PRO- AND ANTI-TUMOR IMMUNITY WITHIN A MOUSE MODEL OF
SPORADIC COLORECTAL CANCER

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Steven Vincent Davies
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The Undersigned Faculty Committee Approves the

Thesis of Steven Vincent Davies:

Pro- and Anti-Tumor Immunity within a Mouse Model of Sporadic Colorectal Cancer

Kathleen L. McGuire, Chair
Department of Biology

Constantine D. Tsekas
Department of Biology

Mee Young Hong
School of Exercise and Nutritional Sciences

5/11/12
Approval Date
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DEDICATION

This thesis is dedicated to first and foremost to my paternal grandmother, Claudia Davies. I would not be here today if it were not for all your support. I would also like to dedicate this thesis to Xiaoxiao Li for all of her support for the duration of my Masters studies. I look forward to sharing the rest of my life with you. This thesis is also dedicated to my family including my father Steve, mother Teri, Adam, and Alyssa. Thank you for all your support. Finally, I would like to dedicate this thesis to my advisor, Dr. Kathleen McGuire. Your support, patience, and understanding during this project made my time at San Diego State a wonderful experience which I will never forget.
In scientific work, those who refuse to go beyond fact rarely get as far as fact.

-Thomas Huxley
ABSTRACT OF THE THESIS

Pro- and Anti-Tumor Immunity within a Mouse Model of Sporadic Colorectal Cancer
by
Steven Vincent Davies
Master of Science in Biology with a Concentration in Molecular Biology
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Colorectal cancer (CRC) is the 2nd leading cause of cancer death in the western world. There are few mouse models which accurately recapitulate the sporadic, colon-specific tumor formation characteristic of the majority of human colorectal tumors without sacrificing normal immune function. Mutation in a tumor suppressor gene known as APC is often a first event leading to spontaneous development of human CRC. Recently, a single allele APC conditional knockout mouse (CAC;APC580D+/+) has been generated that forms sporadic CRC specifically in the large intestine. The CAC;APC580D/+ mice were sacrificed at varying time points to allow for the study of the progression of tumor formation within the context of a normal immune response over time. Recent studies have indicated that human CRC patients that have a strong cytotoxic immune response within the tumor microenvironment have a better overall prognosis than those who do not have a strong cytotoxic immune response. Epidemiological studies have shown an inverse correlation between blood serum vitamin D levels and incidence of CRC which may be explained by vitamin D’s immunomodulating effects. In this study, 41 CAC;APC580D/+ FFPE tumor samples were acquired from the laboratory of Dr. James Fleet at Purdue University. Subject groups were mice receiving no treatment, 2% DSS, or 2% DSS + vitamin D. Dextran sodium sulfate (DSS) causes inflammation in the colon and exacerbates the development of CRC in mice. Samples were cut in 5 μm sections, immunohistochemically stained for CD3 and granzyme B, photographed at 10X objective lens magnification, and quantitatively assessed. Preliminary results suggest that administration of the inactive form of vitamin D3 has no statistically significant effect on intratumoral CD3+ or granzyme B+ cell infiltrates; however there is an increase in granzyme B+ cell density up to 150 IU of vitamin D dosage. The results also suggest that mice at 10 weeks of age have higher intratumoral CD3+ and granzyme B+ cell densities as compared to the later time points, possibly indicating tumor evasion of the immune response over time. The results reported herein provide evidence that the CAC;APC580D/+ mouse is an appropriate model for studying and understanding not only the progression of sporadic CRC but also the tumor’s interaction with and evasion of the immune response.
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CHAPTER 1

INTRODUCTION

Few mouse models of sporadic colorectal cancer exist which accurately recapitulate the human condition. In this study, a recently developed mouse model which forms sporadic colorectal adenomas specifically in the large intestine is used to study the evolution of the immune response to the adenomas over time. The anti-tumor immune response is now widely known to result in a better prognosis among human individuals. It is now important for researchers to elucidate the effects and mechanisms varying treatments have on modulating the immune response in order to take advantage of our body’s intrinsic host defense systems to target and eradicate tumorigenic cells.

1.1 COLORECTAL CANCER STATISTICS

Colorectal cancer (CRC) is the second leading cause of cancer death in many industrialized nations including the United States [1]. It is the third most common malignancy in the world with approximately 1,000,000 new cases and 500,000 deaths per year [2]. An estimated $8.4 billion is spent on the treatment of CRC each year in the United States alone. [3]

1.2 ORIGINS OF COLORECTAL CANCER

CRC arises from progressive mutations or epigenetic expression changes in tumor suppressor genes, oncogenes, or mismatch repair genes and can be spontaneous or familial in origin [4]. The majority of spontaneous CRC cases result from chromosomal instability which is characterized by an allelic imbalance within several chromosomal loci; whereas a minority of sporadic CRC cases results from genetic or epigenetic changes in mismatch repair genes. Most cases of CRC begin with benign adenomatous polyps protruding into the colonic lumen. If left untreated, these polyps will form progressive mutations in genes such as KRAS and p53 leading to the formation of advanced carcinoma and subsequent metastasis (Figure 1.1) [5]. Risk factors for spontaneous CRC development include increasing age, male sex, chronic inflammatory bowel disorders (Crohn’s disease and ulcerative colitis), as well
as environmental factors such as the consumption of red-meat, a high-fat diet, low fiber intake, obesity, sedentary lifestyle, smoking, diabetes, and a high consumption of alcohol. [6]

An estimated 15-20% of CRC cases result from a familially inherited germline mutation in tumor suppressor genes or mismatch repair genes [7]. The two most common and well-understood hereditary CRC disorders are Lynch syndrome and Familial Adenomatous Polyposis (FAP). Lynch syndrome is characterized by mutations in mismatch repair genes, mainly in hMSH2 and hMLH1, resulting in nucleotide insertions or deletions at microsatellite sites following DNA replication. FAP is characterized by a germline mutation in the Adenomatous Polyposis Coli (APC) gene which is part of the WNT signaling pathway and encodes a tumor suppressor protein that is vital for the regulation of cell division. [8]

Microsatellites are characterized by short tandem nucleotide repeats and are hotbeds for errors during DNA replication. Normally, the mismatch repair (MMR) system will identify and repair nucleotide insertions-deletions (IDLs) at these sites; however individuals with mutations in their MMR system will not recognize such errors resulting in frameshift mutations in a number of genes (including proto-oncogenes and tumor suppressors) which may result in tumorigenesis [9]. In mammals, the initiation of repair of single base pair mismatches as well as IDLs are conducted by the MSH2-MSH6 (MutSα) complex; whereas the initiation of repair of 2-4 base pair matches or IDLs are conducted by the MSH2-MSH3 (MutSβ) complex. The MutS complex then works in conjunction with the MutL and MutH complexes to accomplish DNA mismatch repair. Since the MLH1 protein is found in all

three of the MutL homologues, approximately 50% of MMR CRCs occur as a result of hypermethylation of the MLH1 promoter region leading to microsatellite instability (MSI) and subsequent frameshift mutations in any number of possible proto-oncogenes or tumor suppressor proteins. [9]

1.3 Mouse Models of Colorectal Cancer

Studies involving the induction of colon and intestinal cancer in mice have a long history dating back approximately 80 years to Lorenz et al. who first reported tumorigenesis in the intestine and forestomach of mice following feeding with methylcholanthrene, a polycyclic aromatic hydrocarbon [10]. Subsequent studies further validated the role of environmental factors such as various chemicals found to be carcinogenic. As the understanding of these chemical carcinogens improved, researchers discovered that certain chemicals could induce tumorigenesis in organ-specific regions of the mouse. In a 1963 population study by Laqueur et al., Guamanians who ingested cycad flour displayed a higher incidence of tumor progression in the colon and intestinal tract [11]. These observations were applied to rats in which large amounts of cycad flour were introduced into their diet resulting in a high incidence of colon cancer. Further studies of the toxicity of cycad flour showed that it contained cycasin, which is a form of methylazoxymethanol (MAM). [12]

Following these studies, numerous other carcinogenic chemicals have been discovered but none are more utilized than azoxymethane (AOM) which is a known carcinogen causing random genetic mutations within the epithelial cells lining the gastrointestinal tract [12, 13]. AOM is usually administered to mice in conjunction with a non-genotoxic compound known as dextran sulfate sodium (DSS) to promote gastrointestinal inflammation resulting in an increased incidence of intestinal tumorigenesis [13]. While the combined administration of AOM/DSS results in effective intestinal tumor formation, the type and number of genetic mutations leading to neoplasia is highly variable and cannot be controlled in order to effectively mimic the specific types of mutations leading to CRC formation within the human population. [12]

Numerous mouse models have been produced to mimic human tumor progression, but do not exhibit all of the phenotypic characteristics of the human disease. Most importantly, deaths from human colon cancer are usually associated with invasive tumors
and metastasis to the liver, lungs, and lymph nodes. Most mouse models of CRC utilize mutations in the Wnt, K-ras, and mismatch repair associated proteins which do not show the aggressive behaviors of invasion and metastasis as seen in human CRC. Therefore, models utilizing grafts of human tumor cells into Nude or SCID mice are beneficial in understanding the mechanisms underlying tumor invasion and metastasis. While grafting tumor cells into immunocompromised mice allows for the *in vivo* study of tumor growth, metastasis, and chemotherapeutic interventions; it is not useful for studying tumor progression within the context of a normal immune response which is lacking in the Nude and SCID mouse models.

Numerous mouse models which attempt to recapitulate MMR-deficient CRC have been generated with mutations in the known components of the MMR system including the genes encoding MSH2, MSH3, MSH6, MSH4, PMS1, and PMS2. While MMR knockout mouse models do resemble human MMR-deficient CRC in that they develop gastrointestinal tumors, there are some differences in the phenotypic characteristics of the MMR-deficient mice when compared to MMR-deficient humans [1]. For example, single allele knockouts in *MSH2*, *MSH6*, and *MLH1* do not result in early-life tumorigenesis as seen in humans with homologous mutations. Further, MMR double-allele knockout mice usually succumb to aggressive lymphomas before developing recognizable gastrointestinal tumors making the study of CRC tumorigenesis in these mice difficult. The differences in MMR-deficient mice as compared to MMR-deficient humans most likely arise from the mouse’s shorter lifespan making them less prone to somatic loss of the remaining wildtype allele which is required for tumorigenesis. Recently, a *Cdx2-NLS-cre* conditional MSH2 knockout mouse has been generated which forms tumors solely in its large intestine. Conditional mouse models such as this are required to better model human MMR-deficient tumorigenesis. [1]

### 1.4 APC and WNT Signaling

A unique feature of CRC is that a mutation in the *APC* gene is found in >80% of human sporadic CRC cases [14]. The *APC* gene is evolutionarily conserved and present in both humans and mice. It resides on chromosome 5q in humans and chromosome 18 in mice. It encodes a large protein, 2850 amino acids, with several domains responsible for a variety of functions vital to cellular maintenance and control [15]. As shown in Figure 1.2 [16], the
APC protein forms a complex with axin and helps glycogen synthase kinase-3β (GSK-3β) to phosphorylate N-terminal serine/threonine residues of β-catenin effectively accelerating its rapid degradation through ubiquitination [17]. Truncation mutations of the C-terminal end of the APC protein prevents GSK-3β from phosphorylating β-catenin leading to its accumulation within the cytoplasm. As stabilized β-catenin accumulates, it translocates to the nucleus and activates the Wnt gene targets T cell factor (TCF)/LEF transcription factors initiating aberrant cellular growth and division. [1]

**Figure 1.2. WNT signaling and the role of the APC protein. Mutated APC results unchecked cellular proliferation and adenoma formation. Source: Eisenmann D (2005) Wnt signaling. WormBook 1: 1-17.**

**1.5 APC MUTANT MICE**

Since the mouse APC protein shares 90% amino acid homology with the human APC protein, numerous attempts have been made to recapitulate the human CRC condition through the use of mouse models containing various APC-specific mutations [18]. The first APC mutant mouse was discovered in a colony of randomly mutagenized mice. Genotypic analysis of this mouse revealed a truncation mutation at codon 850 on the APC gene. Mice with this mutation were identified as having multiple intestinal neoplasms (MIN) prompting
investigators to refer to these as \( \text{APC}^{\text{min}} \) mice. Further targeted gene knockouts produced truncation mutations at codons 716 and 1638 giving rise to \( \text{Apc}^{\Delta716} \) and \( \text{Apc}^{\Delta1638} \) mice, respectively. Although both of these mutations produce benign polyps in both mice within the small intestine, they differ in the number of polyps with \( \text{Apc}^{\Delta716} \) mice produced ~300 polyps while \( \text{Apc}^{\Delta1638} \) mice produced ~3 polyps on the same C57BL/6J background [19].

The conventional mouse \( \text{APC} \) gene knockouts do not accurately recapitulate human CRC cases since the majority of human CRC cases result in polyps developing in the large intestine while the majority of murine \( \text{APC} \) knockout models result in polyps developing primarily in the small intestine. Furthermore, the majority of \( \text{APC} \) single-allele full knockout mouse models show defects in proper cellular maintenance and control leading to progressive loss of immature and mature T cells, splenic natural killer cells, immature B cells, and B progenitor cells in the bone marrow making these mice a poor model for immune response studies. [1]

1.6 \( \text{APC}^{580D/+} \) Mice

Homozygous mutation of the \( \text{APC} \) gene is embryonically lethal requiring single \( \text{APC} \) allele mutations and loss of heterozygosity of the remaining allele for sporadic tumor formation to occur. These single allele knockout mice are effective models for sporadic tumorigenesis [20]. Through homologous recombination, Shibata et al. generated a mouse with loxP sites flanking exon 14 of a single \( \text{APC} \) allele resulting in an \( \text{APC}^{580S} \) mouse [21]. Floxed genes require the expression of a Cre recombinase protein in order to delete the gene region flanked by the loxP sites. Recently, Xue et al. have generated a mouse model which expresses a \( \text{Cre recombinase} \) (Cre) gene downstream of the carbonic anhydrase I (CAC) gene promoter. The \( \text{CAC} \) gene is expressed solely in the large intestine allowing for the colon-specific inactivation of a single allele of the \( \text{APC} \) gene. The \( \text{CAC};\text{Cre} \) mouse was crossed with an \( \text{APC}^{580S/580S} \) mouse resulting in a \( \text{CAC};\text{APC}^{580D/+} \) mouse with the 580D representing a frameshift mutation at codon 580 within the \( \text{APC} \) gene. These mice developed colon-specific, single allele \( \text{APC} \) mutations resulting in adenomatous polyp formation within 10 weeks of age. [22]

Histological examination of the large intestine of these mice revealed microadenomas and adenomatous polyps located primarily in the distal colon. No gross abnormalities or
lesions consistent with tumor formation were found in any other organs examined. The addition of 2% DSS resulted in a 66.7% incidence of tumor formation as compared to 20% within the non-DSS group. Within the CAC;APC<sup>580D/+</sup> mice, the accumulation of β-catenin and the increase in Ki-67-stained cells outside the crypt base were apparent only after obvious changes in the colonic epithelial cell morphology took place suggesting loss of heterozygosity of the remaining wildtype APC allele was necessary for adenoma formation to occur (Figure 1.3) [22]. These observations provide evidence that sporadic adenoma formation is occurring solely in the large intestine in a manner similar to human sporadic CRC and is dependent upon APC inactivation. The CAC;APC<sup>580D/+</sup> mouse model is the first which limits adenoma formation to the large intestine and will be used in our study to characterize the immune response to these tumors over time. [22]

### 1.7 Role of Adaptive Immunity in Colorectal Cancer

Previous studies have provided mounting evidence that the immune system is capable of recognizing and mounting a cytotoxic response to colorectal tumors leading to the destruction of tumor cells and an overall better patient prognosis [23, 24, 25]. Evidence to support this proposition derive from observations that Th1-mediated cytotoxic cell infiltration of the tumor microenvironment leads to an increased probability of disease-free survival in human patients [26]. Furthermore, there are over 10 tumor associated antigens as well as 35 major histocompatibility complex (MHC) class I epitopes related to CRC indicating that various antigens exist which give rise to the activation of cytotoxic T lymphocytes (CTLs). Also, many CRC tumors (~73%) undergo partial or complete loss of their MHC class I complexes which is an effective strategy to evade the immune system’s cytotoxic response [24]. In order for colorectal tumors to escape the host immune response, numerous alterations of the tumor’s genetic and cellular expression profiles must occur to shift the balance of the immune microenvironment away from a cytotoxic immune response which would then be more conducive to tumor growth and progression. [25, 27]

A tumor immune microenvironment can be characterized as either pro- or anti-tumoral depending on the types of T helper cells and cytokines present [23]. A T helper 1
Figure 1.3. Ki-67 negative and cytoplasmically-localized β-catenin in normal cells (N) as compared to ki-67 positive and nuclear-localized β-catenin in microadenoma (M) and adenoma tissues. Source: Xue Y, Johnson R, DeSmet M, Snyder PW, Fleet JC (2010) Generation of a transgenic mouse for colorectal cancer research with intestinal cre expression limited to the large intestine. Mol Cancer Res 8: 1095-1104.

(Th1) immune response is characterized by the activation of CD8+ CTLs and the presence of Th1-related cytokines such as interleukin-12 (IL-12), interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) [23]. CTLs are the primary effector cell of the Th1 immune response and are activated by antigen presenting cells expressing non-self antigen on their class 1 major histocompatibility complex (MHC) cell surface proteins. Upon activation by the class 1 MHC, CTLs directly target and bind cells expressing the same non-self antigen on their class 1 MHC complex in order to release perforin and granzyme B directly into the target cell. Perforin acts to form a pore in the target cell’s membrane allowing for directed entry of the serine protease, granzyme B. Granzyme B is a granular serine protease which cleaves caspase 3 upon entry into the target cell causing programmed cell death [28]. It is
now widely accepted that activated tumor-specific CTLs play an important role in the host anti-tumor immune response allowing for the detection and destruction of tumorigenic CRC cells. [26, 27]

A T helper 2 (Th2) immune response is characterized by the expression of cytokines IL-4, IL-5, IL-10, and IL-13 and is primarily involved in IgE antibody production, eosinophil recruitment, and clearance of parasites [29]. Th2 immune responses are largely responsible for clearing various extracellular pathogens such as parasites, bacteria, and viruses but have no effect on intracellular pathogens or stressed/damaged cells. Therefore, a shift to a Th2-dominated tumor immune microenvironment has been described as potentially pro-tumoral in that a Th2 immune response does not actively target and destroy tumorigenic cells [23]. While Th1 and Th2 cells are both found in the tumor microenvironment, the dominance of a particular T helper subtype may determine whether the immune response is pro- or anti-tumoral which has important consequences for disease-free survival. [23, 26]

Another T helper cell subset involved in tumor immunity is known as the T helper 17 (Th17) cells which produce a number of cytokines (TNF, IL-1, IL-6, granulocyte colony-stimulating factor, and granulocyte-macrophage colony stimulating factor) and chemokines (CXCL1, CXCL5, IL-8, CCL2, and CCL7) resulting in the recruitment of various inflammatory monocytes and neutrophils to the area of interest [30]. Intratumoral inflammation can have a paradoxical role in that it can be either pro- or anti-tumoral. Inflammation is required for immune-mediated, tumor-specific recognition and destruction of tumorigenic cells; however, it can also be detrimental to the host in that it can promote an inflammatory microenvironment which is not conducive to active tumor recognition and subsequent cytotoxicity. Furthermore, chronic inflammation can lead to an increased incidence of CRC as evidenced by the observation that individuals suffering from inflammatory bowel disease have a greater than 20% chance of developing CRC within 30 years of disease onset [31]. Not only can chronic inflammation lead to a poorly organized anti-tumor immune response, it can also lead to DNA damage through the macrophage- and neutrophil-mediated production of reactive oxygen species and reactive nitrogen intermediates. [32]

The cell membrane protein, CD3, is found on all T cells and associates with the T cell receptor. It is involved in T cell signal transduction upon T cell receptor binding to its
cognate antigen [33]. In this study, we utilize CD3 as a marker of T cells while using granzyme B to assess the anti-tumor cytotoxic immune response. An increase in the CD3:granzyme B ratio indicates increased intratumoral T cell inflammation without a concomitant increase in anti-tumor cytotoxic activity.

1.8 COLORECTAL CANCER AND INNATE IMMUNITY

In order for cells of the adaptive immune response to become activated, members of the innate immune response such as macrophages need to be activated. While the majority of tumor immunity studies have focused on the role the adaptive immune response plays in tumor immunity, it is also necessary to evaluate the roles macrophages play. The roles macrophages play in tumor immunity is paradoxical in that some macrophages (M1 macrophages) will present tumor antigens and produce IL-12 leading to the activation of the Th1 cytotoxic response whereas other macrophages (M2 macrophages) will produce TGFβ and IL-10 resulting in the promotion of Th2 immune responses, angiogenesis, tissue remodeling, and a general suppression of anti-tumor activities. [34]

Natural killer (NK) cells are members of the innate immune system and can recognize tumor cells through an MHC-independent manner. This is important since many cancer cells down-regulate their MHC molecules in order to avoid detection by cytotoxic T lymphocytes. NK cells kill by two mechanisms including granule-dependent cytotoxicity which is mediated by perforin and granzyme B and the triggering of an apoptosis pathway in the target cell through the expression of TNF-related apoptosis-inducing ligand (TRAIL) or Fas ligand (FasL). NK cells can recognize their targets through three primary mechanisms including the recognition of pathogen-encoded molecules, self-proteins that are up-regulated in transformed or infected cells, and self proteins that are expressed by normal cells but down-regulated in infected or transformed cells. While tumor cells have the capacity to evade apoptosis by NK cells, they are a potent weapon used by the immune system to detect and destroy cancerous cells. [35]

1.9 PHYSIOLOGICAL PRODUCTION OF VITAMIN D

Vitamin D is a fat-soluble secosteroid hormone which plays a role in a variety of cellular processes in humans. Exposure to sunlight is the main mechanism by which humans obtain vitamin D. Skin exposure to ultraviolet B sunlight within the wavelength range of 290
- 315 nm results in the photochemical conversion of 7-dehydrocholesterol to pre-vitamin D₃. Body heat then isomerizes pre-vitamin D₃ to vitamin D₃ which is then transported to the liver (bound by the vitamin D binding protein) where the enzyme, 25-hydroxylase, produces 25-hydroxyvitamin D₃ or 25(OH)D₃ [36]. The 25(OH)D₃ form of vitamin D is then transported to the kidneys where it is once again hydroxylated by the enzyme CYP27B1 to form the physiologically active form of vitamin D (1,25(OH)₂D₃) [37]. Here, the term vitamin D will refer to the physiologically active form of vitamin D unless otherwise noted. The vitamin D binding protein acts as a chaperone to transport vitamin D throughout the blood. Upon the transportation of vitamin D across a target cell’s membrane, the vitamin D binding protein is released. Once vitamin D enters the cytoplasm of the cell, it binds to the cytoplasmically-localized vitamin D receptor. Upon ligand binding, the vitamin D receptor forms a heterodimer with the retinoid x receptor and translocates to the nucleus. This heterodimer acts as a transcription factor binding to various vitamin D response elements which in many cases results in the inhibition of immune or cancer cell proliferation. [38]

1.10 Serum Vitamin D Sufficiency

Serum 25(OH)D₃ level is the best indicator of vitamin D status. While controversial, many investigators and health professionals have agreed that serum vitamin D levels within the range of 120-225 nmol/L is sufficient to avoid health problems associated with vitamin D deficiency [39]. In humans, a vitamin D serum level below 80 nmol/L is considered deficient while levels greater than 500 nmol/L have been associated with toxicity. Normal vitamin D levels can be accomplished by the dietary supplementation of 1,000-2,000 IU of vitamin D per day [40]. Studies involving vitamin D toxicity in mice have shown that serum vitamin D levels (25(OH)D₃) above 2500 nmol/L result in hypercalcemia and other pathological symptoms which result from high calcium/potassium levels [41]. Serum vitamin D levels of the mice used in this study are shown in Figure 1.4 [37] which illustrates that the mice used in this study have normal serum vitamin D (25(OH)D₃) levels.

Numerous epidemiological studies have shown an inverse correlation between serum vitamin D (25(OH)D₃) levels and cancer incidence/mortality. A profound inverse correlation was recently established in the National Health and Nutrition Examination Survey (NHANES) III cohort in which individuals with serum vitamin D levels of >80 nmol/L had
Figure 1.4. Serum vitamin D (25(OH)D$_3$) levels of the CAC;APC$^{580D/+}$ mice at varying vitamin D diet groups. Source: Fleet JC, DeSmet M, Johnson R, Li Y (2012) Vitamin D and cancer: a review of molecular mechanisms. J Biochem 441: 61-76.

approximately ¼ the risk of CRC mortality as compared to vitamin D deficient (<50 nmol/L) individuals. [42]

1.11 CRC, IMMUNITY, AND VITAMIN D

Vitamin D has long been known to be involved in mineral homeostasis, bone and skin metabolisms, cellular proliferation including cancer progression, and regulation of the endocrine system. With regard to CRC, vitamin D has been shown to reduce tumor angiogenesis and promote the inhibition of cellular proliferation. Vitamin D can suppress the proliferation of cancerous cells through various mechanisms including the induction of G1 cycle arrest, the interference of growth factor release, and the induction of apoptosis [43]. Increasing evidence has suggested that vitamin D plays an important role in the modulation of the immune system. Evidence to suggest vitamin D’s role in the regulation of the immune
Many immune cells such as Th1, Th2, Th17, B cells, neutrophils, macrophages, and dendritic cells express the vitamin D receptor and appear to be affected by its administration both \textit{in vivo} and \textit{in vitro}. Within neutrophils and macrophages, vitamin D appears to increase chemotaxis and phagocytosis while decreasing the expression of toll-like receptors 2 and 4 (TLR-2 and -4) resulting in a hyporesponsiveness to pathogen associated molecular patterns in order to prevent excessive activation and inflammation at the later stages of infection. Other studies have provided evidence that vitamin D administration decreases B cell differentiation and proliferation of their associated antibodies [45]. Furthermore, vitamin D administration appears to increase the suppressive activity of T regulatory cells while increasing the anti-inflammatory cytokine, interleukin-10 (IL-10). [46]

Studies involving the effect of vitamin D administration on T helper cells suggest that vitamin D administration tends to suppress the production of the Th1 cytokines, IFN-\(\gamma\) and IL-12, while increasing the production of Th2-related cytokines such as IL-4. However, these studies have been conducted primarily \textit{in vitro} and require further verification with an \textit{in vivo} model system [44, 47, 48, 49]. Further evidence to support vitamin D’s role in inhibiting Th1 immunity is shown by vitamin D knockout mice which exhibit an increased production of IFN-\(\gamma\) and have an increased propensity to develop inflammatory bowel disease. [50]

A chronic inflammatory response has recently been considered as an emerging hallmark of cancer [51, 52]. While a Th1-mediated intratumoral immune response has been shown to have protective effects, a Th2 or a Th-17 dominated intratumoral immune response has been characterized as pro-tumorigenic and resulting in a poorer prognosis [25, 53]. Th17 cells secrete IL-17 which is a proinflammatory cytokine that recruits various monocytes and neutrophils to the inflammatory region. IL-17 has also been known to promote angiogenesis through the induction of vascular endothelial growth factor expression. Taken together, these observations denote IL-17 as a pro-tumoral cytokine which encourages tumoral evasion of the immune response and tumor growth [30]. An overview of the various effects vitamin D administration has on the immune system is shown in Figure 1.5. [45]

Recent studies have provided convincing evidence that vitamin D administration results in decreased IL-17 production [54, 55]. A mechanism to explain this process was
Figure 1.5. The various effects vitamin D has on varying immune cell types. Source: Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu G (2010) Vitamin D: modulator of the immune system. Curr Opin Pharmacol 10: 482-96.

postulated by Chang et al. who showed that increased administration of vitamin D led to the expression of C/EBP Homologous Protein which is known to be involved in the post-transcriptional inhibition of IL-17 [56]. The reduction in IL-17 levels could result in a reduced chronic inflammatory response which may explain a mechanism by which vitamin D reduces overall cancer incidence and survival. Together, these studies support the role of vitamin D in regulating directed and efficient clearing of harmful cells/pathogens through the elimination of harmful inflammatory and autoimmune reactions to healthy tissue.

Numerous studies depicting the effect of vitamin D on the immune response have been conducted with varying results. Some studies suggest that vitamin D decreased IFN-γ and IL-17 while increasing IL-4 cytokine production. Still others show no effect of vitamin D administration on IFN-γ or IL-4 production. However, the majority of studies do seem to agree that vitamin D decreases IL-17 production. The variability in these results is most likely explained by the different types of antigenic stimulation and cell types present in
culture. Furthermore, very few studies of this nature have been conducted in vivo which is a much more complex environment than those set up in culture [57]. The few mouse in vivo experiments that have been performed show no effect on IFN-γ production while some human in vivo studies have shown an increase in NK cell cytotoxicity upon increased vitamin D administration [57-61]. These results provide evidence that the in vivo immune environments are much different than those that exist in vitro and more studies are required to fully evaluate the effect of vitamin D administration on the immune response within its native in vivo environment.

1.12 HYPOTHESIS

In this study, we aimed to determine the intratumoral CD3+ and granzyme B+ cell densities over time, in the presence and absence of 2% DSS administration, and at varying levels of vitamin D administration. As previously described, tumors become successful when they are able to effectively evade the immune response. We hypothesize that over time, there will be a decrease in the overall intratumoral granzyme B+ cell density as the tumors find ways to evade the anti-tumor immune response [30]. It is well known that 2% DSS results in increased inflammation throughout the gastrointestinal tract, so we hypothesize that mice receiving DSS will have an increase in the intratumoral CD3+ cell density. [13]

It is also known that vitamin D has immunomodulating effects and therefore could play a role in tumor immunity. It has been observed that individuals who have greater serum vitamin D levels tend to have a decreased incidence/mortality of CRC so we chose to investigate whether vitamin D administration results in an increased anti-tumoral cytotoxic response [42]. From the studies that have been reported so far, there have been numerous conflicting results regarding the effect vitamin D administration has on the immune response. Some studies show either no effect or a decrease in Th1 immunity while others show an increase in the cytotoxic response.

The variation in these results most likely occurs based on the conditions of the experiments as well as the immune stimulating agents used such as PMA/ionomycin, anti-CD3, or various antigenic peptides. Since the studies conducted in vitro show a decrease in Th1 immunity (as evidenced by a decrease in IFN-γ production) while the studies conducted in vivo show an increase in NK cell-mediated cytotoxicity; we hypothesize that the
intratumoral cytotoxic immune response will increase as vitamin D administration increases in a dose-dependent manner. Further, the studies (whether or not conducted in vivo) seem to agree that vitamin D administration decreases IL-17 cytokine production and increases T regulatory cell populations. Together, these data provide compelling evidence that vitamin D is able to modulate the immune system towards a more cytotoxic immune response within the tumor microenvironment under in vivo conditions.
CHAPTER 2

MATERIALS AND METHODS

2.1 SAMPLES

In this study, 41 formalin-fixed, paraffin-embedded APC^{580D/+} mouse colon tumor samples were obtained from the laboratory of James Fleet at Purdue University. These mice had a single allele deletion at exon 14 of the APC gene, rendering the protein functionally inactive following spontaneous mutation of the remaining wildtype allele. The mice either received no treatment, treatment with 2% DSS, or treatment with 2% DSS + vitamin D (Table 2.1). 2% DSS was administered in drinking water ad libitum at ten weeks of age for seven or five days for mice sacrificed at fifteen and twenty weeks of age, respectively. Unhydroxylated vitamin D₃ was administered ad libitum using the AIN-93G diet at weaning for the duration of the lifespan. Samples were cut in 5 μm sections and mounted on positively-charged slides for immunohistochemical staining with rabbit anti-mouse CD3 (DAKO cat # A0452), rabbit anti-mouse granzyme B (Abcam cat# ab4059), and rabbit anti-mouse IL-17 (Santa Cruz cat# sc-7927) antibodies.

2.2 IMMUNOHISTOCHEMICAL STAINING

Samples were deparaffinized and rehydrated using xylene and gradually decreasing concentrations of ethanol. 3% H₂O₂ was administered to samples for 30 minutes at room temperature in order to quench any endogenous peroxidase activity. Antigen retrieval for samples stained with CD3 was accomplished using 10 minutes of boiling in the microwave using 10 mM citrate acid buffer, pH 6.0. Antigen retrieval for samples stained with granzyme B was accomplished with 4 minutes of heating in a pressure cooker in 1X Target Retrieval Solution, pH 6.0 (DAKO cat# S2369). Antigen retrieval with IL-17 was achieved with 90 seconds in the pressure cooker in 10 mM citric acid buffer, pH 6.0. 1% bovine serum albumin was administered to CD3 and granzyme B samples for 30 minutes at room temperature in order to block non-specific antibody binding. 5% BSA 5% goat serum was used to block non-specific antibody binding during IL-17 staining. CD3 antibodies were
Table 2.1. Summary of the CAC;APC<sup>580D/+</sup> Mouse Treatment Groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>10 Weeks</th>
<th>15 Weeks</th>
<th>20 Weeks</th>
<th>30 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>2% DSS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% DSS + 25 IU vitamin D</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2% DSS + 150 IU vitamin D</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2% DSS + 1000 IU vitamin D</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>2% DSS + 10000 IU vitamin D</td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

diluted 1:500 in blocking buffer and covered in a humidity chamber for 16 hours at 4°C. Granzyme B antibodies were diluted 1:500 in antibody diluent (DAKO cat # S0809). IL-17 antibodies were diluted 1:50 in the 5% BSA 5% goat serum blocking buffer. Following primary antibody incubation, samples were covered with horseradish peroxidase (HRP)-conjugated goat α-rabbit IgG antibody (DAKO cat # K4002) for 30 minutes at room temperature in order to bind the primary antibody. 3,3’-Diaminobenzidine (DAB) was administered to samples for 8 minutes (5 minutes for IL-17) at room temperature. DAB is a type of peroxide which is cleaved by HRP to form a brown precipitate at the site of specific primary antibody binding. Samples were then counterstained with Mayer’s hematoxylin and mounted with a cover slip. Representative images of tumors stained with CD3 and granzyme B are shown in Figures 2.1 and 2.2, respectively.

2.3 Data Acquisition and Statistical Analysis

The immunohistochemically stained tumor samples were photographed at 10X objective lens magnification using the Nokia Eclipse E600 microscope camera at Moore’s Cancer Center. Photographed tumor areas were manually outlined and calculated using SPOT Advanced software. Representative image of photographed and outlined whole CD3-
stained tumor sample is shown in Figure 2.3. Total positively stained intratumoral CD3 and granzyme B cells were manually counted by two blinded independent observers and reconciled to ensure similarity in counts to within 10%. A representative image of a CD3-stained tumor containing both tumor and normal tissue is shown in Figure 2.4. Statistical differences between age, DSS, and vitamin D dosage groups were analyzed using the Mann-Whitney U test. The Mann-Whitney U test was employed as a result of low sample size and a
non-normal distribution of data. The Mann-Whitney U Test is a non-parametric test and is appropriate for analyzing the differences among two independent groups based on each group’s data point ranking. The Mann-Whitney U Test is appropriate for this study because assumptions about the data are not required and can be used to test significance with a non-normal distribution of data and a low sample size [62]. The result of the Mann-Whitney U Test is a p-value which determines the probability of the difference between two groups occurring by chance. A p-value of 0.05 indicates there is a 5% probability the difference observed between the two groups occurred by chance. In this study, p-values less than or equal to 0.05 were considered statistically significant. A table including all samples used in this study and their respective intratumoral CD3 and granzyme B densities is included in the Appendix.
Figure 2.4. 10X objective lens magnification of CD3-stained mouse APC KO tissue (C62T1A). The tumor tissue (red arrow) is adjacent to the normal tissue (blue arrow).
CHAPTER 3

RESULTS

3.1 INTRATUMORAL CD3+ CELL DENSITY AND TEMPORAL PROGRESSION

In this study, it was of interest to track the intratumoral CD3+ cell density over time. CAC;APC^{580D/+} mice were sacrificed at 10, 15, 20, and 30 weeks of age to determine tumor formation and intratumoral CD3+ cell density. Intratumoral CD3+ cell density did not differ significantly between age groups although there does appear to be a decreasing trend in the mean values over time (Figure 3.1). A mean increase in CD3+ cell density is observed at the 20 week time point; however this group had a particularly low sample size and one of the samples must be considered an outlier since it contained many more CD3+ cells than any other sample in this study. The decrease observed between the 10 and 30 week time points is not statistically significant (p=0.133). Further studies are required to confirm these results.

3.2 INTRATUMORAL CD3+ CELL DENSITY IN THE PRESENCE AND ABSENCE OF DSS

In order to determine the effect DSS has on intratumoral CD3+ immune infiltrates, 2% DSS was administered to a subset of CAC;APC^{580D/+} mice at 10 weeks of age for five or seven days. DSS administration was then stopped and mice receiving seven days of 2% DSS administration were sacrificed at 15 weeks of age whereas mice receiving five days of 2% DSS administration were sacrificed at 20 weeks of age. At 15 weeks of age, mice receiving 2% DSS administration had an increase (p = 0.05) in overall CD3+ cell density (Figure 3.2). Among mice receiving DSS administration, there is a statistically significant decrease (p = 0.006) in CD3+ cell density at 20 weeks of age as compared to 15 weeks of age (Figure 3.2). This data is consistent with evidence suggesting that several cycles of DSS administration are required for chronic inflammation to occur. [13, 63]
Figure 3.1. Intratumoral CD3+ cell densities at various time points. Numbers indicate mean values within each age group. The p-value between the 10 and 30 week time points is 0.133.

Figure 3.2. Intratumoral CD3+ cell densities in the absence and presence of 2% DSS administration. Values shown are mean values in each group.
3.3 Intratumoral Granzyme B+ Cell Density and Temporal Progression

The mere presence of intratumoral CD3+ cells does not necessarily result in effective tumor killing therefore it is important to assess the intratumoral granzyme B+ cell density since it is the primary enzyme used by the immune system for destroying tumorigenic cells. At 10 and 15 weeks of age, the CAC;APC^{580D+} mice have very similar granzyme B+ cell densities (p=0.57). However, the granzyme B+ cell densities at 20 and 30 weeks of age decline in a statistically significant manner as compared to the 10 and 15 week time points (p=0.021) (Figure 3.3).

![Figure 3.3](image)

**Figure 3.3.** Intratumoral granzyme B+ cell densities at various time points. Values shown are mean values for each age group.

3.4 Intratumoral Granzyme B+ Cell Density in the Presence and Absence of DSS

It is well known that DSS results in increased tumor progression as well as increased CD3+ cell inflammation; however to our knowledge the effect of DSS administration on granzyme B activity has not been reported. As shown in Figure 3.2, mice at 15 weeks of age that received DSS administration appeared to have a statistically significant increase in
overall CD3+ cell density which may be the result of DSS-induced inflammation. However, mice at 15 weeks of age that received DSS administration showed a decrease in granzyme B+ cell density as compared to mice that did not receive DSS (Figure 3.4). Although DSS administration results in an increase in CD3+ cell infiltrates, there is no concomitant increase in granzyme B+ cell density suggesting that the increased CD3+ cell infiltrates are not active cytotoxic cells. The resulting pro-inflammatory tumor microenvironment is not conducive to cytotoxic cell killing of tumor cells. Mean intratumoral granzyme B+ cell densities in mice at 20 weeks of age in the absence or presence of DSS administration are very similar suggesting that DSS-mediated inflammation has cleared by this time and has no apparent effect on intratumoral granzyme B+ cell infiltrates (Figure 3.4).

![Figure 3.4](image)

**Figure 3.4.** Intratumoral granzyme B+ cell densities in the presence and absence of 2% DSS administration. Values shown are mean values of each group.

**3.5 CD3:GRANZYM B CELL RATIOS AND TEMPORAL PROGRESSION**

Intratumoral cytotoxicity is an important indicator of immune recognition and destruction of tumor cells. A high concentration of intratumoral CD3+ cells does not necessarily indicate a robust and effective anti-tumor immune response. In order to determine
the ratio of functionally cytotoxic CD3+ cells, the CD3+ cell density was compared to the density of granzyme B+ cells at the 10, 15, 20, and 30 week time points. When comparing the CD3:granzyme B cell ratios in the absence of DSS administration over time, it is apparent that there is no significant difference among age groups. Even though there does appear to be an increase at 20 weeks of age, this is due to the outlier sample within this age group (Figure 3.5).

![Figure 3.5. Intratumoral CD3:Granzyme B ratios over time. Values shown are mean values within each age group. No statistical difference between 10 and 30 week groups (p=0.267).](image)

### 3.6 CD3:Granzyme B Cell Ratios in the Presence and Absence of DSS

It was also of interest for us to evaluate the effect DSS administration had on the CD3:granzyme B cell ratios. At 15 weeks of age, mice receiving DSS administration had a significantly higher CD3:granzyme B cell ratio suggesting that DSS increases CD3+ cell densities without a corresponding increase in granzyme B (Figure 3.6). This data indicates that DSS-mediated inflammation results in an increase in T cells expressing CD3 without a concomitant increase in cytotoxicity. Mice at 20 weeks of age have very similar CD3:granzyme B cell ratios (p=0.29) which may have resulted from a clearance of
intratumoral CD3+ cell inflammation as well as a decrease in the granzyme B+ cell density by the 20 week time point (Figure 3.6).

3.7 EFFECT OF VITAMIN D ADMINISTRATION ON INTRATUMORAL CD3+ CELL DENSITIES

A subset of CAC;APC<sup>580D/+</sup> mice reared in the James Fleet lab at Purdue University were given 2% DSS, varying concentrations of physiologically active vitamin D, and sacrificed at 20 weeks of age in order to determine the effect vitamin D has on tumor formation and intratumoral immune cell densities. Vitamin D administration does not appear to have a significant impact on intratumoral CD3+ cell density, however there is a statistically significant increase (p = 0.008) when comparing the 0 IU vitamin D dosage group to the 10,000 IU vitamin D dosage group (Figure 3.7).

3.8 EFFECT OF VITAMIN D ADMINISTRATION ON INTRATUMORAL GRANZYME B+ CELL DENSITIES

Since it is known that individuals with high blood serum vitamin D concentrations have decreased CRC mortality, we postulated that vitamin D administration may result in
increased functional intratumoral cytotoxic cell density. While no statistically significant increase in intratumoral granzyme B+ density occurred as vitamin D dosage increased, there was a trend of increasing intratumoral granzyme B+ cell density up to the 150 IU vitamin D group with an increase that may be approaching statistical significance (p = 0.056) (Figure 3.8).

3.9 EFFECT OF VITAMIN D ADMINISTRATION ON INTRATUMORAL CD3:GRANZYM E B CELL RATIOS

Since vitamin D administration may result in an increased cytotoxic immune response within the tumor microenvironment, we thought it would be of interest to calculate the CD3:granzyme B ratios at each level of vitamin D dosage. When comparing the intratumoral CD3:granzyme cell ratios, it became apparent that (while not significant; p=0.452) there is a decreasing trend in the CD3:granzyme B cell ratios up to the 1,000 IU dosage group indicating an increase in the proportion of functional cytotoxic cells as opposed to other CD3+ cell types (Figure 3.9). A decreasing CD3:granzyme B ratio may be the result of decreasing non-cytotoxic CD3+ cells, an increase in functional cytotoxic cells, or both. While not significant, this data supports the role that vitamin D aids in coordinating an
Figure 3.8. Intratumoral granzyme B+ cell densities at varying levels of vitamin D administration. Values shown are mean values of each vitamin D dosage group.

Figure 3.9. Intratumoral CD3:Granzyme B cell ratios at varying levels of vitamin D administration. Values shown are the means of each vitamin D dosage group.
intratumoral immune microenvironment that is conducive to tumor cell recognition and killing. While the immune coordinating role of vitamin D is still under investigation; these results, nonetheless, suggest that vitamin D could have an important impact on effectively coordinating the anti-tumor immune response.

3.10 Effect of Vitamin D Administration on Intratumoral IL-17+ Cell Densities

Since previous studies have indicated that increased vitamin D administration results in a decrease in intratumoral IL-17 levels, it was of interest to evaluate the effect vitamin D administration has on the intratumoral IL-17 densities in our samples. A subset of mice in this study received 2% DSS + varying doses of vitamin D administration and was sacrificed at 20 weeks of age. IHC staining using rabbit α-mouse IL-17 antibody in the group of mice that received no vitamin D administration had little to no detectable intratumoral IL-17 activity (Figure 3.10). Therefore, it would be impossible to detect whether vitamin D administration decreases intratumoral IL-17 activity since there is no IL-17 present in the 0 IU vitamin D diet group mouse adenoma. The IL-17 antibody used in this study is a polyclonal antibody which recognizes a region of IL-17 that is conserved in mice, rats, and humans and has been reported to work in these organisms [64-66]. The αIL-17 antibody used in this study binds to an amino acid region that is conserved among humans, mice, and rats. So, in order to show that the lack of IL-17 was due to a true lack of IL-17 presence within the mouse adenoma as opposed to a non-binding antibody or a non-functional IHC staining method, Figure 3.11 shows IHC staining using the same anti-IL-17 antibody in human CRC as a positive control.
Figure 3.10. 10X objective lens magnification of an adenoma from a 20 week old mouse (B60T1A) that received no vitamin D administration and that was stained with IL-17.

Figure 3.11. 10X objective lens magnification of an IL-17 stained human CRC sample from the North Carolina Colon Cancer Study.
CHAPTER 4

DISCUSSION

CD3+ T cells are members of the adaptive immune system and play a major role in constructing the intratumoral immune microenvironment. Through the tracking of CD3+ cell densities over time, in the absence or presence of DSS administration, and in the presence of vitamin D administration it was possible to not only detect that there are T cells present in the tumor but how their levels change in response to the varying conditions present in this study. While no concrete conclusions can be made given the lack of availability of samples in certain groups, these studies provide intriguing evidence for role CD3+ cells play in tumor immunity.

With regards to time, the intratumoral CD3+ cell densities stay consistent with the exception of the 20 week time point which is the result of the outlier (Figure 3.1). Further, DSS administration appears to increase the CD3+ density at 15 weeks of age as compared to the non-DSS group which appears to validate previous claims that DSS results in increased inflammation throughout the gastrointestinal tract (Figure 3.2). The DSS group of mice was administered a single cycle of DSS at 10 weeks of age resulting in acute inflammation. At 20 weeks of age, the CD3+ cell density appears to drop below the non-DSS baseline level which is in agreement with previous studies demonstrating that more than one cycle of DSS administration is required for chronic inflammation to occur [67]. It is possible that the difference in CD3+ cell densities between the 15 and 20 week old mice that received 2% DSS administration is the result of the 20 week old mice receiving only five days of DSS administration whereas the 15 week old mice received seven days of DSS administration. Furthermore, it is possible that DSS-mediated inflammation is detrimental to long term CD3+ cell responses. It would be of interest if future studies could allow the mouse populations to live for longer periods of time in order to further evaluate the interplay of the tumor and the immune response over a longer period of time.

The use of granzyme B is a major mechanism by which the immune system destroys tumorigenic cells. The evaluation of the intratumoral granzyme B+ cell density provides a strong indicator for the effectiveness of the anti-tumor immune response. A higher granzyme
B cell density indicates a more efficient recognition and killing of the adenomatous cells by cytotoxic immune cells.

The statistically significant reduction (p=0.021) in granzyme B+ cell density at 20 and 30 weeks of age as compared to the densities at 10 and 15 weeks of age suggest tumoral evasion of the immune response over time (Figure 3.3). Mice that received DSS administration had less granzyme B+ cytotoxic activity than the non-DSS group at 15 and 20 weeks of age indicating that the acute inflammation caused by DSS administration results in a decreased intratumoral cytotoxic immune response (Figure 3.4). To our knowledge, we are the first to report reduced intratumoral granzyme B densities upon DSS administration which is in agreement with previous observations that inflammation can result in a less robust anti-tumor immune response and a pro-tumor immune microenvironment. [25, 53]

The CD3:granzyme B ratio indicates the number of T cells present as compared to the number of cells expressing granzyme B within the tumor microenvironment. This is not an indicator of the number of CD3+ cells expressing granzyme B since natural killers cells are known to express granzyme B but do not necessarily express the CD3 cell surface protein (with the exception of natural killer T cells) [68]. Regardless, the CD3: granzyme B ratio is a strong indicator of the strength of the anti-tumor immune response within the context of the intratumoral T cell density. A low CD3:granzyme B ratio indicates a strong anti-tumor immune response whereas a high CD3:granzyme B ratio would indicate a poor anti-tumor immune response with a low density of intratumoral cytotoxic cells.

When tracking the CD3:granzyme B cell ratios over time, it is apparent that no significant difference occurred except those that were due to the outlier at the 20 week time point (Figure 3.5). When comparing the group of mice that received DSS against those that did not receive DSS administration, it is apparent at the 15 week time point that DSS significantly increases the CD3:granzyme B ratio with a p-value of .05. Further, at 20 weeks of age, there is no difference between CD3:granzyme B ratios of mice that did and did not receive DSS which may indicate clearance of T cell inflammation by this time point (Figure 3.6).

Among mice receiving vitamin D, there was no significant difference in the intratumoral CD3+ cell densities at the various dosage levels except between the 0 and 10,000 IU dosage groups (Figure 3.7). While previous studies have been controversial with
regard to the effect of vitamin D has on T cell populations, our study appears to support the notion that vitamin D increases intratumoral CD3+ cell populations.

Intratumoral granzyme B densities were assessed among mice receiving varying doses of vitamin D. The granzyme B cell density tends to increase up to the 150 IU dosage group with a difference that may be approaching statistical significance (p=0.056) (Figure 3.8). These data lends support to the hypothesis that vitamin D can increase intratumoral cytotoxic activity under physiologically relevant levels of vitamin D. We believe that this provides compelling evidence that vitamin D studies need to be conducted in vivo in the future in order to take the complexity of the immune response into account which cannot be evaluated under any simple in vitro conditions. At the 1,000 and 10,000 IU vitamin D dosage levels, the granzyme B activity appears to decrease (Figure 3.8). This may be due to the proliferation limiting ability vitamin D has on T cells at very high levels. It would be of interest to continue these studies with other dosage levels between 150 and 1,000 IU of vitamin D to determine the level of serum vitamin D in which intratumoral granzyme B levels begin to decrease in order to ascertain optimal vitamin D serum levels for proper immune function.

Vitamin D certainly has effects on other cells besides CD3+ and granzyme B+ cells. In an effort to address these, IHC staining using anti-CD8 and anti-IFNγ antibodies were attempted but through numerous attempts, we were not able to acquire properly stained adenoma tissue samples. There are also many other cytokines and cell types that could have been evaluated in this study; namely, IL-10, IL-4, FoxP3 T regulatory cells, and B cells all of which serve roles in tumor immunity. The increase in any of these cytokines or cell types are not associated with cytotoxicity and would therefore not be conducive to the evaluation of the anti-tumor cytotoxic response. However, an increase in any of these cytokines or cell types would indicate a transformation away from an anti-tumor immune microenvironment. It would be of interest to evaluate the role vitamin D plays on each of these cytokine/cell types in order to evaluate whether it decreases them in vivo in an effort to address the possible roles vitamin D plays in modulating the cytotoxic immune response. While numerous in vitro studies appear to contradict the in vivo studies, it is possible this is the result of an unexplained mechanism by which active recognition of foreign or transformed cell antigen is required within the context of the in vivo immune microenvironment for a
sustained cytotoxic response. Studies have shown that activated T cells up-regulate the vitamin D receptor in order to inhibit excessive cytotoxicity but no studies have evaluated whether this still occurs during a normal and healthy active cytotoxic response. Further, as stated earlier, \textit{in vivo} models of vitamin D expression support an increase in NK cell cytotoxic activity which may be another role vitamin D plays in decreasing CRC incidence/mortality [60, 61]. It would be of interest if future studies could evaluate the effect vitamin D has on intratumoral NK cell densities in order to elucidate a possible mechanism for vitamin D’s role in increasing anti-tumor cytotoxicity within an \textit{in vivo} intratumoral immune microenvironment.

Furthermore, it would also be of interest to observe the effect vitamin D administration has on limiting chronic inflammation. Previous studies have provided convincing evidence that vitamin D reduces chronic inflammation which may explain the inverse correlation between sun exposure and CRC incidence/mortality [54, 55]. The results of this study provide evidence that mice receiving 2% DSS administration had a reduction of inflammation to baseline levels by 20 weeks of age (Figure 3.6). Furthermore, immunohistochemical staining using a rabbit α-mouse IL-17 antibody revealed no intratumoral IL-17+ cells among 20 week mice receiving no vitamin D administration (Figure 3.10). Additionally, a human CRC sample was stained with the same IL-17 antibody used in the mouse study and showed a high amount of IL-17 activity indicating that the 0 IU vitamin D mice truly did not have intratumoral IL-17 activity rather than a lack of staining from a dysfunctional antibody or experimental method (Figure 3.11). Since the mice that did not receive vitamin D administration had no intratumoral IL-17 activity, it would be impossible to determine whether vitamin D administration would decrease the intratumoral IL-17+ cell density. We then hypothesized that mice needed a longer period of time for inflammation to occur in the colonic adenomas, however IL-17 staining of 30 week old mice also revealed no intratumoral IL-17 activity (data not shown). It would be of interest if future studies could subject the mice to more than one cycle of DSS administration to induce chronic inflammation that would still be present by the 20 week time point [67]. It would then be possible to determine the effect vitamin D has on decreasing IL-17-mediated intratumoral inflammation and provide evidence for another possible mechanism by which vitamin D reduces CRC incidence and mortality. Moreover, allowing the mice to have a
longer lifespan would allow for the further study of the effect age has on IL-17 activity and the resulting tumor progression as well as providing valuable insight into the inflammatory and cytotoxic immune response to the tumor over a longer period of time.
REFERENCES


APPENDIX

CD3+ AND GRANZYMME B+ CELL DENSITIES OF THE CAC; AP C₅₈₀D/+ MOUSE TUMOR SAMPLES
<table>
<thead>
<tr>
<th>Sample</th>
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<th>2% DSS</th>
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