$ T cells traffic into the colon cancer microenvironment. We analyzed the expression of cell trafficking-associated molecules including chemokine receptors and integrins on IL-22$^+$CD4$^+$ T cells in blood and colon cancer. We sorted blood CD4$^+$ T cells into C-C chemokine receptor type 6 (CCR6) and CCR6$^+$ populations, and subsequently examined IL-22 expression. We did not perform intracellular staining because this affects the detection of surface antigens. We found that CCR6$^+$, but not CCR6$^-$, cells expressed high amounts of IL-22 mRNA (Figure 2.2F) and protein (Figure 2.2G).

We then asked whether IL-22$^+$ T cells could migrate toward primary tumor tissues through chemokine (C-C motif) ligand 20 (CCL20), the ligand for CCR6. We observed that T cells efficiently migrated in response to CCL20 (Figure 2.2H), and that the migrating cells were enriched for IL-22$^+$CD4$^+$ T cells (Figure 2.2I), expressing CCR6 and CD49D (Figure 2.2H).

We further investigated the potential effects of primary colon cancer associated IL-22$^+$CD4$^+$ cells on colon cancer stemness. To this end, colon cancer cell sphere assay was performed with autologous colon cancer associated CD4$^+$ T cells. We showed that these T cells enhanced primary colon cancer cell-sphere formation, whereas anti-IL-22 abrogated this effect (Figure 2.2J). In line with this, these T cells also increased core stem cell gene expression (Figure 2.2K) and ALDH1 activity (Figure 2.2L) in colon cancer cells. This increase in stemness was reduced with IL-22 blockade (Figures 2.2K and 2L). Thus, IL-22$^+$CD4$^+$ cells traffic into the tumor, and promote colon cancer stemness via secreting IL-22 in the colon cancer microenvironment.

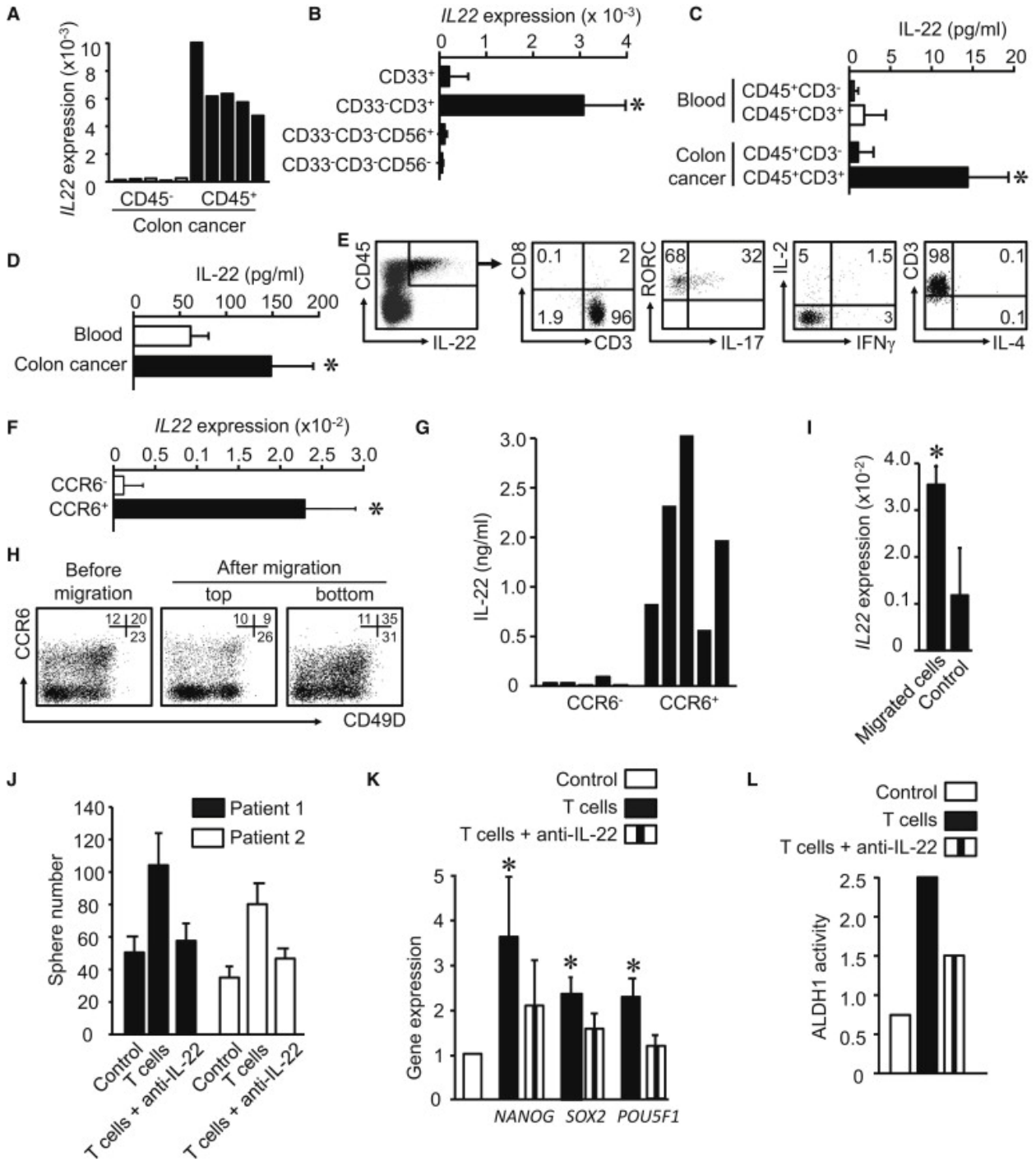

Figure 2.2 IL-22^{+}CD4^{+} T cells traffic into the tumor via CCR6-CCL20 and promote cancer stemenss via IL-22. (A and B) Different cell populations were sorted from colon cancer tissues and IL-22 expression was measured by real time PCR (5–10 donors, $p < 0.05$). (C) Different CD45^{+} immune subsets (10^{6}/ml) were sorted from colon cancer and blood, and cultured for 12 hr. IL-22 was detected via ELISA. (n = 5, *$p < 0.05$). (D) CD45^{+} subsets (10^{6}/ml) were sorted from colon cancer and blood, and were activated with anti-CD3 and anti-CD28 for 2 days. IL-22 was detected by ELISA (n = 5, *$p < 0.05$). (E) Single colon cancer environmental cells were stained

with anti-CD3, CD8, CD45, RORgc, IL-2, IL-4, IL-17, IL-22, and IFNγ antibodies. The phenotype and the expression of indicated cytokines were analyzed by FACS. Right panels were gated on IL-22$^+$CD45$^+$ cells. One of three independent experiments is shown. (F and G) CD4$^+$ T cells were sorted based on CCR6 expression and activated with anti-CD3 and anti-CD28 and antigen-presenting cells. IL-22 mRNA was detected by real-time PCR (F) and IL-22 protein by ELISA (G) (5 different donors, $p < 0.05$). (H and I) Migration assay was conducted with CD4$^+$ T cells for 4 hr in the presence of CCL20. The phenotype of migrated cells, nonmigrated cells, and control (before migration) was analyzed by FACS (H). IL-22 expression was quantified with real-time PCR in the migrated and nonmigrated cells. Results are expressed as the mean relative expression (I) ($n = 3$, $p < 0.05$). (J) Sphere assay was performed with autologous colon tumor cells in the presence of activated colon cancer-associated T cells in a transwell system. Anti-IL-22 or isotype mAb was added in the assay. Results are shown as the mean numbers of spheres in triplicates (2 of 5 patients are shown. $p < 0.01$). (K and L) Primary colon cancer associated T cells were sorted and activated for 3 days. DLD-1 colon cancer cells were cultured with these T cell supernatants in the presence of anti-IL-22 or isotype mAbs. The mRNA of stem cell core genes was detected by real-time PCR after 6 hr (K) and ALDH activity was detected by FACS after 48 hr (L) ($n = 5$, *$p < 0.05$, compared to control and T cells with anti-IL-22).

2.4c IL-22 promotes colon cancer stemness via STAT3 activation

Next, we dissected the molecular mechanisms by which IL-22 promotes colon cancer stemness. Signal transducer and activator of transcription 3 (STAT3) plays a key role in the crosstalk between cancer and immune cells in the tumor microenvironment (Lee et al., 2009, Yu et al., 2007). In line with previous reports (Lejeune et al., 2002, Pickert et al., 2009), we observed that IL-22 activated STAT3 in colon cancer cells (Figure 2.3A). We next examined whether the effect of IL-22 on colon cancer stemness was STAT3-dependent. To this end, we manipulated STAT3 expression either with specific STAT3 knockdown (sh-STAT3) or forced expression of a STAT3 active domain (STAT3C) in colon cancer cells. Sh-STAT3 resulted in reduced colon cancer sphere numbers (Figures 2.3B and C). In the absence of IL-22, expression of STAT3C had minimal effects on colon cancer sphere formation, but addition of IL-22 increased colon cancer sphere formation in STAT3C-expressing cells compared with controls (Figure 2.3D). The data suggest that IL-22-mediated STAT3 activation is necessary and relatively specific in promoting colon cancer stemness. However, in the absence of IL-22, forced STAT3 activation alone is insufficient to strongly induce cancer stemness (Figure 2.3D).

We further explored the effects of IL-22-activated STAT3 in expression of stemness-related genes. STAT3 knockdown reduced core stem cell gene expression (Figures 2.3E-G). We speculated that STAT3 might directly bind to the promoters of core stem cell genes and subsequently induce their expression. We found several predictions for STAT3 binding on the SOX2 promoter region (http://www.sabiosciences.com/chipqpcrsearch/php). Chromatin immunoprecipitation (ChIP) demonstrated that IL-22 increased STAT3 binding in several sites on the SOX2 promoter area (Figures 2.3H and I) and suggests that STAT3 might directly activate

stemness genes. Thus, IL-22 promotes colon cancer stemness via STAT3 activation and its associated signaling genes.

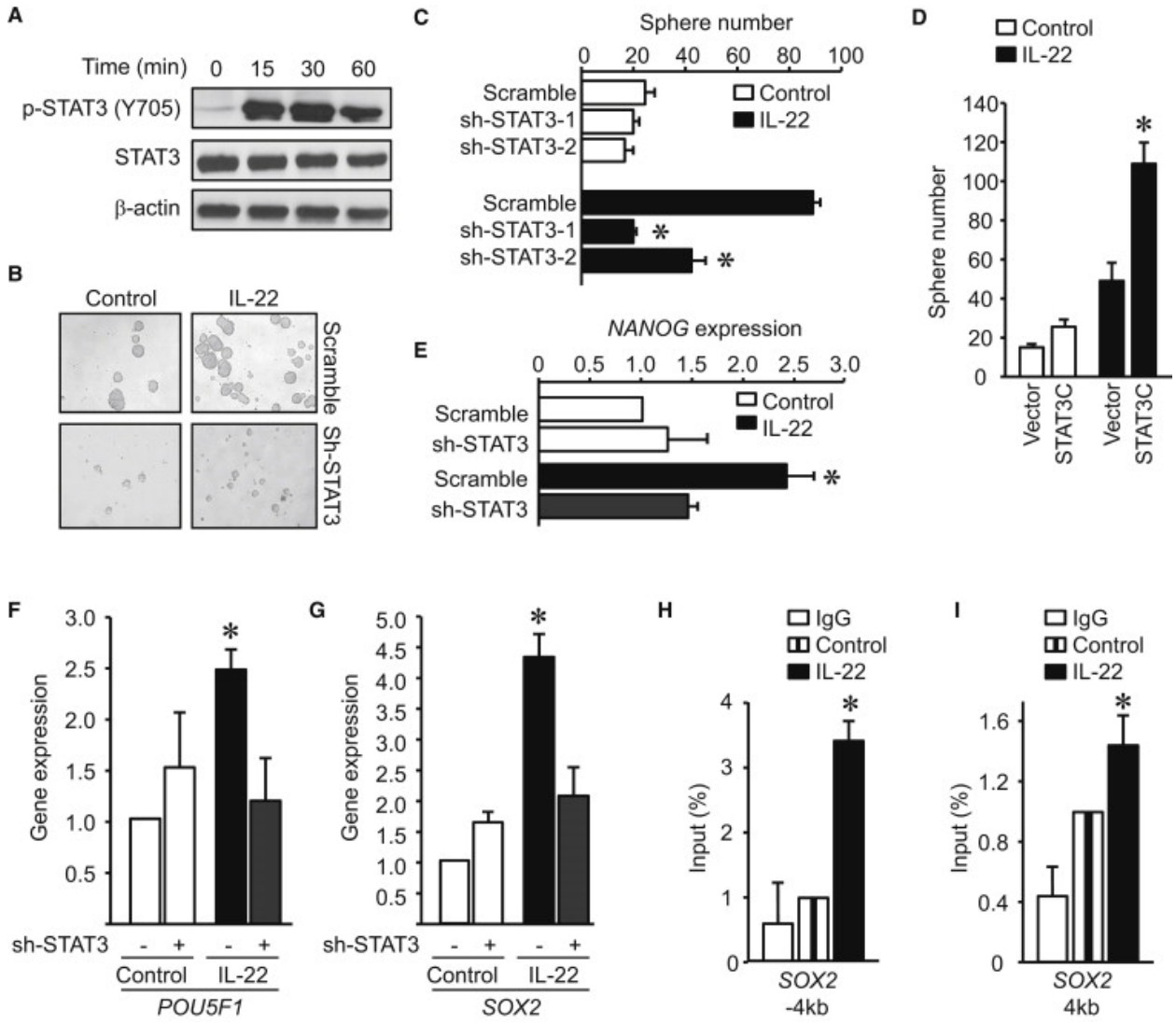

Figure 2.3 IL-22 Promotes Colon Cancer Stemness via STAT3 Activation. (A) Colon cancer cells were treated with IL-22 for different time points. The amount of phosphorylated STAT3 and STAT3 protein was detected by immunoblotting. (B–D) Sphere assay was performed with shSTAT3 (B and C) or STAT3C (D) expressing colon cancer cells in the presence of IL-22. Results are shown as sphere images (B) and the mean numbers of spheres in triplicate (C and D). (n = 5. *p < 0.01). (E–G) Colon cancer cells expressing shSTAT3 or scrambled vector were cultured with IL-22. Stem cell core gene mRNAs were detected by real-time PCR after 6 hr (n = 5, *p < 0.05). (H and I) STAT3-ChIP assay was performed DLD-1 cells cultured with or without IL-22 (mean ± SEM, n = 3, *p < 0.05).

2.4d DOT1L regulates IL-22 dependent colon cancer stemness via H3K79 methylation

Epigenetic modifications of chromatin and their crosstalk with transcription factors play an important role in the regulation of gene expression. STAT3 binding to the promoters of core stem cell genes is highly context dependent (Hutchins et al., 2013). Thus, STAT3 activation might not completely explain the increase in stemness. Because transcription factors and epigenetic modifications often guide external signals to a specific genetic response, we wondered whether epigenetic control, including histone modifications, is involved in controlling IL-22-induced stemness gene expression. To this end, we examined the global changes in several histone marks in IL-22-treated colon cancer cells. Among several histone marks, we observed that IL-22 selectively increased the dimethylation of histone 3 lysine 79 (H3K79me2) (Figure 2.4A). Disruptor of telomeric silencing 1-like (DOT1L) is the sole H3K79 methyltransferase (Min et al., 2003, Ng et al., 2002). EPZ004777 (Daigle et al., 2011, Yu et al., 2012), a selective inhibitor of DOT1L, suppressed DLD-1 (Figure 2.4B) and primary colon cancer (Figure 2.4C) sphere formation. Several proteins recruit DOT1L to mediate methylation of H3K79 including MCEF (AFF4), AF9 (MLLT3), and AF10 (MLLT10) (Mohan et al., 2010). IL-22 consistently promoted the expression of DOT1L and AFF4, but not MLLT10 in DLD-1 (Figure 2.4D) and primary colon cancer cells (Figure 2.4E). To further determine the role of DOT1L in colon cancer stemenss, we knocked down DOT1L expression with sh- DOT1L and forced ectopic DOT1L expression. Similar to EPZ004777, DOT1L knockdown reduced colon cancer sphere formation (Figure 2.4F). These data suggest that DOT1L signaling activation potentiates the colon cancer stemness program.

We also explored whether IL-22 regulates H3K79me2 on core stem cell gene promoters. ChIP assays with H3K79me2 revealed an increase in H3K79me2 on the proximal promoter areas of NANOG (Figure 2.4G), SOX2 (Figure 2.4H), and POU5F1 (Figure 2.4I) in an IL-22 dependent manner. Moreover, EPZ004777 treatment resulted in reduced H3K79me2 on the stem cell gene promoter sites (Figure 2.4J-L). In support of these observations, IL-22 administration caused the activation of STAT3, H3K79 dimethylation, and core stemness gene activation in human colon cancer cells in the NSG model in vivo (Figure 2.4M). These data indicate that IL-22 regulates colon cancer stemness in a DOT1L- and H3K79me2-dependent manner.

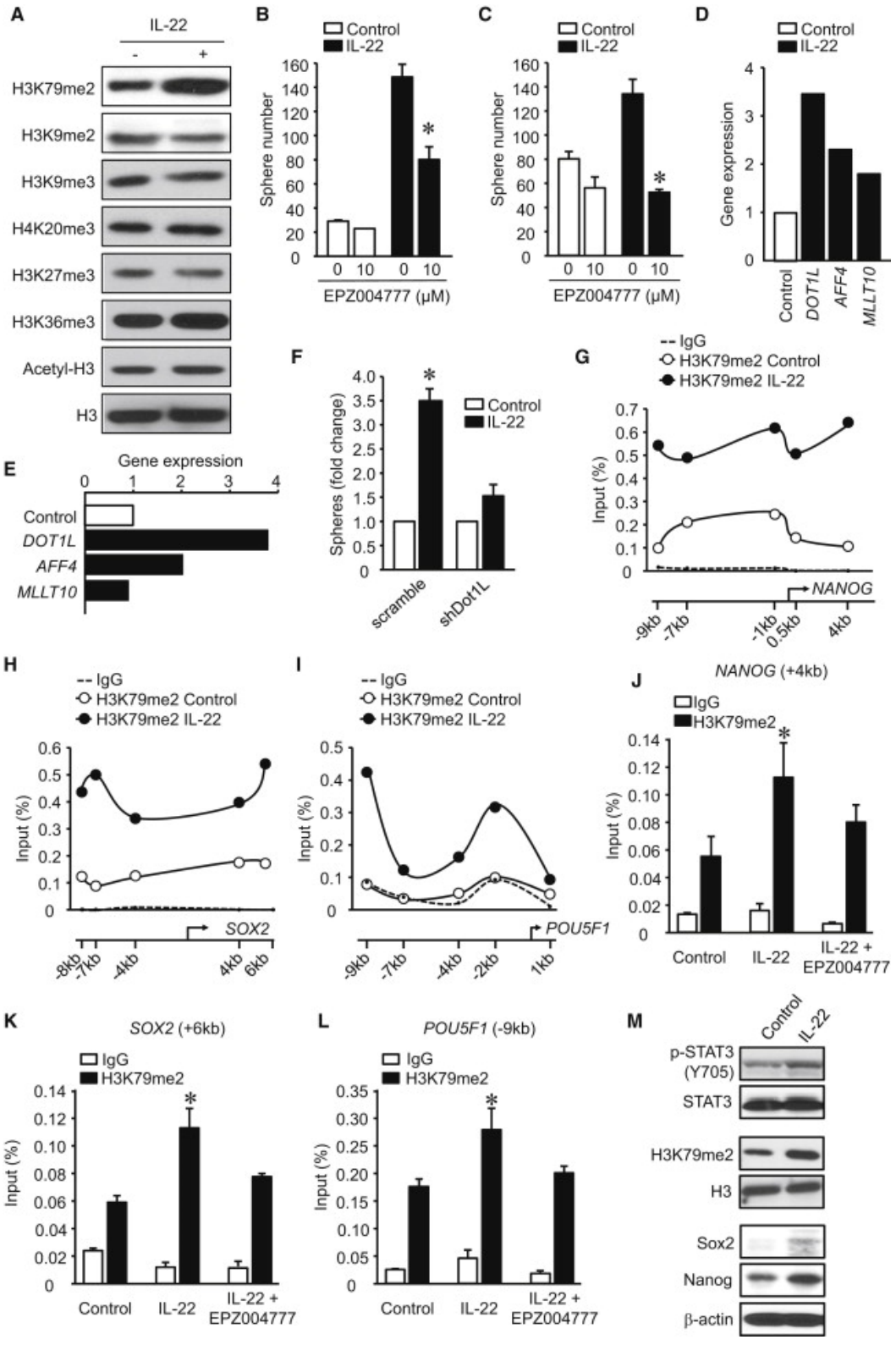

Figure 2.4 IL-22 Promotes Colon Cancer Stemness via DOT1L and H3K79me2. (A) Colon cancer cells were treated with IL-22 for 48 hr. Histone modifications were analyzed by

immunoblotting. (B and C) Colon cancer sphere assay was performed in the presence of IL-22 and the DOT1L inhibitor EPZ004777. Sphere numbers were recorded. DLD-1 cells (B), primary colon cancer cells (C) ($n = 5$, $p < 0.05$). (D and E) Colon cancer cells were cultured for 12 hr with IL-22. Expression of the genes encoding members of the DOT1L complex was quantified by real-time PCR. DLD-1 cells (D), primary colon cancer cells (E) ($n = 3$, $p < 0.05$). (F) Colon cancer sphere assay was performed with colon cancer cells expressing sh-DOT1L or vector in the presence of IL-22. Sphere numbers were recorded ($n = 5$, $p < 0.05$). (G–I) H3K79me2-ChIP assay was performed to examine H3K79me2 at the core stem cell genes promoters in DLD-1 colon cancer cells cultured with IL-22. One of three experiments is shown. (J–L) H3K79me2 ChIP was performed to examine occupancy at core stem cell genes in DLD-1 colon cancer cells cultured with or without IL-22 and EPZ004777. One of three experiments is shown. (M) IL-22 (0.5ug) was injected into the DLD-1 tumor. After 12–48 hr, tumor tissues were extracted for immunoblotting analysis for the STAT3, H3K79me2, SOX2, and NANOG proteins. One of three experiments with triplicate sections is shown.

2.4e STAT3 induces DOT1L expression and controls IL-22-induced cancer stemness

Both STAT3 (Figure 2.3) and DOT1L-H3K79 signaling (Figure 2.4) are involved in the control of IL-22-induced cancer stemness. We hypothesized that IL-22-activated STAT3 causes H3K79 methylation. IL-22 treatment increased STAT3 binding to the DOT1L promoter area (Figure 2.5A). As a confirmatory experiment, we showed that IL-22 also augmented the binding of p300 to the promoter area of DOT1L (Figure 2.5D). Thus, IL-22 promotes the binding of the transcription factor STAT3 to the promoter area of the DOT1L complex and controls colon cancer stemness.

In addition to the interaction of STAT3 on the DOT1L promoter, we explored whether STAT3 could directly impact the amount of H3K79me2 via DOT1L. Enhanced activation of STAT3 in STAT3C transduced cells caused a genome-wide increase in H3K79 dimethylation (Figure 2.5E), but no increase in sphere formation (Figure 2.3D) in the absence of IL-22. IL-22 treatment moderately augmented H3K79 dimethylation (Figure 2.5E) and dramatically increased sphere formation (Figure 2.3D). Thus, STAT3 activation could promote H3K79me2 via DOT1L.

To determine whether STAT3 regulates core stem cell gene expression through DOT1L-dependent H3K79 methylation, we performed ChIP with H3K79me2 in sh-STAT3 IL-22-treated colon cancer cells. STAT3 knockdown abrogated IL-22-induced H3K79 methylation on the stem cell core gene promoters of NANOG, SOX2, and POU5F1 (Figures 2.5F–5H). The data further solidify the notion that STAT3 is essential for H3K79 dimethylation and IL-22 potentiates potent cancer stemness via STAT3 mediated-H3K79 dimethylation. DOT1L knockdown (Figure 2.5I) or overexpression (Figure 2.5J) had no effects on IL-22-mediated STAT3 activation. This suggests that IL-22-induced STAT3 phosphorylation is DOT1L independent. Altogether, the results

indicate that IL-22-activated STAT3 directly regulates DOT1L expression and subsequently induces H3K79 methylation at the stemness genes, facilitating and accelerating stemness gene activation.

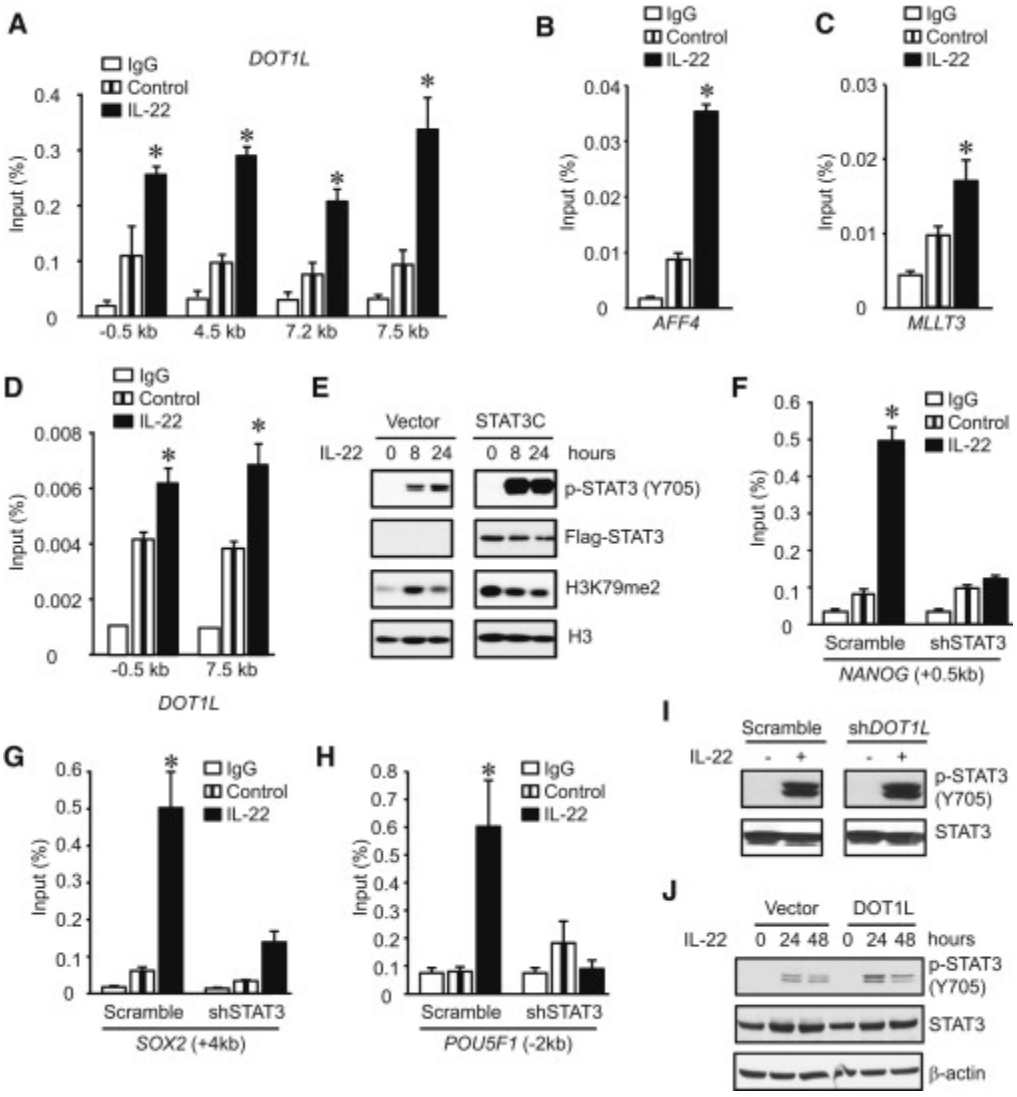

Figure 2.5 STAT3 stimulates DOT1L expression and promotes IL-22-induced cancer Stemness. (A–C) STAT3-ChIP assay was performed in DLD-1 colon cancer cells cultured with or without IL-22 to examine STAT3 occupancy at DOT1L (A), AFF4 (B), and MLLT3 (C) promoters. One of three experiments is shown. (D) p300-ChIP assay was performed in DLD-1 colon cancer cells cultured with or without IL-22 to examine p300 occupancy at DOT1L promoter. (n = 3 with duplicates, *p < 0.05). (E) DLD-1 cells were transduced with STAT3C expressing or control lentiviral vectors, and cultured with IL-22. H3K79me2 was detected by immunoblotting. One of three experiments is shown. (F–H) H3K79me2-ChIP was performed in colon cancer cells cultured with IL-22 for 24 hr to examine H3K79me2 occupancy at the promoter areas of core stem cell genes (n = 3 with duplicates, *p < 0.05). (I and J) DLD-1 cells were transduced with shDOT1L (I) or DOT1L expressing (J) or control lentiviral vectors, and cultured with IL-22. STAT3 and STAT3 phosphorylation were detected by immunoblotting. One of three experiments is shown.

2.4f High amounts of colon cancer H3K79-DOT1L predict poor patient survival

Finally, we examined the clinical relevance of the IL-22-DOT1L signaling pathway in colon cancer patients. To this end, we first analyzed the relationship between IL-22, DOT1L, and stem cell gene transcripts in patients with colorectal cancer from the National Center for Biotechnology Information Gene Expression Omnibus database (GSE17536) (Smith et al., 2010). The GSE17536 database includes 177 colorectal cancer patients with clinic and pathological information. We found that IL-22 expression correlated with DOT1L (Figure 2.6A). Moreover, the expression of DOT1L correlated with SOX2 (Figure 2.6B). Furthermore, when we divided patients into ‘‘low’’ and ‘‘high’’ groups based on the median value of SOX2, we observed that high SOX2 expression was associated with poor patient survival (Figure 2.6C). Then, we quantified nuclear DOT1L and H3K79me2 via immunohistochemistry in paraffin fixed colorectal cancer tissues from patients with available clinical and pathological information. The expression of DOT1L correlated highly with that of H3K79me2 in the same tumor (Figure 2.6D). Furthermore, based on the median values of DOT1L intensity, we divided patients into ‘‘low’’ and ‘‘high’’ groups. Overall survival was shorter in patients with high DOT1L staining compared to low DOT1L expression (Figure 2.6E). Age and tumor stage (TNM) were important prognostic factors for colon cancer survival. After adjusting for the clinical factors, overall survival remained shorter in patients with high DOT1L expression. The data strongly suggest that increased tumor DOT1L abundance is a significant and independent predictor of poor survival in colorectal cancer.

We further analyzed the relationship between tumor H3K79me2 expression and survival. Similar results were observed with H3K79 methylation (Figure 2.6F). Overall survival

was shorter in patients with high H3K79 dimethylation (Figure 2.6F). Therefore, DOT1L expression and H3K79me2 could be an oncogenic predictor for poor survival in colorectal cancer. Altogether, IL-22^{+}CD4^{+} T cells produce IL-22 and shape colon cancer stemness through a STAT3-DOT1L-mediated stem cell core signaling pathway.

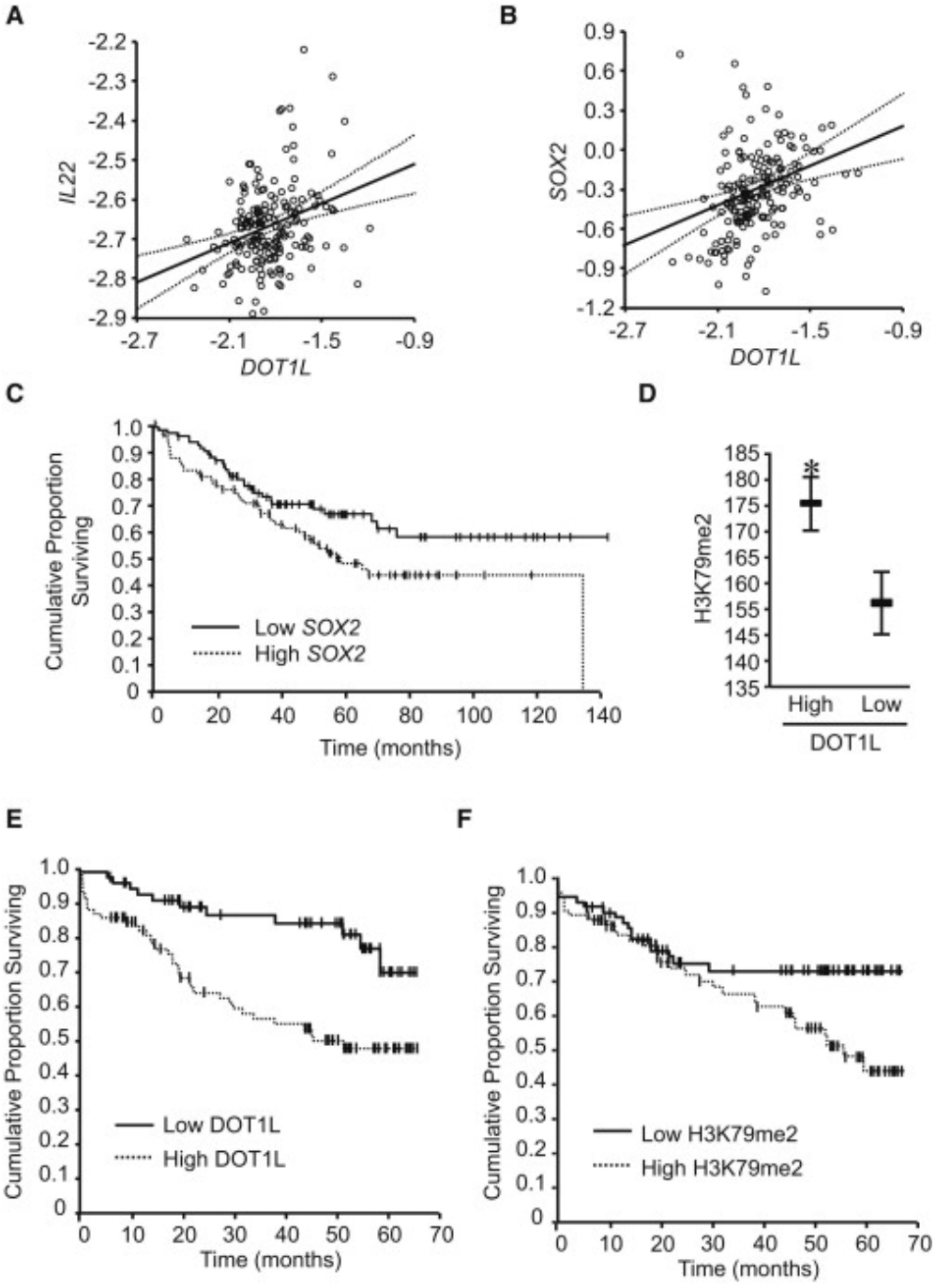

Figure 2.6 High amounts of colon cancer H3K79-DOT1L predict poor patient survival. (A and B) The correlation betweenDOT1L, IL22 and SOX2 transcripts in patients with colorectal cancer was examined analyzing 177 colorectal cancer patients (GSE17536). R = 0.31, p = 0.000032 (A); R = 0.38, p = 0.00000024 (B). (C) The association between SOX2 transcripts and patient survival. The analyses were conducted in 177 colon cancer patients (GSE17536), p = 0.046). (D) The correlation between DOT1L and H3K79me2 in patients with colon cancer. n = 144, R = 0.23, p = 0.005 (E and F). The relationship between tumor DOT1L (E), H3K79me2 (F) expression and colon cancer overall survival was evaluated. n = 151, p = 0.001 (E), n = 144, p = 0.039 (F).

2.5 Discussion

The capacity of immune cells to modulate the cancer phenotype has been the subject of intensive investigation. The immune surveillance model proposes that immunoediting contributes to cancer dormancy in a mouse model (Dunn et al., 2002, Matsushita et al., 2012). It is also well known that immune cell subsets, when chronically activated, directly foster tumor development (Ben-Neriah and Karin, 2011, Coussens et al., 2013), and promote cancer progression (Ben-Neriah and Karin, 2011, Coussens et al., 2013, Cui et al., 2013). As an extension of this model, we demonstrate that IL-22$^+$ immune cells select and sustain the invasive phenotype of human colon cancer: cancer stemness.

Th22 cells and IL-22 have been reported to be a protective immune element in infection, inflammation (Aujla et al., 2008, Basu et al., 2012, Hanash et al., 2012, Pickert et al., 2009, Zheng et al., 2008), and thymic repair (Dudakov et al., 2012). Colon cancer associated IL-22$^+$CD4$^+$ T cells stimulate cancer stem cell core gene expression and promote cancer stemness. Cancer stemness and the invasive tumor phenotype are thought to be a cell-autonomous process specified by the genetic and epigenetic signature of cancer cells. Our data indicate that IL-22$^+$CD4$^+$ T cells, a crucial T cell immune component in the colon cancer microenvironment, functions as an environmental extrinsic signal, directly targets cancer cells, and defines their stemness. We focus on patients with advanced colon cancer due to practical and ethical reasons. Given the defined roles of IL-22 in cancer stemness, it is possible that ILC22 or/and other IL-22$^+$ cells employ a similar mechanism and regulate cancer initiation and development in early phases of colon cancer. Nonetheless, it is reasonable to conclude that IL-22$^+$CD4$^+$ T cells participate in colon cancer stemness.

STAT3 activity is required for small-intestine crypt stemcell survival in mice (Matthews et al., 2011). We have demonstrated that both STAT3 activation and DOT1L-H3K79 signaling are essential for IL-22-induced cancer stemness. STAT3 and DOT1L facilitate core stem cell gene activation, and are indispensable in IL-22-induced cancer stemness. Although understanding the dynamic interaction between the transcription factor STAT3 and the histone mark H3K79me2 warrants further investigation, our work addresses a central question: do histone modifications cue and instruct transcription or support and correlate with gene activity (Henikoff and Shilatifard, 2011)? Our findings demonstrate an instructive role for methylated H3K79 in colon cancer stem cell gene activation stimulated by IL-22. Thus, our work reveals a relationship between a key transcription factor (STAT3) and an important epigenetic mark (H3K79) in determining cancer stemness. DOT1L is the sole H3K79 methyltransferase (Min et al., 2003, Ng et al., 2002). We have shown that IL-22 regulates colon cancer stemness via DOT1L-H3K79. The amount of DOT1L expression in colon cancer independently predicts poor patient survival. Notably, it has been reported that DOT1L recruitment through Mixed Lineage Leukemia (MLL) fusion proteins is strongly associated with MLL transformation (Chang et al., 2010, Jo et al., 2011). It remains to be determined whether DOT1L is an oncogene in human epithelial cancer, and whether it is biochemically and functionally linked to the well-defined colon oncogenes. Nonetheless, our study is the first to demonstrate that DOT1L contributes to human epithelial carcinoma, including its involvement in colorectal tumorigenesis and its regulation by IL-22^{+}CD4^{+} T cells. Therefore, our work has revealed epigenetic mechanisms by which IL-22^{+}CD4^{+} T cells and IL-22 control human colon cancer stemness and tumorigenesis. Similar mechanisms might apply for other types of human cancers. Because DOT1L inhibition is

a proposed strategy for targeted therapy of leukemia with MLL translocation (Daigle et al., 2011), our work suggests that DOT1L might be a marker for colon cancer progression, and targeting this pathway might be meaningful for colon cancer treatment.

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Chapter 3

PRC2 Epigenetically Silences Th1-Type Chemokines to Suppress Effector T-Cell Trafficking in Colon Cancer[2]

3.1 Abstract

Infiltration of tumors with effector T cells is positively associated with therapeutic efficacy and patient survival. However, the mechanisms underlying effector T-cell trafficking to the tumor microenvironment remain poorly understood in patients with colon cancer. The polycomb repressive complex 2 (PRC2) is involved in cancer progression, but the regulation of tumor immunity by epigenetic mechanisms has yet to be investigated. In this study, we examined the relationship between the repressive PRC2 machinery and effector T-cell trafficking. We found that PRC2 components and demethylase JMJD3-mediated histone H3 lysine 27 trimethylation (H3K27me3) repress the expression and subsequent production of Th1-type chemokines CXCL9 and CXCL10, mediators of effector T-cell trafficking. Moreover, the expression levels of PRC2 components, including EZH2, SUZ12, and EED, were inversely

[2]Excerpts taken from: Nagarsheth N et al. PRC2 epigenetically silences Th1-type chemokines to suppress effector T cell trafficking in colon cancer. Can Res. 76(2):275-82. PMID: 26567139.

associated with those of CD4, CD8, and Th1-type chemokines in human colon cancer tissue, and this expression pattern was significantly associated with patient survival. Collectively, our findings reveal that PRC2-mediated epigenetic silencing is not only a crucial oncogenic mechanism, but also a key circuit controlling tumor immunosuppression. Therefore, targeting epigenetic programs may have significant implications for improving the efficacy of current cancer immunotherapies relying on effective T-cell–mediated immunity at the tumor site.

3.2 Introduction

Effector T cells are indispensible for protective tumor immunity and therapeutic efficacy of cancer treatment (Zitvogel et al., 2008, Rosenberg et al., 2004, Park et al., 2010). In colon cancer, the presence of $CD8^+$ T cells and its cytotoxic antitumor molecules is a parameter for improved patient survival and tumors without signs of metastasis (Fridman et al., 2012). Th1-type chemokines CXCL9 and CXCL10 mediate the trafficking of the main antitumor immune cells, including Th1 and $CD8^+$ T cells, into the tumor microenvironment. However, it is not well understood how Th1-type chemokine expression is controlled in the tumor and subsequently how it affects effector T cell trafficking into the human cancer microenvironment.

Epigenetic regulation, including histone modifications, can mediate gene repression (Greer and Shi, 2012). One epigenetic repressive machinery involves the polycomb repressive complex 2 (PRC2), which trimethylates histone 3 lysine 27 (H3K27me3) (Cao et al., 2002, Margueron and Reinberg, 2011). H3K27me3 is a repressive histone mark associated with facultative heterochromatin that functions to recruit regulatory proteins to repress gene transcription (Margueron and Reinberg, 2011). PRC2 component, enhancer of zeste homolog 2

(EZH2) is highly expressed in multiple cancers (Varambally et al., 2008, Mimori et al., 2005). Its role in cancer cell proliferation, invasiveness, and differentiation has been widely studied (Varambally et al., 2008, Mimori et al., 2005). However, it is not known if EZH2 and the other PRC2 proteins are involved in the regulation of human T cell tumor trafficking, and, in turn, tumor immunity. Cancer epigenetic studies suggest that an abnormal evolution of repressive epigenetic marks including histone modification may directly contribute to cancer development and progression (Schlesinger et al., 2007, Feinberg et al., 2006). However, the nature of cancer epigenetic repression is involved in the control of cancer immunity remains unanswered. Given the relevance of effector T cells and their tumor homing in anti-tumor immunity (Zitvogel et al., 2008, Rosenberg et al., 2004, Fridman et al., 2012), we hypothesized that the cancer epigenetic repressive PRC2 machinery represses Th1-type chemokines in colon cancer, and in turn, alters effector T cell tumor migration and effective anti-tumor immunity. The validation of this hypothesis will lead to a notion that cancer epigenetic repressive machinery-mediated Th1-type chemokine repression is a novel immune evasive mechanism and the repressive machinery may be a target for novel cancer immunotherapy. Thus, in the current work, we tested this hypothesis in the context of human colon cancer at the molecular and clinical level.

3.3 Materials and Methods

Human subjects and colon cancer tissues

Patients diagnosed with colon carcinomas were recruited in the study. All usage of human subjects in this study was approved by local Institutional Review Boards. 6-9 fresh colon tissues were collected from patients with colon cancer and ulcerative colitis. Primary colon cancer

cells, colon epithelial cells, and all the *in vitro* functional assays were performed with single cells from fresh colon cancer and colitis tissues as previously described (Kryczek et al., 2014). In addition, patients with colorectal carcinoma were evaluated from datasets in Oncomine.org.

Cell culture

Primary colon cancer cell lines (C1) were established from fresh colon cancer tissue. Routine short tandem repeat analysis is done to confirm the uniqueness of the primary line (Kryczek et al., 2014). DLD-1 and SW480 cell lines (ATCC) were used in the experiments. Single colon epithelial cells were made from fresh colon tissues from patients with colon cancer and ulcerative colitis. Colon cancer cells were treated with recombinant IFNγ (R&D systems), DZNep (Sigma), GSK-126 (GlaxoSmithKline), and GSK-J4 (GlaxoSmithKline), for different time points and concentrations.

Real-Time reverse-transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from the cells by Trizol (Ambion) and converted to cDNA using reverse transcriptase PCR (cloned AMV reverse transcriptase, Invitrogen). The mRNA was then quantified by real-time RT-PCR using StepOnePlus (Applied Biosystems). Fast SYBR Green Master Mix (Applied Biosystems) was used to detect fluorescence. Relative quantification was calculated according to the comparative Ct method with normalization to *GAPDH*. Unless otherwise noted, fold change with normalization to control is shown in the figures.

Lentiviral transduction and transfection

The lentiviral vectors, pGIPz or pGreen, encoding gene specific shRNAs were used to transduce colon cancer cells to establish stable cell knockdowns. Lentiviral shRNAs were from the Vector Core at the University of Michigan or provided by Arul Chinnaiyan (Varambally et al., 2008). The lentiviral transduction efficiency was confirmed by GFP which was co-expressed by the lentiviral vector. The knockdown efficiency was assessed by Western blotting and real-time PCR. For transfections, Fugene HD (Promega) was used to transfect colon cancer cells with PKH3 (empty vector) and pCMV HA JMJD3 (Addgene (#24167)) according to the manufacturer's protocol. The overexpression was confirmed by Western blotting.

Enzyme-Linked Immunosorbent Assay (ELISA)

The protein levels of CXCL9 and CXCL10 were detected by ELISA (R&D) from the supernatants of treated colon cancer cells or single cells from fresh colon cancer and colitis tissue.

Western Blot

Western blotting was performed with specific antibodies against Histone H3 (9715, Cell Signaling), β-Actin (A5441, Sigma), EZH2 (612667, BD), SUZ12 (46264, Santa Cruz), EED (28701, Santa Cruz), and H3K27me3 (07449, Millipore). Signals were detected by ECL reagents (GE Healthcare, Buckinghamshire, UK).

T cell migration assays

In vitro migration assay was performed in a Transwell system with a polycarbonate membrane of 6.5-mm diameter with a 3-μm pore size as described (Curiel et al., 2004, Curiel et al., 2003).

Activated T cells were treated with anti-human CXCR3 or isotype, and added to the upper chamber. Supernatant from the cultured colon cancer cells was added to the lower chamber. After incubation at 37°C for 12 hours, the phenotype and number of T cells in the upper and lower chambers were determined by FACS (LSRII, BD).

Chromatin immunoprecipitation (ChIP)

ChIP was performed and as previously described (Cui et al., 2013, Kryczek et al., 2014). Crosslinking was performed with 1% formaldehyde or 1% paraformaldehyde for 10 minutes. Sonication was performed with the Misonix 4000 water bath sonication unit at 15% amplitude for 10 minutes. Protein/DNA complex was precipitated with specific antibodies against H3K27me3 (6002, Abcam) and IgG control (Millipore). ChIP-enriched chromatin was used for RT-PCR with SYBR Green Master Mix, normalizing to input.

Statistical analysis

Dependent on data distribution and experimental design, paired or unpaired Student's t-test and Mann-Whitney U-tests were used. Correlation coefficients (Spearman correlation) denoted by r, together with a p-value, were computed to measure correlation between different genes. Survival functions were estimated by Kaplan-Meier methods using genes classified as high or low based on mean or median expression values. Data was censored at the last follow-up for patients who were disease-free or alive at the time of analysis. All analyses were done using SAS 9.3 software. $p < 0.05$ was considered significant.

3.4 Results

3.4a Inverse correlation exists between Th1-type chemokines and PRC2 in colon cancer

Th1-type chemokines CXCL9 and CXCL10 mediate effector $CD8^+$ T cell tumor trafficking. $CD8^+$ T cell tumor infiltration is associated with improved cancer patient survival. Th1-type chemokines are correlated with effector T cell density in some human tumors, including colon cancer, and positively associated with cancer patient survival (Zhang et al., 2003, Kryczek et al., 2009). However, it is poorly understood how Th1-type chemokine expression is controlled in human colon cancer. We found that the levels of CXCL9 and CXCL10 mRNA (Fig. 3.1A) and protein (Fig. 3.1B) were higher in colitis colon compared to colon cancer tissue. The chemokines CXCL9 and CXCL10 can be stimulated by interferon gamma (IFNγ). When we treated single epithelial cells from the microenvironments of colon cancer, adjacent colon tissue, and colitis with IFNγ, the levels of *CXCL9* and *CXCL10* were higher in adjacent tissues (Fig. 3.1C) and colitis tissues (Fig. 3.1D) than colon cancer tissues. Patients with ulcerative colitis are at increased risk for developing colorectal cancer. The data suggest that Th1-type chemokine expression in colon cancer may evolve and become repressed when going from inflammatory tissue to cancer.

The PRC2 complex (including EZH2, embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (SUZ12)) represses gene transcription through methylation of H3K27 (Tan et al., 2007, Cao et al., 2002). We hypothesized that the PRC2 complex repressed Th1-type chemokines in colon cancer. To test this hypothesis, we analyzed a colon cancer tissue microarray from Oncomine.org (Khambata-Ford et al., 2007) for potential correlations between PRC2 and Th1-type chemokines, CXCL9 and CXCL10. In support of our hypothesis, we found

significant negative correlations between the PRC2 complex components and CXCL9 and CXCL10 (Fig. 3.1E-I). The data suggests that the high levels of PRC2 may control and repress Th1-type chemokine expression in colon cancer.

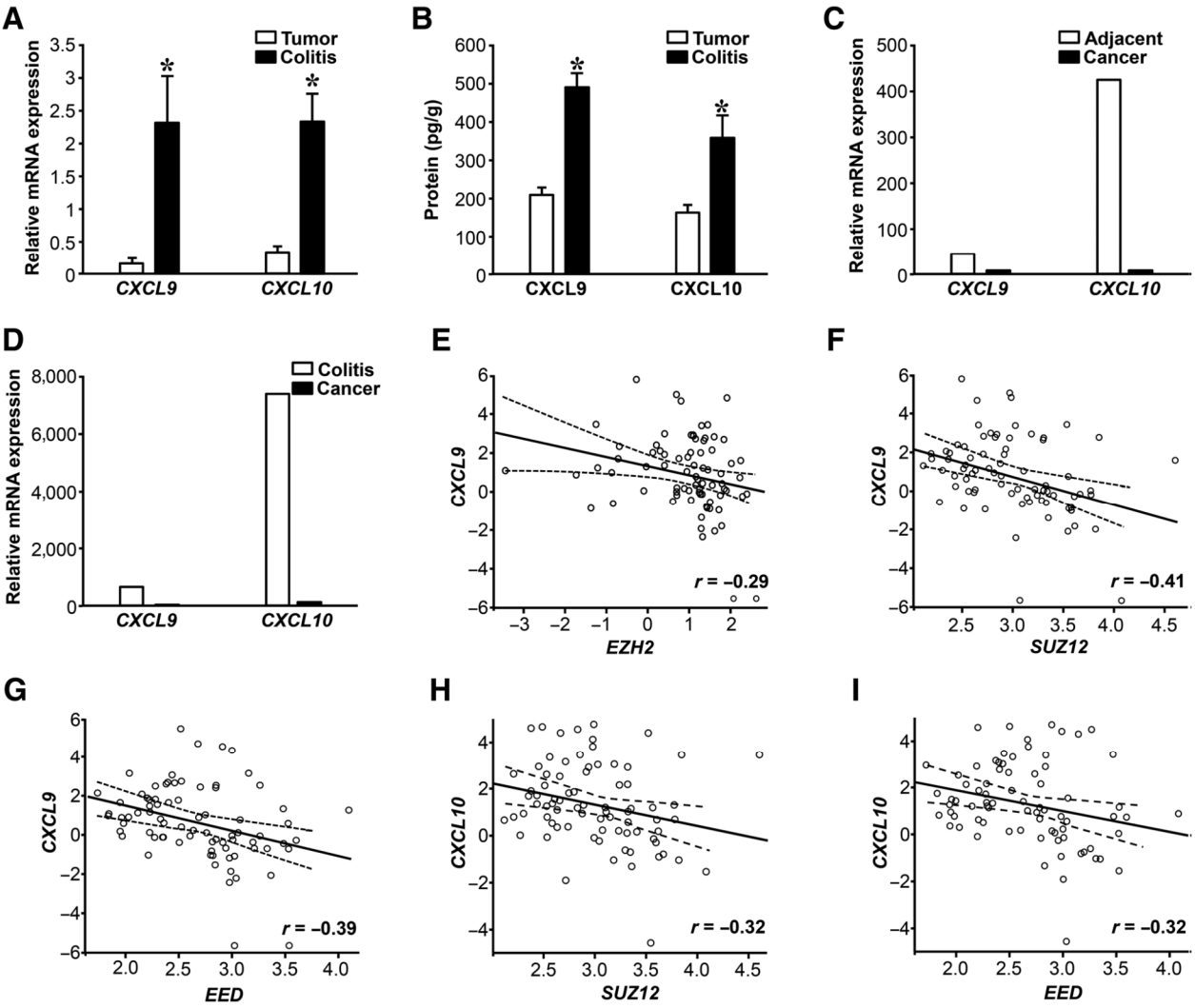

Figure 3.1. Inverse correlation exists between Th1-type chemokines and the PRC2 complex in colon cancer. (A): CXCL9 and CXCL10 mRNA in colitis tissue and colon cancer tissue. CXCL9 and CXCL10 mRNA levels were detected by real-time PCR in human colon cancer and colitis tissue (n= 6 tissues; $^{*}p < 0.05$). (B): CXCL9 and CXCL10 protein in colitis tissue and colon cancer tissue. CXCL9 and CXCL10 protein was detected by ELISA in 1 gram of human colon cancer and colitis tissue (n=6 tissues; $^{*}p < 0.05$). (C and D): CXCL9 and CXCL10 mRNA in IFNγ -treated colitis tissue, colon cancer tissue, and tissue adjacent to colon cancer. Single epithelial cells were prepared from colon cancer tissue (C and D), tissue adjacent to colon cancer (C) and colitis tissue (D), and treated with IFNγ (5 ng/mL). CXCL9 and CXCL10 mRNA levels were detected by real-time PCR. One representative of three independent experiments is shown. (E–I): correlation between Th1-type chemokines and PRC2 components in patients with colon cancer. Numbers represent r values with significance ($p < 0.05$). Spearman analysis for correlation were conducted from Khambata–Ford Colon database from Oncomine (oncomine.org; n=80).

3.4b PRC2 represses Th1-type chemokine expression in colon cancer

Given the inverse relationship between PRC2 and Th1-type chemokines (Fig. 3.1), we investigated if PRC2 machinery represses Th1-type chemokine expression in colon cancer. We initially examined the potential effect of 3-Deazaneplanocin A (DZNep), a pharmacological PRC2 inhibitor (Tan et al., 2007), on Th1-type chemokine expression in human colon cancer cells. In response to IFNγ treatment, we observed that treatment with DZNep led to higher levels of *CXCL9* and *CXCL10* expression in a primary colon cancer cell line (C1) (Fig. 3.2A, B), DLD-1 (Fig. 3.2C, D) and SW480 (Fig. 3.2E, F) colon cancer cells. As expected, DZNep reduced the expression of EZH2, SUZ12, and EED (Fig. 3.3A). However, the effect of DZNep was specific to chemokines as other IFNγ associated genes, including IFNγ receptor (*IFNGR2*) (Fig. 3.3B) and HLA-B (Fig. 3.3C) was not affected.

We next genetically knocked down EZH2 expression with lentivirus-based short hairpin RNA for EZH2 (shEZH2) in primary colon cancer C1 cells. shEZH2 specifically reduced the expression of EZH2 and SUZ12 (Fig. 3.3D), and resulted in elevated Th1 type chemokine mRNA (Fig. 3.2G,H) release in response to IFNγ stimulation (Fig. 3.2G, H). In addition, specific knockdown of EZH2 with shEZH2 and SUZ12 with shSUZ12 in DLD-1 cells (Fig. 3.3E, F) also increased Th1-type chemokine protein expression (Fig. 3.3F). Similar results were observed in shEED expressing DLD-1 cells (Fig. 3.3G). Thus, the PRC2 complex mediates Th1-type chemokine repression in colon cancer cells.

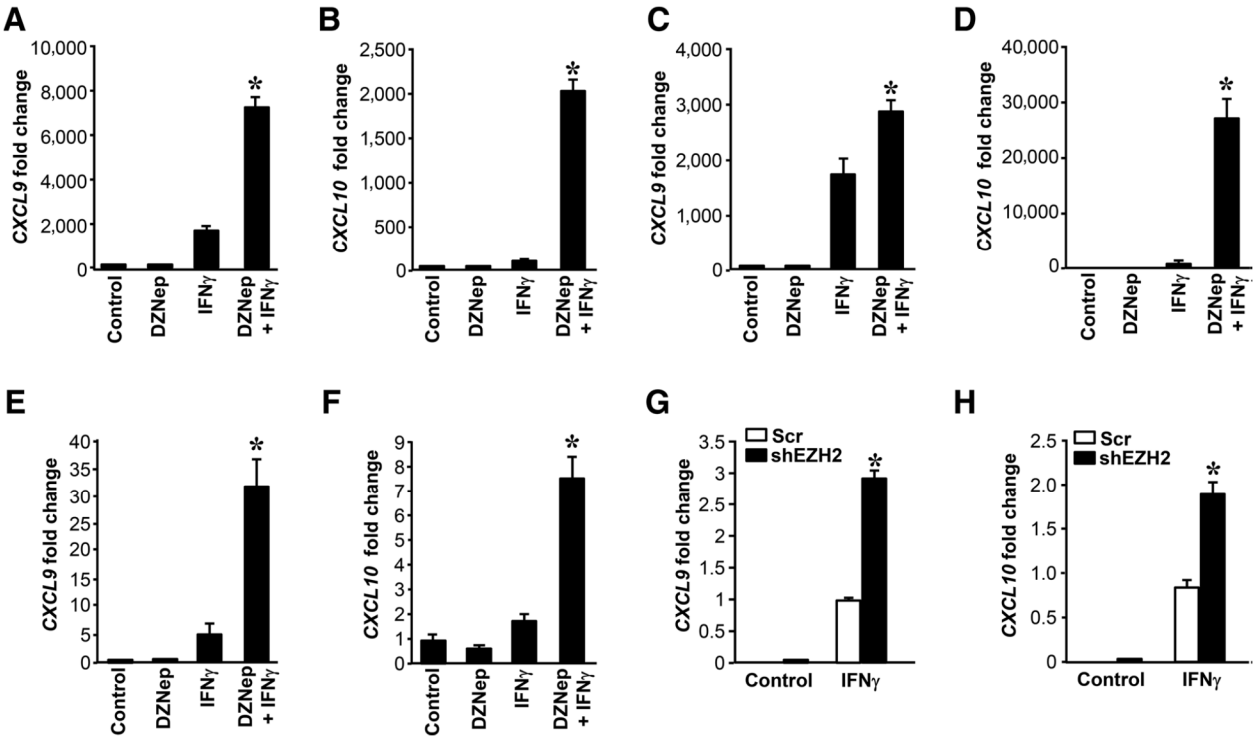

Figure 3.2 PRC2 represses Th1-type chemokine expression in colon cancer. (A–F): effects of DZNep on IFNγ -stimulated Th1-type chemokine production. CXCL9 and CXCL10mRNAlevels were detected by real-time PCR in C1 cells (A and B), DLD-1 (C and D), and SW480 (E and F) cells treated with IFNγ (0.5 or 10 ng/mL) and DZNep (0.5, 2, or 5 mmol/L; three independent experiments are shown; $^{*}p < 0.05$). (G and H): effects of shEZH2 on IFNγ -mediated chemokine production. C1 cells expressing shEZH2 or scr vector were cultured with IFNγ (0.5 ng/mL), and CXCL9 (G) and CXCL10 (H) mRNA was detected by real-time PCR (three independent experiments are shown; $p < 0.05$); fold change shown relative to Scr with IFNγ.

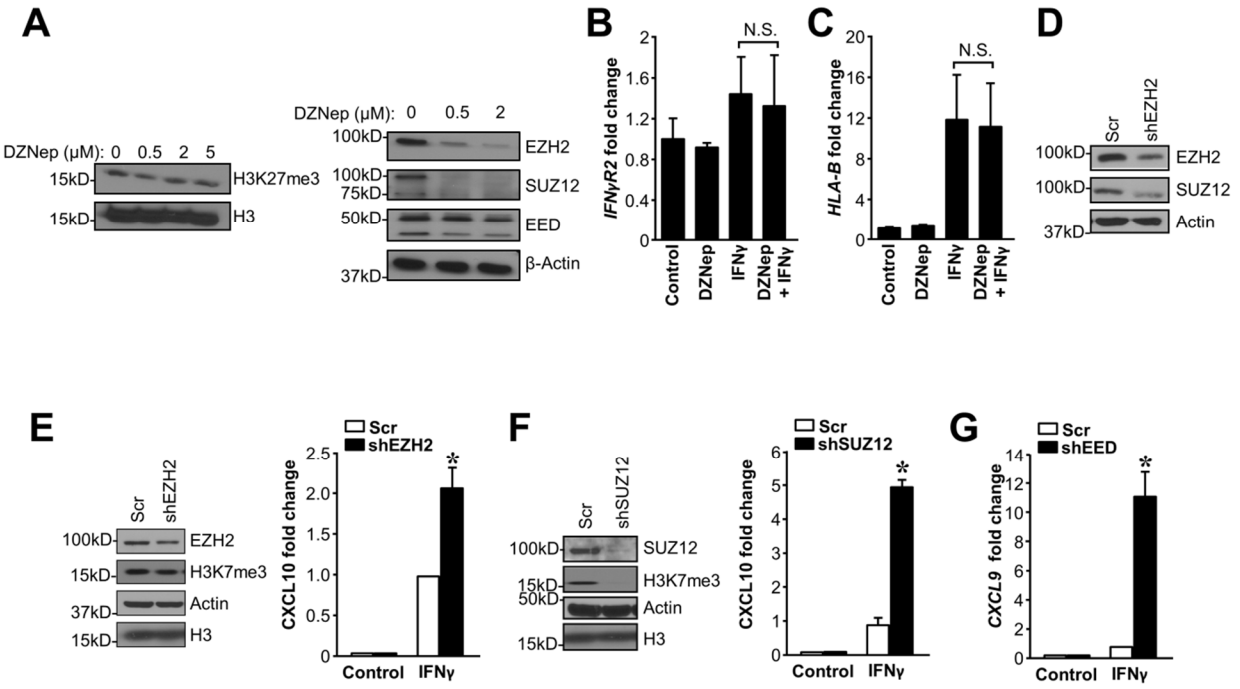

Figure 3.3 Effects of the PRC2 complex on the IFNγ-mediated Th1-type chemokine production in colon cancer cells. (A): Effects of DZNep on the PRC2 complex expression in colon cancer cells. DLD-1 cells were treated with different concentrations of DZNep for 12 hours. H3K27me3, EZH2, SUZ12, and EED levels were detected by Western blotting. One of 3 experiments is shown. (B and C): Effects of DZNep on the expression of the IFNγ receptor (IFNGR2) (B) and HLA-B (C) in colon cancer cells. SW-480 cells were treated with IFNγ (10 ng/ml) and/or DZNep (0.5 uM) for 19 hours. IFNGR2 and HLA-B mRNA was detected by real-time PCR in SW-480 (N.S. not significant, 3 independent experiments shown). (D): Effects of shEZH2 on the expression of EZH2 and SUZ12 in C1 cells. SUZ12 and EZH2 levels were detected by Western blotting. One of 3 experiments is shown. €: Effects of shEZH2 on CXCL10 chemokine production. Left: EZH2 and H3K27me3 levels were detected by Western blotting. One of 3 experiments is shown. Right: DLD-1 cells expressing shEZH2 or scr vector were cultured with IFNγ (5 ng/ml), and CXCL10 protein was detected by ELISA (5 independent experiments shown, *p<0.01); fold change shown relative to scr with IFNγ. (F): Effects of shSUZ12 on CXCL10 chemokine production. Left: SUZ12 and H3K27me3 levels were detected by Western blotting. One of 3 experiments is shown. Right: shSUZ12 DLD-1 cells were cultured with IFNγ (10 ng/ml) and CXCL10 protein was detected by ELISA (3 independent experiments shown, *p<0.01); fold change shown relative to scr with IFNγ. (G): Effects of shEED on CXCL10 chemokine production. shEED DLD-1 cells were cultured with IFNγ (0.5 ng/ml) and CXCL10 mRNA was detected by real-time PCR (3 independent experiments shown, *p<0.05); fold change shown relative to scr with IFNγ.

3.4c Th1-type chemokine repression is mediated via H3K27 methylation

The PRC2 complex mediates H3K27 methylation (Cao et al., 2002, Margueron and Reinberg, 2011). We next examined whether Th1-type chemokine repression mediated by PRC2 depends on H3K27me3 changes at the promoter level of the chemokine genes. To test this possibility, we performed specific chromatin immunoprecipitation (ChIP) assay to detect H3K27me3 marks, based on the ENCODE database for H3K27me3 in GM12878 cells (Harth-Hertle et al., 2013) (Fig. 3.4A). We observed high levels of H3K27me3 marks on the promoter of *CXCL9* (Fig. 3.5A), the intergenic regions in between the *CXCL9* and *CXCL10* genes (Fig. 3.5B) and the promoter of *CXCL10* (Fig. 3.5C). Knockdown of SUZ12 with shSUZ12, importantly, removed H3K27me3 on these areas (Fig. 3.5A-C) and increased Th1-type chemokine expression (Fig. 3.3F). *GAPDH and HOXB1* were used as a negative and positive control, respectively (Fig. 3.5D). Thus, H3K27me3 may be involved in the Th1-type chemokine gene silencing in colon cancer.

GSK126 is a highly selective, potent small molecule inhibitor of EZH2 methyltransferase activity (McCabe et al., 2012). 5uM GSK126 treatment abolished the global level of H3K27me3 without inhibiting EZH2 (Fig. 3.4B). GSK126 treatment led to higher levels of IFNγ-induced *CXCL10* (Fig. 3.5E) expression in primary colon cancer C1 cells. Similar results were observed in DLD-1 cells (Fig. 3.5C, D).

Jumonji C (JmjC)-domain-containing protein, JMJD3, is an H3K27 specific demethylase (Agger et al., 2007). As an activator of gene expression, ectopic expression of JMJD3 increased Th1-type chemokine expression (Fig. 3.5F). GSK-J4, a small molecular catalytic site inhibitor, selectively targets the H3K27-specific JmjC demethylase subfamily (Kruidenier et al., 2012). GSK-J4 treatment led to reduced *CXCL9* and *CXCL10* expression in primary colon cancer C1 cells

(Fig. 3.5G, H). Thus, H3K27me3 specific methyltransferase and demethylase regulate Th1-type chemokine repression in colon cancer cells.

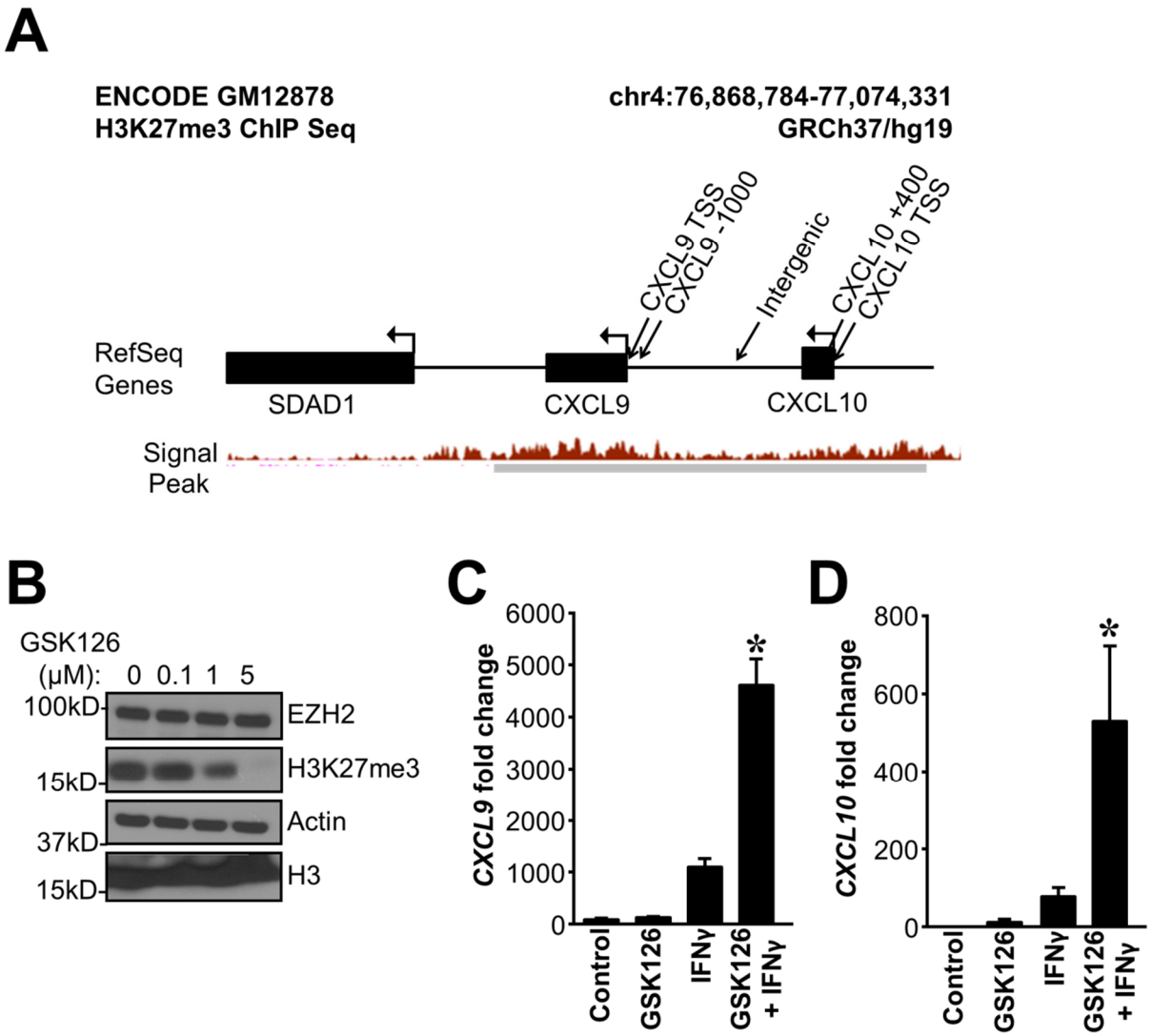

Figure 3.4 Effects of H3K27me3 on Th1-type chemokine production in colon cancer cells. (A): Schematic representation of the chemokine promoter area indicating the H3K27me3 signal and peaks according to H3K27me3 ChIP-sequencing in GM12878 cells (ENCODE.org)**. (**B): Effects of GSK126 on global H2K27me3 and EZH2 expression in colon cancer cells. C1 cells were treated with different concentrations of GSK126 for 72 hours. EZH2 and H3K27me3 levels were detected by Western blotting. One of 3 experiments is shown. (C and D): Effects of GSK126 on CXCL9 and CXCL10 chemokine expression in colon cancer cells. DLD-1 cells were treated with 5 uM GSK-126 in the presence of IFNγ (0.5 and 5ng/ml) and CXCL9 and CXCL10 mRNA was detected by real-time PCR (3 independent experiments shown,*$p<0.05$).

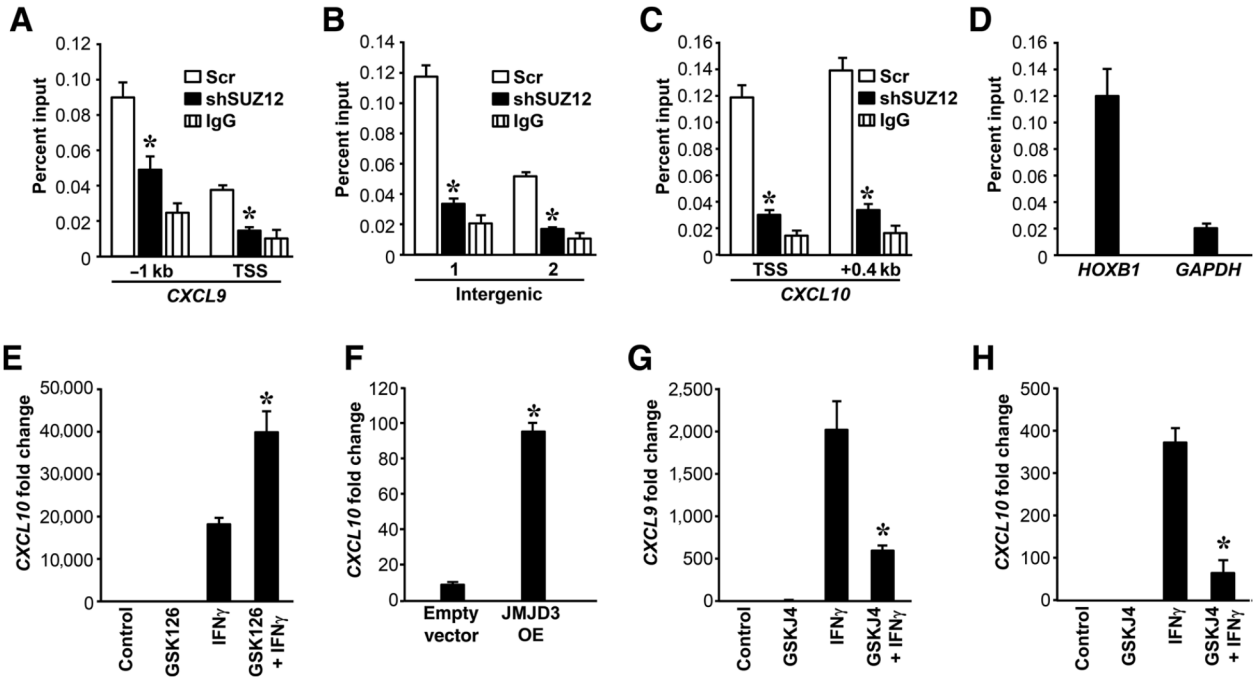

Figure 3.5 Th1-type chemokine repression is mediated via H3K27 methylation. (A–C): H3K27me3 occupancy in the promoter area of CXCL9 and CXCL10 in colon cancer cells. H3K27me3-ChIP assay was performed in DLD-1 colon cancer cells for the promoter areas of CXCL9 (A), the intergenic region of CXCL9 and CXCL10 (B), and the promoter areas of CXCL10 (C; three independent experiments shown; *p < 0.01). kb, kilobase; TSS, transcription start site. (D): ChIP-positive and -negative control. H3K27me3-ChIP assaywas performed in DLD-1 colon cancer cells for HOXB1 and GAPDH (three independent experiments are shown). (E): effects of GSK126 on the IFNγ -mediated chemokine production. C1 cells were treated with IFNγ (5 ng/mL) and 0.5 mmol/L GSK126. CXCL10 mRNA was detected by real-time PCR (three independent experiments are shown; *p < 0.05). (F): effects of JMJD3 overexpression on IFNγ -mediated chemokine production. DLD-1 cells were transfected with PKH3 (empty vector) and pCMV HA JMJD3 (JMJD3 OE) before IFNγ (0.5 ng/mL) was added and RT-PCR was performed for CXCL10 (four independent experiments are shown; *p < 0.05). (G and H): effects of GSK-J4 on IFNγ -mediated chemokine production. C1 cells were treated with IFNγ (0.5 ng/mL) and 5 mmol/L or 10 mmol/L GSK-J4, and CXCL9 and CXCL10 mRNA was detected by real-time PCR (three independent experiments are shown; *p < 0.05).

3.4d PRC2 affects T cell migration toward colon cancer

Next, we examined whether the PRC2 complex can affect T cell migration via controlling CXCL9 and CXCL10 expression. We collected supernatants from colon cancer cells transfected with shEZH2 and control lentiviral vectors. We showed that $CD4^+$ (Fig. 3.6A) and $CD8^+$ (Fig. 3.6B) T cells more efficiently migrated towards shEZH2 supernatant than control. The migration was blocked by monoclonal antibody (mAb) against CXCR3, the receptor for CXCL9 and CXCL10 on T cells (Fig. 3.6A, B). Thus, the PRC2 component, EZH2, controls T cell trafficking toward colon cancer-derived CXCL9 and CXCL10.

Finally, we assessed the clinical relevance of this phenomenon by examining the association of chemokines and the PRC2 complex with patient survival. We first performed survival analysis on chemokines by combining multiple datasets of colorectal carcinoma patients from oncomine.org. The patients were stratified in groups based on the mean expression level of each gene. When we divided patients with high or low expression of both *CXCL9* and *CXCL10,* high *CXCL9/CXCL10* associated with better patient overall survival (Fig. 3.7A). As a confirmation, we also observed positive correlation with *CD8* and *CXCL9* and *CXCL10* in different cohorts. This suggests that higher chemokines may lead to higher $CD8^+$ T cell tumor infiltration (Fig. 3.7B). To examine the association between the 3 immune gene signature (CD8, CXCL9, and CXCL10) and the 3 PRC2 gene components (EZH2, EED, and SUZ12) with patient survival, we divided patients with high or low expression of these genes. We found that high *CD8/CXCL9/CXCL10* (Fig. 3.6C) and low *EZH2/SUZ12/EED* (Fig. 3.6D) associated with better patient overall survival. We also analyzed the relationship between PRC2 complex component transcripts and *CD4* and *CD8* mRNA in patients with colorectal cancer. We found that *CD4* and

CD8 mRNA expression negatively correlated with the PRC2 complex components, *EZH2, SUZ12*, and *EED* mRNA (Fig. 3.6E-G). Altogether, the data suggests that PRC2 controls T cell trafficking via repressing Th1-type chemokines and impacts colon cancer pathology.

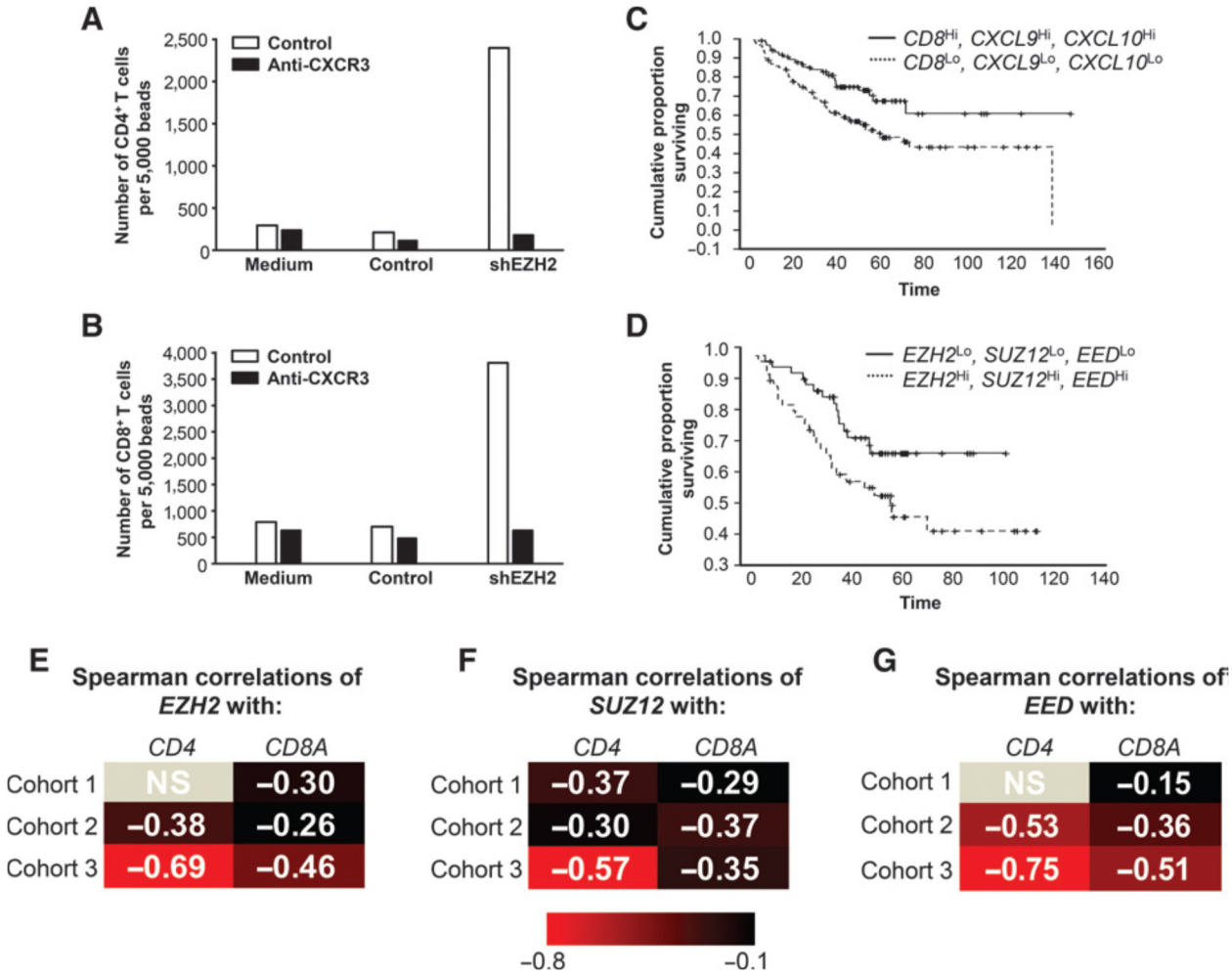

Figure 3.6 PRC2 affects CD8⁺ T-cell migration toward colon cancer. (A and B): effect of shEZH2 on migration of CD4⁺ (A) and CD8⁺ (B) T cells. shEZH2 and control vector expressing DLD-1 cells were treated with 5 ng/mL IFNγ and the supernatants were collected for T-cell migration assay. Activated T cells were subject to the migration toward the supernatants in the presence of anti-human CXCR3 or control. The migrated T cells were assessed by flow cytometry analysis. One representative of three independent experiments is shown. (C): the association between CD8/CXCL9/CXCL10 transcripts and survival in patients with colorectal carcinoma (Smith colorectal, Smith colorectal 2, Jorissen Colorectal 3, and Staub colorectal). The analyses were conducted by combining multiple databases from Oncomine (oncomine.org; n=185, p=0.0089). (D): the association between EZH2/SUZ12/EED transcripts and survival in patients with colorectal carcinoma (Smith colorectal, Smith colorectal 2, Jorissen Colorectal 3, and Staub colorectal). The analyses were conducted by combining multiple databases from Oncomine (oncomine.org; n=105; p=0.037). (E–G): correlation between PRC2 components and CD4 andCD8A mRNAin patients with colorectal carcinoma. Numbers represent r values with significance ($p < 0.05$). Spearman analysis for correlation was conducted from multiple databases (Khambata-Ford Colon, Staub Colorectal, Smith Colorectal 2, TCGA, and Jorissen Colorectal 3) from Oncomine (oncomine.org). NS, not significant.

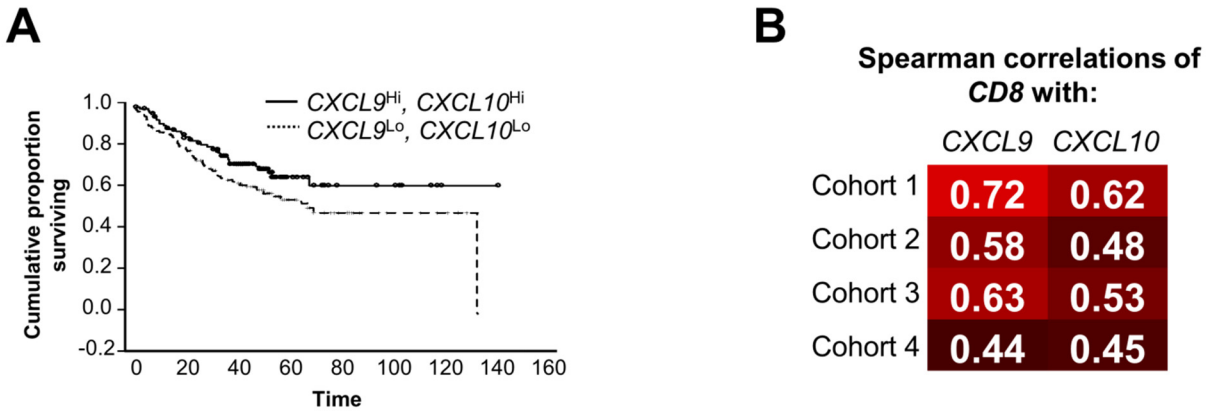

Figure 3.7 Clinical relevance of CXCL9 and CXCL10 expression in colon cancer (A): Association between *CXCL9/CXCL10* transcripts and patient survival in patients with colorectal carcinoma (Smith colorectal, Smith colorectal 2, Jorissen Colorectal 3, and Staub colorectal). The analyses were conducted by combining multiple databases from Oncomine (oncomine.org) (n=247, p=0.0494). (B): Correlation between *CD8* and *CXCL9 and CXCL10* mRNA in patients with colorectal carcinoma. Numbers represent r-values with significance ($p<0.05$). Spearman analysis for correlation was conducted from multiple databases (Staub Colorectal, Smith Colorectal 2, Smith Colorectal 1, and Jorissen Colorectal 3) from Oncomine (oncomine.org).

3.5 Discussion

Epigenetic changes are biologically vital and often linked to cancer proliferation, progression and metastasis (Jones and Baylin, 2002, Varambally et al., 2008, Mimori et al., 2005, Fussbroich et al., 2011). Our studies have shown that the PRC2 components in colon cancer cells silence the tumor production of Th1-type chemokines, potentially restrain effector T cell tumor infiltration, and in turn lessen anti-cancer immunity. Thus, PRC2 and H3K27me3-mediated Th1-type chemokine silencing is a novel immune evasion mechanism in human colon cancer. We propose a unifying mechanistic model of cancer in which epigenetic silencing plays both biological and immunological roles in supporting tumor progression.

Previous reports show that the expression of CXCR3 and CXCL10 correlates with metastasis in certain cancer including colon cancer (Wightman et al., 2015, Murakami et al., 2013, Zipin-Roitman et al., 2007). However, whether the expression of CXCR3 in the tumor cells leads to CXCL10 dependent metastasis is not fully elucidated. Nonetheless, our data shows that there is a positive correlation between *CD8* and *CXCL9 and CXCL10*, and the expression of CD8, CXCL9 and CXCL10 is positively associated with colon cancer patient survival. In support of this observation, it has been shown that $CD8^+$ T cells and Th1-type chemokines positively predict colon cancer patient outcome (Fridman et al., 2012, Galon et al., 2006, Mlecnik et al., 2011, Pages et al., 2005).

A critical question is what drives the epigenetic changes in the first place. Several reports suggest microRNA involvement of EZH2 regulation (particularly loss of microRNA101) in prostate cancer (Varambally et al., 2008). Further studies will be needed to demonstrate the drivers of this repression in cancer cells versus normal epithelium and if epigenetic changes are

one of the earliest events in cancers, leading to immune evasion. We assume that this mechanism progressively evolves in cancer cells as colon inflammatory tissues express high levels of Th1-type chemokines. In contrast to irreversible genetic mutations, epigenetic alterations can be manipulated, making them a valuable target for therapy (Yoo and Jones, 2006). To this end, it is crucial to understand the immune associated epigenetic mechanisms in the human cancer and to dissect how different epigenetic modifiers differentially affect cancer immune signature gene expression. In this regard, we have noticed that GSK126, a compound acting as a direct histone methyltransferase (HMT) inhibitor and DZNep, a compound acting as an indirect HMT inhibitor have shown certain differences in potencies in promoting cancer Th1-type chemokine expression. Perhaps additional mechanisms of DZNep, such as proteasomal degradation of PCR2 subunits, inhibition of other methylations, and reactivation of thioredoxin-binding protein 2 (TXNIP) which causes disruption of PCR2 could explain this difference (Zhou et al., 2011).

Current cancer immunotherapies (Rosenberg et al., 2008, Scholler et al., 2012, Pardoll, 2012) and classic therapies (Zitvogel et al., 2008, Park et al., 2010) largely rely on efficient T cell tumor trafficking and T cell-mediated tumor immunity. Epigenetic reprograming may unlock Th1-type chemokine repression, transform the tumor from poor T cell infiltration to rich T cell infiltration, and, ultimately, improve the effectiveness of any given cancer therapy.

3.6 References

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Chapter 4

Protein Arginine Deaminase 4 (PAD4) signaling regulates effector cytokine production, proliferation, and survival in T cells

4.1 Abstract

The protein arginine deaminase (PAD) family of enzymes are responsible for deamination of arginine residues (in histones and other proteins) into citrulline. Protein citrullination can affect protein stability, protein interaction, and gene regulation and is involved in many important pathways including cell death, stemness, proliferation, terminal epithelial cell differentiation, and, recently, in neutrophil extracellular trap (NET) formation. Due to its role in NET formation, the role of PADs has been explored in neutrophils and antigen presenting cells in autoimmunity. In this chapter, we investigate the role of PAD in T cells. We observed that PAD expression, particularly PAD4, and citrullination is induced very early on in T cell activation. Using an inhibitor of PAD4, F-Amidine, we found that PAD4 inhibition leads to increased effector cytokines, proliferation, and survival of T cells upon activation. Thus, PAD4 seems to play an important role in T cell activation, affecting T cell effector ability, proliferation, and survival.

4.2 Introduction

Peptidylarginine deaminase (PAD or PADI) is a family of enzymes that mediate the deamination of arginine to citrulline, in a calcium-dependent manner (Arita et al., 2004). This deamination converts a positive arginine to a neutral amino acid on histone and proteins, thus affecting histone and protein function and interaction (Wang et al., 2004). There are 5 PADs that are expressed in various tissues and cells and each specifically target different substrates (Rus'd et al., 1999, Guerrin et al., 2003, Ishigami et al., 2002, Kanno et al., 2000, Chavanas et al., 2004). PAD-mediated citrullination is involved in many cellular processes including cell death, gene regulation, protein post-translational modifications, and terminal epithelial cell differentiation (Witalison et al., 2015b). Recent findings indicate PAD4 is critical in autoimmunity. Not only is PAD4 indispensable for neutrophil NET formation (Li et al., 2010b, Wang et al., 2009) but antibodies against citrullinated peptides, including self-peptides, are present in autoimmune diseases as in rheumatoid arthritis (Klareskog et al., 2008, Mowen and David, 2014, Nguyen and James, 2016).

PAD4 has a nuclear localization signal (Asaga et al., 2001) and thus can act as a transcriptional regulator. As a chromatin modifying enzyme, PAD4 can regulate gene transcription by citrullination of histones. For example, PAD4 regulates key stem cell genes expression in stem cells by citrullinating histone H1, which loosens chromatin compaction for gene expression (Christophorou et al., 2014). Another example is PAD4 interacting with the transcription factor Tal1 (translocated in leukemia-1) in lineage differentiation of hematopoietic stem cells (Kolodziej et al., 2014). PAD4 binds with Tal1 on the promoter of *IL6ST* and acts as coactivator by counteracting protein arginine methyltransferase 6 (PRMT6) mediated

repressive histone marks. PAD4 can also act as repressor, as in c-myc (Nakashima et al., 2013), p53-dependent genes like p21 (Li et al., 2008), miRNA 16 (Cui et al., 2013), and OKL38 (Yao et al., 2008) in various cancers. In these cases, PAD4 binds with other transcription factors, like p53, and negatively and positively regulate gene expression, through regulation of histone marks. p53 often coordinates and localizes with PAD4 for p53-mediated negative gene regulation through PAD4 deamination (Li et al., 2008).

In addition to targeting histones, PAD4 can mediate post-translational modifications (PTMs) of various proteins, including ING4 (inhibitor of growth 4) (Guo and Fast, 2011), glycogen synthase kinase-3β (Stadler et al., 2013), p300 (Lee et al., 2005) and structural proteins involved in apoptosis such as: nucleophosmin (Hagiwara et al., 2002) and nuclear lamin C (Tanikawa et al., 2012) in which citrullination, and thus the loss of positive charge, can affect the protein function, stability, and interaction with other proteins. In some cases, as in ING4, a well-known binding partner and enhancer of p53 transcriptional activity, citrullination by PAD4 disrupts its interaction with p53, and thus affects downstream gene expression like p21 (Guo and Fast, 2011).

As PAD4 acts as a corepressor with p53 and mediates p53-mediated negative gene regulation, PAD inhibition leads to apoptosis in cancer cells. PAD inhibition suppresses colitis-induced colon cancer (Witalison et al., 2015a), breast cancer (McElwee et al., 2012, Wang et al., 2016), and mouse sarcoma (Wang et al., 2012) *in vivo. In vitro* evidence includes PAD inhibition impeding tumor growth in various cancers such as breast cancer (Wang et al., 2015), and human sarcoma cell lines (Wang et al., 2012) through both p53 independent and dependent effects, respectively. Also, some evidence exists of PAD4 inhibition reducing proliferation,

invasion, migration, and increasing apoptosis in ovarian cancer (Cui et al., 2016). Not surprisingly, PAD4 is overexpressed in many cancers including in blood and tissues while being relatively absent in benign or normal tissues (Chang et al., 2009, Slack et al., 2011). Also, PAD2 overexpressing mice spontaneously developed skin neoplasia with EMT and inflammation properties 37% of the time (McElwee et al., 2014).

Since PAD is involved in tumor biology as well as immunity, we wanted to explore its role in T cells and thus antitumor immunity. Some evidence exists for a protective T cell effect. Padi4 -/- mice undergoing radiation shows resistance to apoptosis in the thymus (Tanikawa et al., 2009). Also, overexpression of PAD2 or PAD4 in Jurkat T cells leads to an increase in apoptosis via increasing p53 and p21 or citrullination of vimentin, respectively (Liu et al., 2006, Hsu et al., 2014). Thus, we hypothesized PAD regulation could negatively affect T cells. However, since most studies were in mice and in an overexpression system, we wanted to manipulate PADs by inhibiting its activity in primary human T cells and study its biology in T cell activation. PADs, due to its various functions, are tightly regulated. We initially observed and detected specific PAD expression and citrullination in T cell activation. We found that PAD4 is predominantly expressed in human T cells and induced early on in T cell activation, along with citrullination. We then manipulated PAD4 activity in T cells by inhibiting its function and observed an increase in T cell proliferation and effector cytokine production as well as a pro-survival effect.

4.3 Materials and Methods:

Human Subjects

Patients diagnosed with ovarian cancer were recruited in this study. All usage of human subjects in this study was approved by local Institutional Review Boards. Ovarian cancer patient blood was used and $CD3^+$ T cells were subsequently isolated as described below.

Isolation and culture of peripheral blood T cells and neutrophils

Human peripheral blood from healthy donors was obtained from Carter BloodCare (Bedford, TX). PBMC's were isolated from peripheral blood by Lymphoprep density gradient (Stemcell Technologies). $CD3^+$ T cells were isolated using human T cell enrichment cocktail (RosetteSep, Stemcell Technologies). Naïve ($CD45RA^+$) and memory ($CD45R0^+$) T cells were then isolated by column based magnetic separation (CD45R0 microbeads, Miltenyi Biotec). Cell purity was confirmed as >97% by flow cytometry (LSRII, BD). Human peripheral blood T cell subsets (at concentration of one million per milliliter of medium) were activated with plate bound anti-CD3 (clone UCHT1, 1.25 μg/ml) and soluble anti-CD28 (clone CD28.2, 0.62 μg/ml) (eBioscience) or CD3/CD28 beads (Dynabeads, Thermo Fisher Scientific) with F-Amidine (Cayman Chemicals) for different time points as indicated in the figure legend. As stated in the figure legend, cisplatin (10uM, Teva Pharmaceuticals) was added the last two days before collection. For naïve T cell cultures, IL-7 and IL-15 was added (5ng/ml). All T cell subsets were cultured in RPMI 1640 medium (HyClone, GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin.

Mouse T cells were isolated from lymph nodes and CD3 T cells were isolated using negative selection (EasySep, Stemcell Technologies). T cells were cultured in RPMI 1640 medium (HyClone, GE Healthcare Life Sciences) supplemented with 10% fetal bovine serum and

1% Penicillin/Streptomycin with plate-bound CD3 (clone 17A2, eBioscience) and soluble CD28 (clone 37.51, eBioscience) with IL-7 and IL-15 (5ng/ml) added every 3 days for 7 or 14 days. Human neutrophils were isolated from the bottom layer of lymphoprep density gradient and further purified by dextran gradient. They were then treated with PMA (100nM) for 2 hours to induce neutrophil NET formation.

Flow cytometry

Cells were immunostained and analyzed on LSRII (BD). Cytokine analysis was determined by intracellular immunostaining (Ebioscience manual and reagents) and analyzed on the LSRII. The following antibodies/reagents were used: Annexin V (FITC; BD Biosciences 556420), 7-AAD (BD Biosciences 559925), human TNFα (PE; BD Biosciences, 554513), human IL-2 (PerCP; eBioscience, 46-7029-42), human IFNγ (PE-CY7; BD Biosciences 557844), BrdU (AF647; BD Biosciences 560209), human Granzyme B (AF700; BD Biosciences 560213), human CD8 (APC-Cy7, BD Biosciences 557760), viability dye (Pacific Blue, ThermoFisher, L34962), mouse Granzyme B (PE, eBioscience 12-8898-82) mouse CD4 (APC-Cy7,eBiosciences 47-0042-82), and mouse CD8 (PE, BD Biosciences 553033). BrdU Staining was performed according to the EBioscience manual. Apoptosis detection was performed according to the BD Biosciences kit.

PAD enzymatic activity test

PAD enzymatic was measured using the BAEE (Nα-Benzoyl-L-arginine ethyl ester) colorimetric method of detection (Sugawara et al., 1998). In detail, cells were lysed in lysis buffer (20mM Tris-HCL pH 7.6, 1mM EDTA, 1mM PMSF, and 1% Triton X-100) on ice for 10 minutes and snap

frozen in liquid nitrogen. 25uL of lysate was then mixed with 50uL of reaction mixture (0.1M Tris-HCL pH 7.6, 10mM CaCl2, 5mM DTT, and 10mM BAEE) and incubated at 50C for one hour. The reaction was stopped with 12.5uL of 5M Perchloric acid. Water was added up to 200ul. Reagent 1 (23.39mM Diacetly monoxime), Reagent 2 (47.8mM Antipyrine), and Reagent 3 (32.5% sulfuric acid and 0.3mM ferric chloride) was mixed in a 75:15:10 (v/v/v) ratio just before use. 600ul of this chromogenic reagent mixture was added to the 200ul of reaction, mixed, and boiled for 25 minutes. The samples were then cooled on ice and read at absorbance 464nm. L-citrulline (Sigma C7629) was diluted in 0.02N HCL and used as the standard to then back-calculate the sample citrulline uM amounts. All reagents were purchased from Sigma Aldrich.

RT-PCR

RNA was isolated from the cells by TRIzol (Ambion) and converted to cDNA using reverse transcriptase PCR (cloned AMV reverse transcriptase, Invitrogen). The mRNA was then quantified by real-time reverse transcriptase (RT)-PCR using StepOnePlus (Applied Biosystems). Fast SYBR Green Master Mix (Applied Biosystems) was used to detect fluorescence. Relative quantification was calculated according to the comparative Ct method with normalization to 18s rRNA.

Western Blotting

Western blotting was performed with the following antibodies against: peptidyl-citrulline (clone F95, MABN328), β-actin (A5441, Sigma), and PAD4 (Abcam 50332). Milk was used for actin and

PAD4 blotting and 2% fish skin gelatin was used for anti-peptidyl citrulline. Signals were detected by ECL reagents (GE Healthcare)

Statistical analysis

Dependent on data distribution (parametric versus nonparametric data), paired Student *t* test and Mann–Whitney *U* tests were used. All analyses were done using R. $p < 0.05$ was considered significant.

4.4 Results

4.4a: Citrullination and PAD4 levels increase after T cell activation

As protein citrullination is an important process for immune cells such as neutrophils NET formation, we wanted to see if citrullination is active in T cells. We used primary human $CD3^+$ T cells isolated from peripheral blood. Using western blotting to observe total citrullination levels, very low citrulline levels were observed in resting inactivated T cells (Figure 4.1A Day 0). However, within one day after T cell activation with CD3 and CD28 beads, total citrullination levels went up (Figure 4.1A). Slight variation was observed between donors, but in general, citrullination increased within one or two days after activation. We confirmed the citrullination levels in T cells by performing a biochemical colorimetric test reflecting PAD enzymatic activity (Figure 4.1C). In principle, PAD citrullinates the substrate BAEE, which can then be detected by a colorimetric assay. As PMA induces citrullination in neutrophils for NET formation, we used PMA-treated neutrophils isolated from blood as a positive control for the

PAD enzymatic test (Figure 4.1B). As calcium is induced early on in T cell activation, early PAD activity in T cell activation is consistent with PAD enzyme being triggered by calcium signaling (Arita et al., 2004).

PADs, consisting of a family of five, are the enzymes responsible for citrullination of arginine residues. We thus performed RT-PCR and western blotting to detect PADs in T cells. We found that PAD4 mRNA was predominantly expressed. Indeed, PAD4 is detected in other immune cells (Foulquier et al., 2007, Jones et al., 2009) and is restricted to hematopoietic stem cells, especially granulocytes. We found that PAD4 mRNA and protein is also induced early in T cell activation (Figure 4.1D, E). Thus, primary human T cells increase citrullination levels upon activation by PAD4 induction.

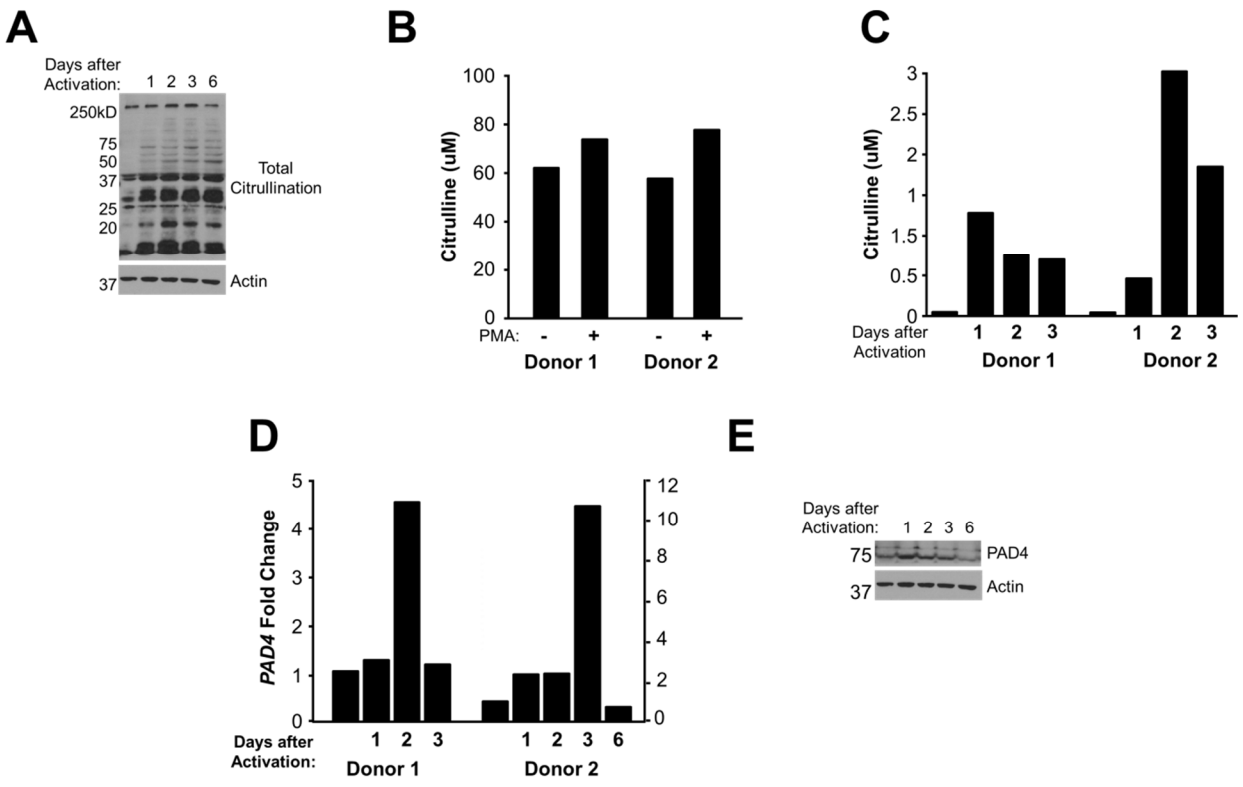

Figure 4.1 Citrullination and PAD4 levels increase after T cell activation. (A) Citrullination levels after T cell activation. Primary $CD3^+$ T cells were activated for the indicated days and western blotting was performed to detect total citrullination levels. Representative image shown (representative image, n=7 donors). (B) PAD enzymatic test control. Human neutrophils were isolated and treated with PMA (100nm) for 2 hours. PAD enzyme test was performed to measure the citrulline amount in uM (n=6 donors). (C) PAD enzymatic activity after T cell activation. Primary $CD3^+$ T cells were activated for the indicated days. PAD enzyme test was performed to measure the citrulline amount in uM (representative images, n=7 donors). (D, E) PAD4 expression after T cell activation. Primary $CD3^+$ T cells were activated for the indicated days and collected for PAD4 detection by RT-PCR and western blotting analysis (representative images, n=7 donors (D), n=4 donors (E)).

4.4b: PAD4 inhibition through F-Amidine treatment leads to increased effector cytokine production in T cells

To test the role of PAD activity in T cells, we used a potent PAD4 inhibitor, F-Amidine. F-Amidine covalently inactivates PAD4 enzymatic activity (Li et al., 2015, Luo et al., 2006). We first confirmed the drug's specificity by testing PAD's enzymatic activity in the presence of F-Amidine. We saw that F-Amidine inhibited PAD enzymatic activity in neutrophils (Figure 4.2A). In addition, in T cells, F-Amidine treatment led to a decrease in PAD4 mRNA (Figure 4.2B).

We then used F-Amidine to test its effect on T cells. When we activated the T cells and simply added F-Amidine, we saw three phenomena. The first was a striking increase in effector cytokines with F-Amidine, after T cell activation. We saw that T cells produced effector cytokines, tumor necrosis factor alpha (TNFα), interleukin (IL)-2, interferon gamma (IFNγ), and Granzyme B (Gran B) upon T cell activation. All four of these cytokines were greatly enhanced in the presence of F-Amidine (Figure 4.2C-G). This was confirmed when starting with both naïve $CD45RA^+$ $CD3^+$ cells (Figure 4.5) or memory $CD45R0^+$ $CD3^+$ T cells (Figure 4.2C-F). In memory cells, when further gating on $CD4^+$ and $CD8^+$ T cell separately, we saw a differential effect (Figure 4.3 and 4.4). Even though both $CD8^+$ cells and $CD4^+$ T cells predominantly and significantly increased all cytokines with F-Amidine treatment, the effects were more pronounced in $CD4^+$ T cells (Figure 4.3 and 4.4). There was also a differential effect of $CD4^+$ versus $CD8^+$ T cells on intracellular cytokine production in naïve T cells treated with F-Amidine (Figure 4.5). Since memory T cells have faster activation compared to naïve cells, we used human memory cells for further experiments. We also confirmed that Granzyme B effector cytokine is significantly increased in mouse T cells from the lymph nodes. The increase in

Granzyme B was observed in both $CD4^+$ and $CD8^+$ T cells (Figure 4.6). Therefore, F-Amidine treatment and thus PAD4 inhibition leads to increased T cell effector function.

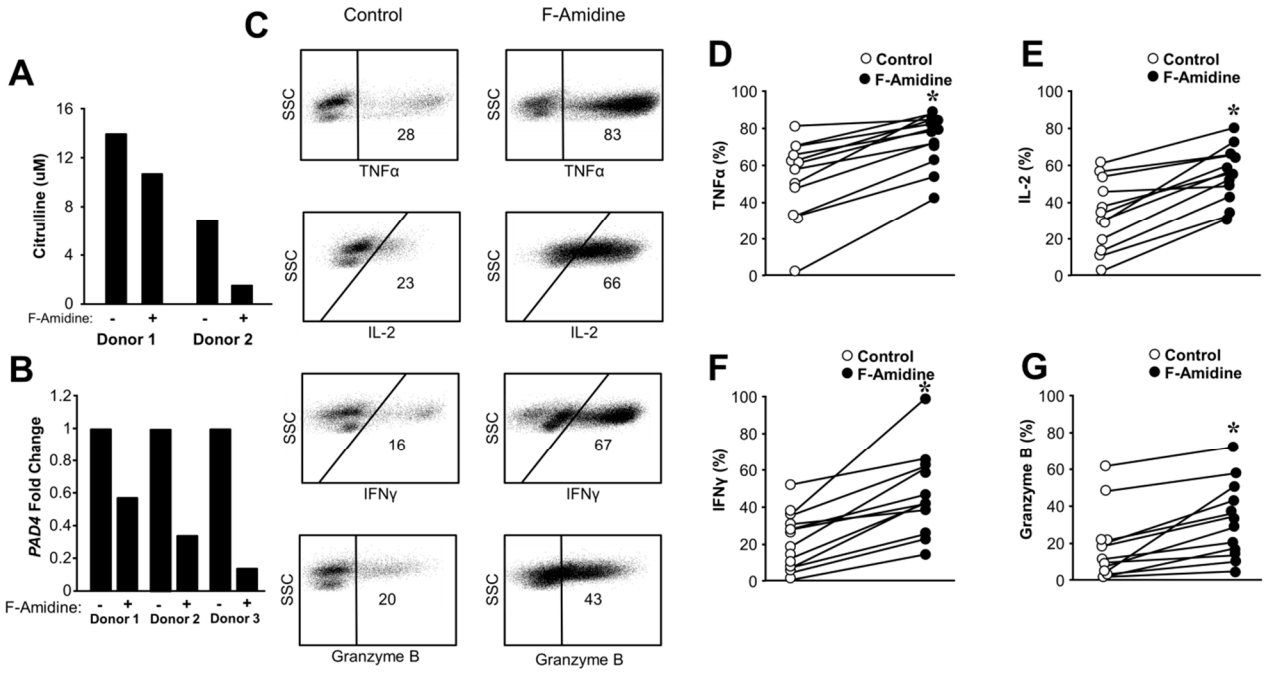

Figure 4.2: PAD4 and citrullination inhibition through F-Amidine treatment leads to increased effector cytokine production in memory T cells. (A): F-Amidine decreases citrullination levels. Human neutrophils were isolated and treated with F-Amidine (500uM) and the PAD enzyme test was performed to measure the citrulline amount. (B): Primary $CD3^+$ T cells were activated for 7 days and collected for PAD4 detection by RT-PCR. (C): $CD3^+$ $CD45R0^+$ memory T cells were sorted from fresh healthy donor PBMC's and activated with plate bound CD3, soluble CD28, and F-Amidine (10uM) for 7 days. Cytokines were detected by flow cytometry after gating for FSC-SSC and viability dye. Representative FACS data are shown (C) as well as the raw data for all donors (D-G) (* $p<0.0002$).

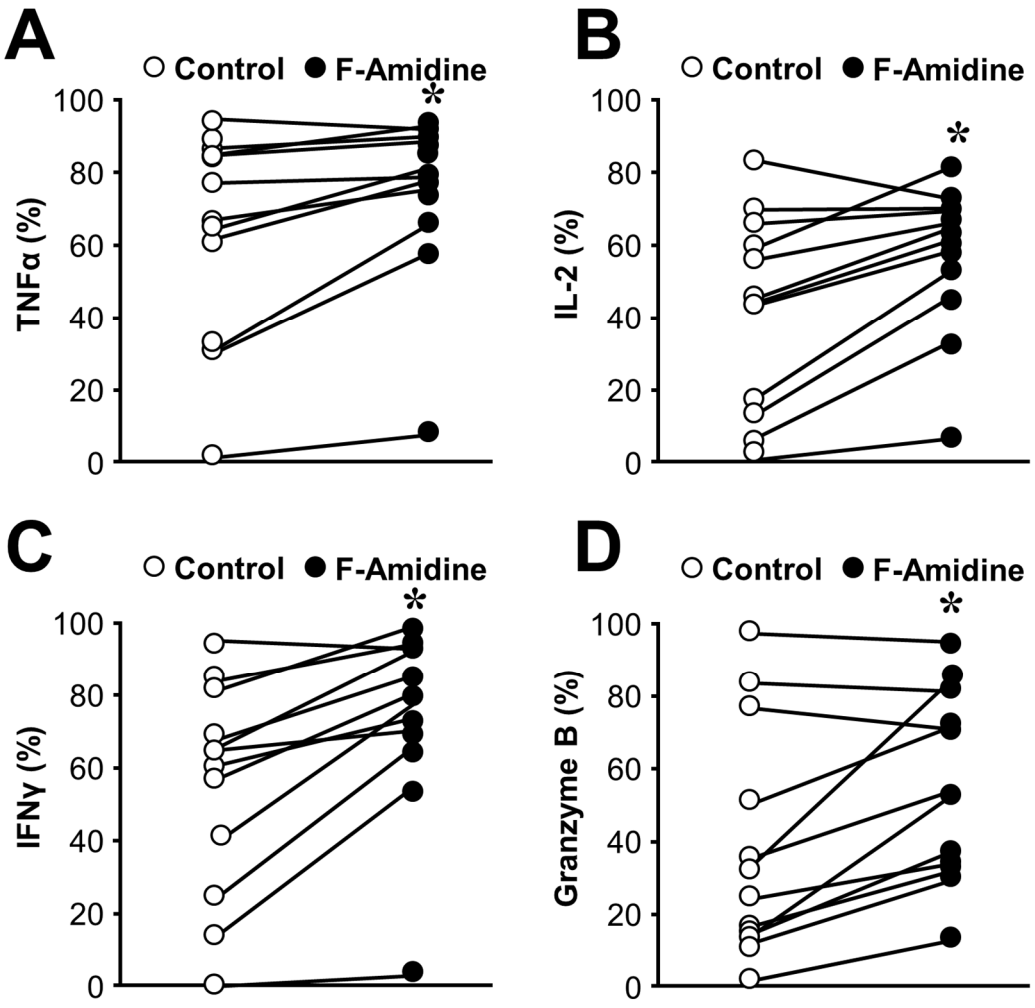

Figure 4.3: F-Amidine treatment leads to decrease in PAD4 levels and increased effector cytokine production in memory CD8$^+$ T cells. (A-D): CD3$^+$ CD45R0$^+$ memory T cells were sorted from fresh healthy donor PBMC's and treated with plate bound CD3, soluble CD28, and F-Amidine (10uM) for 3-5 days. Cytokines were detected by flow cytometry after gating for FSC-SSC-viability dye-CD8+ (*$p<0.004$).

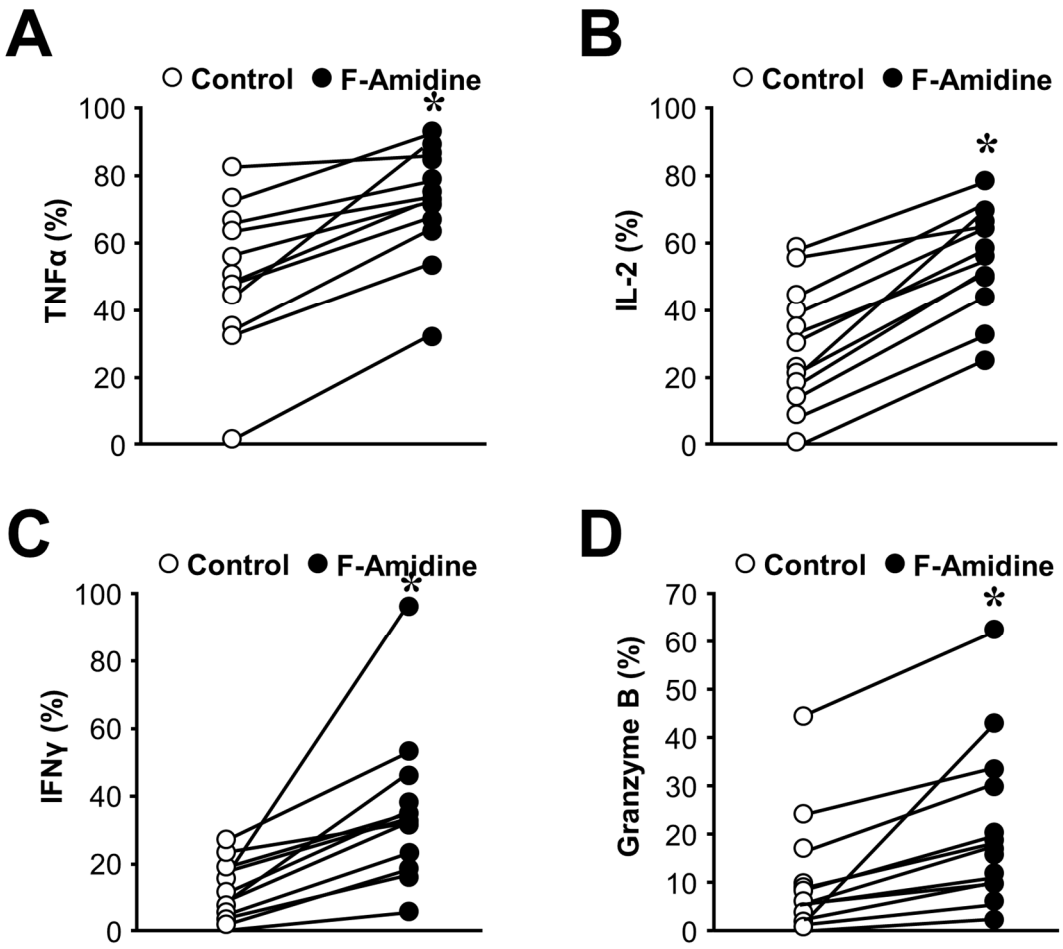

Figure 4.4: F-Amidine treatment leads to increased effector cytokine production in memory CD4$^+$ T cells. (A-D): CD3$^+$ CD45R0$^+$ memory T cells were sorted from fresh healthy donor PBMC's and treated with plate bound CD3, soluble CD28, and F-Amidine (10uM) for 3-5 days. Cytokines were detected by flow cytometry after gating for FSC-SSC-viability dye-CD4+ (*$p<0.002$).

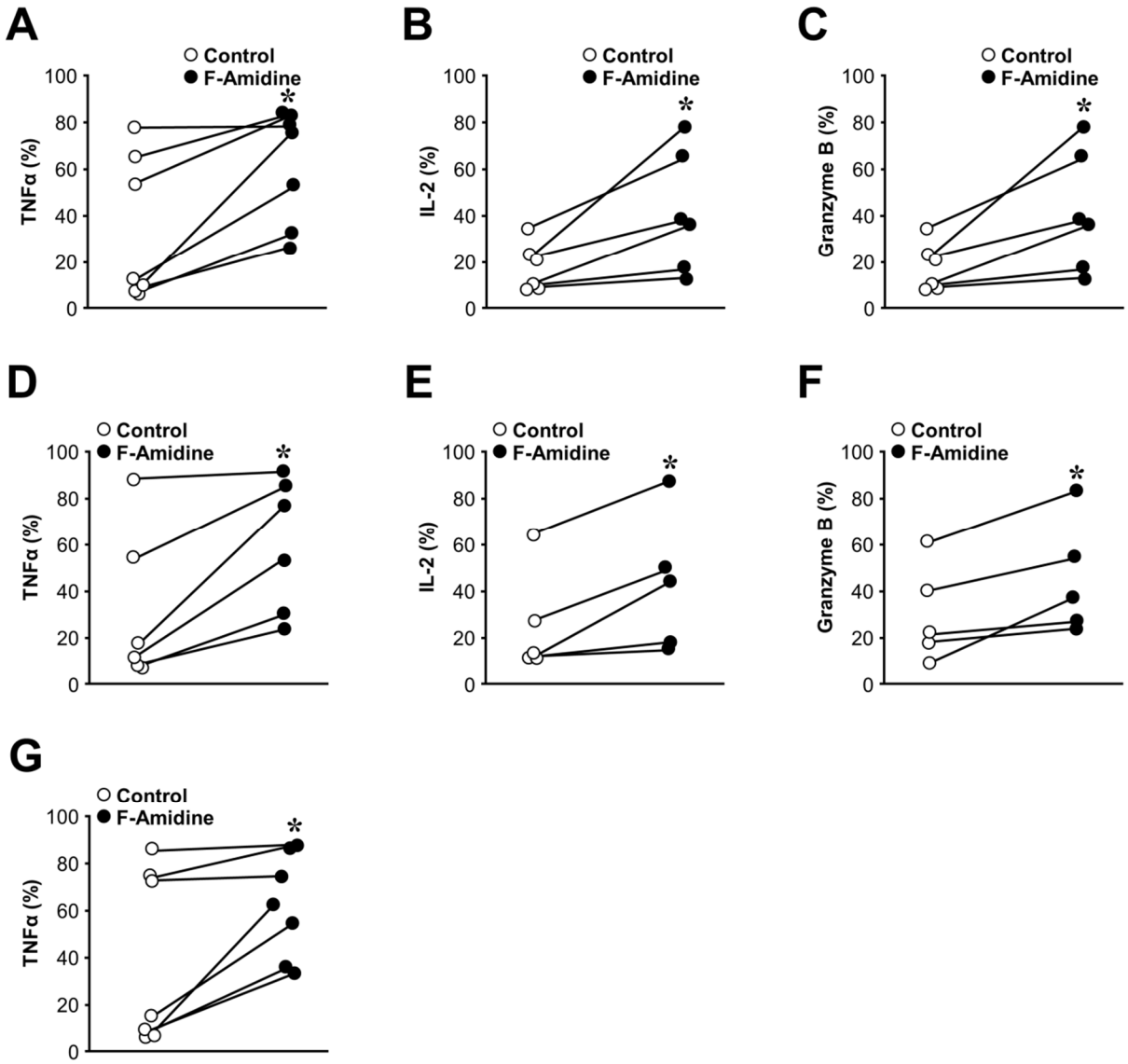

Figure 4.5: F-Amidine treatment leads to increased effector cytokine production in naïve T cells. $CD3^+$ $CD45RA^+$ naïve T cells were sorted from fresh healthy human donor PBMC's and treated with CD3/CD28 Dynabeads (1:20), IL-7 and IL-15 (5ng/ml), and F-Amidine (10uM) for 14 days. Cytokines were detected by flow cytometry after gating for FSC-SSC and viability dye (A-C), FSC-SSC and viability dye-CD4 (D-F), FSC-SSC and viability dye-CD8+ (G) (*$p<0.04$).

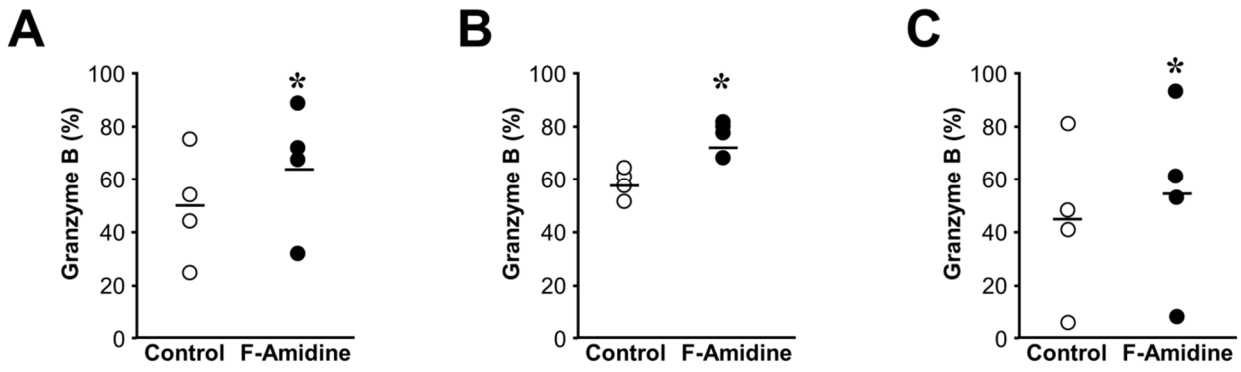

Figure 4.6: F-Amidine treatment increases Granzyme B production in mouse T cells. $CD3^+$ T cells were isolated from mouse lymph nodes and activated with plate bound CD3 and soluble CD28 antibodies and IL-7 and IL-15 for 7 or 14 days. Flow cytometry was performed to analyze Granzyme B cytokine production, with FSC-SSC-viability dye gating-BrdU+ (A-C), FSC-SSC-viability dye gating-BrdU+CD4+ (B), and FSC-SSC-viability dye gating-CD4+ (C) (*$p<0.05$).

4.4c: F-Amidine treatment in T cells leads to increased proliferation

Since Pad4 -/- mice had an increase in proliferation of hematopoietic progenitor cells (Nakashima et al., 2013), we looked at proliferation of T cells. Upon treatment with F-Amidine, and thus PAD inhibition, we observed an increase in BrdU incorporation suggesting an increase in proliferation. This was seen for total $CD3^+$ (Figure 4.7A) and for $CD8^+$ and $CD4^+$ memory T cells (Figure 4.7B and C, respectively). We also confirmed this in mouse T cells from the lymph nodes (Figure 4.7D-E). All in all, PAD4 inhibition increases proliferation in activated T cells.

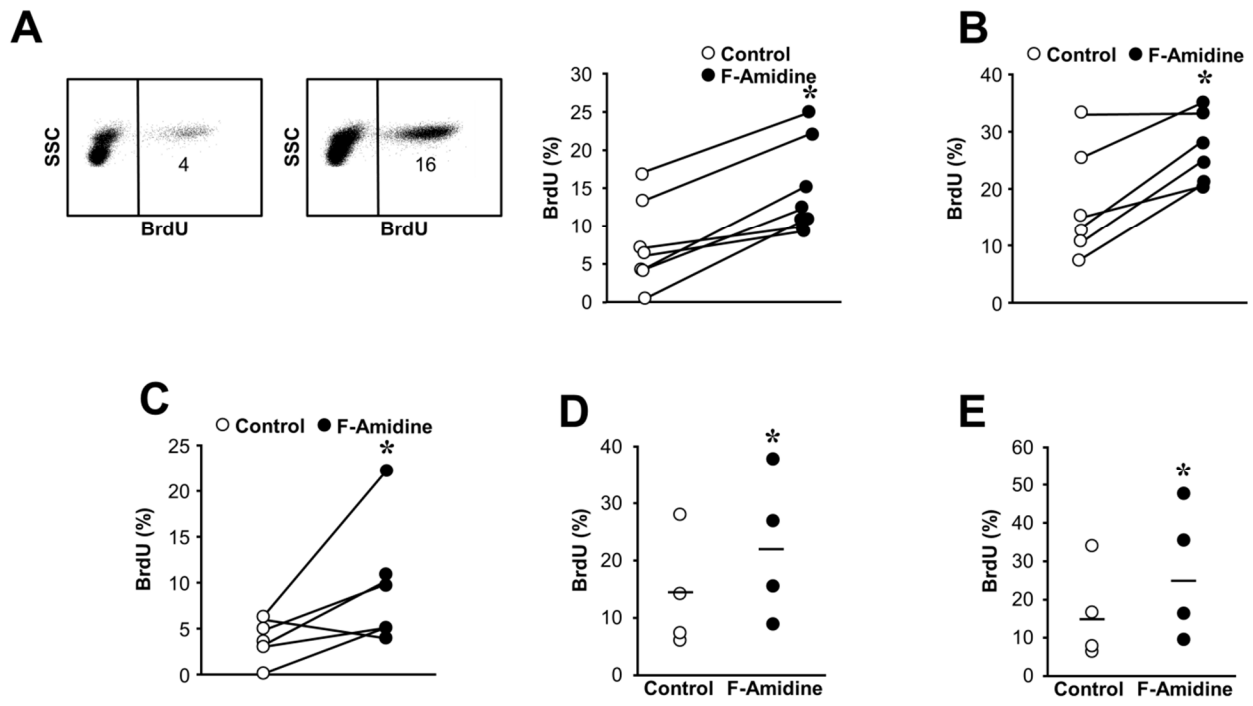

Figure 4.7: F-Amidine treatment in T cells leads to increased proliferation. F-Amidine treatment increases BrdU incorporation. $CD3^+$ $CD45R0^+$ memory T cells treated with plate bound CD3, soluble CD28, and F-Amidine (10uM) for 3-5 days. Flow cytometry was performed to analyze BrdU incorporation, with FSC-SSC-viability dye gating-BrdU+ gating (A), FSC-SSC-viability dye gating-BrdU+CD8+ gating (B), and FSC-SSC-viability dye gating-BrdU+CD4+ gating (C). Representative FACS data are shown (A, left). (D, E): F-Amidine treatment increases BrdU incorporation in mouse T cells. CD3+ T cells were isolated from mouse Lymph nodes and activated with plate bound CD3 and soluble CD28 antibodies and IL-7 and IL-15 for 7 or 14 days. Flow cytometry was performed to analyze BrdU incorporation, with FSC-SSC-viability dye gating-BrdU+ (*$p<0.05$).

4.4d PAD4 inhibition in T cells results in a pro-survival effect

Finally, we observed that the drug F-Amidine confers a protective effect on T cells. This is in agreement with the literature on the survival and antiapoptotic effect of PADs in Jurkat cells and mouse thymus (Hsu et al., 2014, Liu et al., 2006, Tanikawa et al., 2009). Initially, we saw that spontaneous apoptosis induced by T cell activation was reduced in F-Amidine treated T cells (Figure 4.8A). When we used an apoptosis inducing agent, cisplatin, we saw that upon T cell activation and treatment with cisplatin, primary human memory T cells had less Annexin V staining compared to untreated controls (Figure 4.8B). Thus, PAD4 inhibition serves as a general pro-survival function in T cells. We also used blood from ovarian cancer patients. Similar to healthy donor blood, T cells from cancer patients survived better when treated with F-amidine and cisplatin (Figure 4.8C). In concordance with the pro-survival effects of F-Amidine on T cells, we saw that proapoptotic genes Bak1, Bax, and Bim were down-regulated with F-Amidine treatment (Figure 4.8D-F)

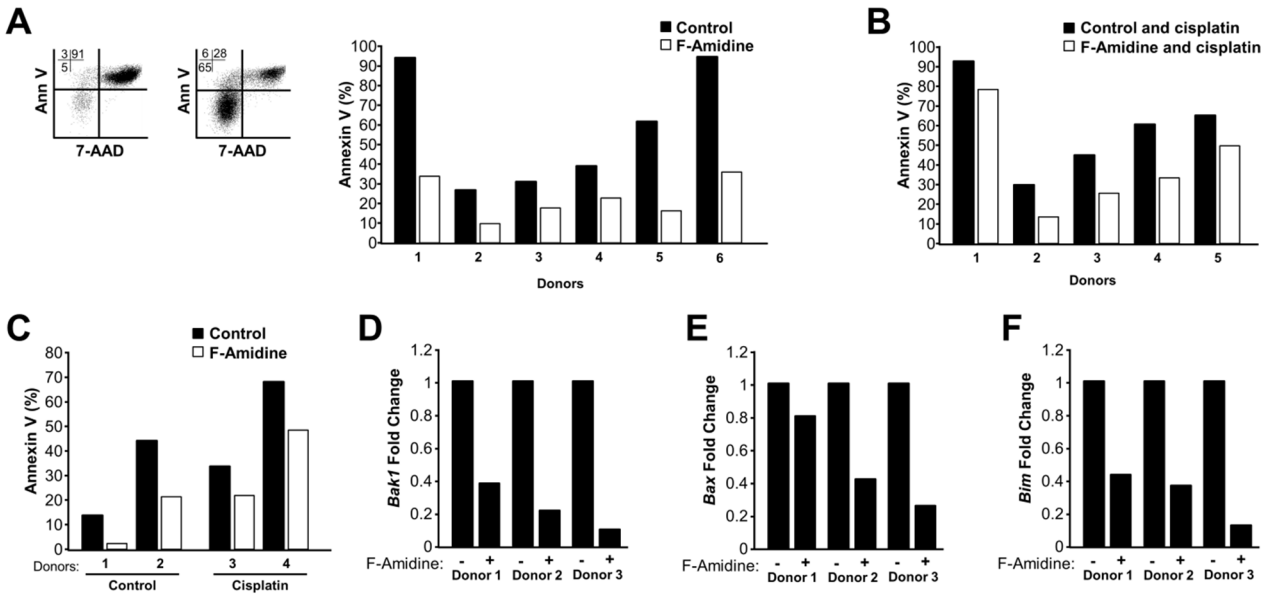

Figure 4.8: PAD4 inhibition in T cells results in a pro-survival effect. A: Spontaneous apoptosis decreases with F-Amidine treatment. Primary CD3 CD45R0$^+$ T cells were activated with soluble CD3 and plate bound CD28 and F-Amidine. Spontaneous apoptosis was detected by Annexin staining via flow cytometry. Representative image shown (left). (B): Cisplatin-induced apoptosis is reduced with F-Amidine treatment. Primary CD3 CD45R0$^+$ T cells were activated with soluble CD3 and plate bound CD28 and F-Amidine (10uM) for 7 days and cisplatin (10uM) was added the last two days. Apoptosis was detected by Annexin staining via flow cytometry. (C): Ovarian cancer blood was activated with CD3 and CD28 with F-Amidine and with or without Cisplatin. Apoptosis was detected by Annexin V staining. (D-F): Primary CD3$^+$ T cells were activated for 7 days and collected for Bak1, Bax, and Bim mRNA detection by RT-PCR.

4.5 Discussion

Adoptive T cell therapy for cancer treatment is becoming well accepted (June, 2007, June et al., 2015). In particular, effector T cells: cytotoxic $CD8^+$ T cells and $CD4^+$ T helper type 1 cells are cultured and activated *ex vivo* and transferred back into patients as these cells directly kill the tumor through apoptosis-inducing molecules or cytotoxic granules. Recent evidence has shown that *in vitro* expansion of T cells with modulating AKT and glycolysis signaling can greatly improve their antitumor properties *in vivo* (Sukumar et al., 2013, Sukumar et al., 2016, Crompton et al., 2015). This project was designed to find other drugs that could be used *in vitro* on T cells to improve T cell stemness properties such as survival, persistence, and effector function. We found that F-Amidine enhances T cell stemness properties, including survival, persistence, and effector function, and could be added to the *ex vivo* T cell culture system. We also demonstrate that PAD4 inhibition has a pro-survival effect in ovarian cancer patient blood cells and this could augment T cell survival when reintroduced into patients. To further explore this option, we will use adoptive T cell mouse models and transfer F-Amidine treated T cells into tumor bearing mice. We will examine tumor burden as well as stemness properties of the T cells upon transfer *in vivo*.

We found that PAD4 inhibition, through F-Amidine, increases T cell: cytokine production, proliferation, and survival, upon activation. This project is still at its exploratory stage and how PAD4 regulates T cell effector function, survival, and proliferation is still unknown. PAD4 seems to be affecting many characteristics of T cell function and probably affects a crucial regulator such as IL-2. IL-2 is dramatically increased with PAD4 inhibition (more pronounced than the other effector cytokines: IFNγ, TNFα, and Granzyme B). IL-2 signaling is

important for T cell proliferation, survival, and cytokine release, explaining all of the phenomena we see (Boyman and Sprent, 2012). Also, like IL-2, PAD4 is induced very early on in T cell activation. I am currently testing this hypothesis and exploring how exactly could PAD4 regulate IL-2 protein expression. The mechanism could be through histone-mediated regulation of IL-2 directly or of key IL-2 regulating protein(s).

PAD4 can bind to various chromatin modifiers, such as histone deactelyase 2 (HDAC2) and other transcription factors, and regulate IL-2 production (Li et al., 2010a). Indeed, nucleosome remodeling is needed for transcription factor accessibility and IL-2 expression upon T cell activation (Kim et al., 2006). In this case, we would hypothesize that PAD4 acts as a repressor of IL-2 expression with other repressors such as HDAC2. To test this hypothesis, we would perform ChIP assays for PAD4, citrullination, and histone marks on the IL-2 promoter as well as use various epigenetic drugs to see what other chromatin modifiers are involved and regulate IL-2 expression with PAD4. The other possibility is PAD4 could regulate key IL-2 regulators. IL-2 expression induced by T cell activation is regulated by critical transcription factors such as NFAT, AP-1, and NF-kB (Kim et al., 2006) and PAD4 could regulate their expression or function through PTM's. We would test this by looking at the critical transcription factors' expression after F-Amidine treatment and T cell activation. However, we cannot rule out that PAD4 may also play an important part in T cell activation through other mechanisms, like p53. Nonetheless, PAD4 function is a critical regulator of basic T cell biology and activation.

4.6 References

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