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PI3K/AKT signaling is essential for communication between tissue infiltrating mast cells, macrophages, and epithelial cells in colitis-induced cancer

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Declaration by candidate

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Summary

Colorectal cancer is one of the leading cause of mortality in USA and worldwide. Colon cancer is a complex world of connective tissue epithelial, inflammatory and endothelial cell interactions. It s long known that patients with persistent colitis are at high risk of developing colon cancer, where the patients passes through a phase from colitis to colitis induced dysplasia and finally invasive cancer. Though a lot of efforts have been poured into understanding the mechanisms underlying the etiology of colon cancer, less has been known with respect to the changes happening at genomic and proteomic levels in the inflammatory compartment of the colon cancer with respect to interaction with the tumor epithelium and subsequent tumor progression.

It is well known that the levels of PI3K/AKT and inflammatory cells mainly mast cells and macrophages are upregulated in the cancer. However, few efforts have been done to understand the pathogenesis in this direction, but they lacked in the correlative study of PI3K/AKT or immune infiltration in colon cancer or colitis and their spatial distribution with respect to cancer prognosis. Hence for the first time in this study a novel investigation was conducted where human patients were studied in the various stages of inflammation associated colonic illness i.e. colitis to dysplasia and invasive cancer and compared it to normal amongst themselves. The human-patient colonic tissue specimens were investigated for the spatial distribution of mast cells, macrophages and pAKT in the histological areas of musoca and submucosa. It was observed that mast cells, macrophages and pAKT levels incrementally rise from colitis to dysplasia to cancer in the submucosal tissues. In mucosal tissue, pAKT levels were found atleast 10 fold higher in the stromal cells in comparison with epithelial cells. The stromal and submucosal $pAKT^{+}$ cells were found to be the macrophages that progressively infiltrate from colitis to dysplasia and invasive cancer**,** also mast cells were found to be high in pAKT activity.

To investigate the role of PI3K/AKT in mast cells and macrophages biology and

their crosstalk with colonic epithelium a pan PI3K/AKT inhibitor used in clinical trials “LY2949002” was used. PI3K/AKT in mast cells was found to be critical for peripheral blood isolated human macrophage migration. LY294002 treatment of human and gut-derived murine mast cells restricted their ability to degranulate in dose-dependent manner. Moreover, inhibition of PI3K/AKT in human and mouse mast cells blocked the release of growth factors from them, and attenuated tumor proliferation and invasion promoting properties of mast cells. Also, PI3K/AKT inhibition in tumor infiltrating leukocytes isolated from colitis-associated patients lowered their soluble growth factor and cell-cell contact based tumor invasion promoting properties.

Treatment of LY294002 using intra-peritoneal injections lowered the incidence of colitis associated invasive cancer development by day 56 in the Piroxicam treated IL-$10^{-/-}$ mice. LY294002 displayed a striking effect on the epithelial proliferation, induced tumor apoptosis and attenuated the pAKT levels. LY294002 showed special predilection for the $pAKT^{+}$ stromal cells in comparison with $pAKT^{+}$ epithelial cells. Moreover, frequencies of mast cells and granulocyte-based inflammation were lowered after LY294002 treatment. Finally, the *in situ* degraunulating potential of mast cells was attenuated by LY294002 treatment.

In conclusion, using *in situ* human patient specimen study, *in vitro* human and murine assays and *in vivo* mouse model system this study confirms the role of PI3K/AKT pathway in mast cells and tumor infiltrating leukocytes in crosstalk with the colonic epithelium and progression of colonic tissue from colitis.

Zusammenfassung

Das kolorektale Karzinom ist eines der häufigsten Todesarten weltweit. Das Kolonkarzinom ist eine komplexe Krankheit aus konvektivem epithelialem Gewebe, Entzündungen und endothelialen Zellinteraktionen. Es ist bekannt, dass Patienten mit persistenter Kolitis ein höheres Risiko haben Darmkrebs zu entwickeln. Diese Patienten gehen durch verschiedene Stadien der Kolitis. Diese entwickeln sich über eine Kolitis, welche die Dysplasie induziert und danach gehen diese zu invasiven Karzinome über. Obwohl viele Arbeitsgruppen zum Verständnis des Mechanismus der Ätiologie des Kolonkarzinoms beitragen, ist bis zum heutigen Zeitpunkt immer noch sehr wenig über die Veränderungen in der Genetik und die Proteomik bekannt. Dies gilt vor allem in den entzündlichen Teilen des Kolonkarzinoms in Bezug auf die Interaktion mit dem Tumorepithelium und der nachfolgenden Tumorprogession.

Es ist bekannt, dass PI3K/AKT und entzündlichen Zellen, welche hauptsächlich Mastzellen und Makrophagen sind, im Karzinom erhöht sind. Jedoch ist bis zum heutigen Zeitpunkt sehr wenig zum Verständnis der Pathogenese in diesem spezifischem Feld beigetragen worden. Bis zum jetzigen Zeitpunkt sind noch keine korrelativen Experimente von PI3K/AKT und der immunen Infiltration im Kolonkarzinom, oder bei Kolitis und deren räumlichen Verteilung in der Karzinom-Prognose durchgeführt wurden. In dieser Arbeit wurden in den Patienten die verschiedenen Phasen der Entzündung, die mit Kolitis assoziiert sind, die danach zur Dysplasie übergehen und zum Schluss in invasiven Karzinom endet, untersucht und mit normalen Gewebe verglichen. Die Patientenproben aus dem Darmgewebe wurden auf die räumliche Verteilung der Mastzellen, Makrophagen und dem pAKT Level in der Mukosa und Submukosa histologisch untersucht. Es konnte gezeigt werden, dass Mastzellen, Makrophagen und das pAKT Level schrittweise in der Kolitis über zur Dysplasie bis hin zum Karzinom im submukosalem Gewebe ansteigen. Im mukosalem Gewebe konnte ein über 10-facher Anstieg des pAKT Levels in den Stromazellen beobachtet werden im Vergleich zu dem Epithelzellen. Die Stroma- und submukosalen pAKT positiven Zellen wurden als Makrophagen identifiziert, welche schrittweise von der Kolitis über Dysplasie bis hin zum invasiven Karzinom infiltrieren. Des Weiteren wurde auch in den Mastzellen eine hohe pAKT Aktivität festgestellt.

Um die Rolle von PI3K/AKT in Mastzellen und Makrophagen in Bezug auf deren Biologie und Wechselwirkung mit dem Kolonepithel zu untersuchen wurde der PI3K/AKT Inhibitor LY294002 verwendet, welcher auch in klinischen Trials zum Einsatz kommt. Das in Mastzellen gefundene PI3K/AKT wirkt sich kritisch auf die

humane Makrophagen Migration aus, die aus peripheren Blut isoliert wurden. Die Behandlung mit LY294002 in humanen und murinen Darm-Mastzellen beschränkt deren Eigenschaften zu degranulieren in einer dosisabhängigen Weise. Darüber hinaus blockiert die Inhibition von PI3K/AKT in humanen und murinen Mastzellen die Freisetzung von Wachstumsfaktoren und verringert die Tumorproliferation und die fördernden Eigenschaften zur Invasion. Außerdem verringert die Inhibition von PI3K/AKT die in Tumoren infiltrierenden Leukozyten, isoliert aus Kolitis-assoziierten Patienten, deren löslichen Wachstumsfaktoren und Zell-Zell-Kontakte basierend an deren fördernden Eigenschaften zur Tumorinvasion.

Die Behandlung mit LY294002 durch intraperitonealen Injektionen verringert die Kolitis Inzidenz assoziiert mit der Entwicklung von invasiven Karzinomen ab dem 56. Tag in IL-$10^{-/-}$ Mäusen, die mit Piroxicam behandelt wurden. LY294002 zeigte eine signifikanten Einfluss auf die epitheliale Proliferation, die Tumorinduzierte Apoptose und die Abnahme von pAKT. Darüber hinaus zeigte LY294002 eine spezielle Präferenz für die pAKT positiven Stromazellen im Vergleich mit den pAKT positiven Epithelzellen. Des Weiteren wurden die Frequenzen von Mastzellen und Granulozyten-basierende Entzündungen nach LY294002 Behandlung verringert. Schließlich wurde das in-situ degranulierende Potential der Mastzellen verringert.

Zusammenfassend bestätigt diese Arbeit die Rolle des PI3K/AKT Signalweg in Mastzellen und Tumor infiltrierende Leukozyten in der gegenseitigen Beeinflussung des Kolonepitheliums und die Progression des Kolongewebes von Kolitis über zum invasiven Karzinoms.

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Abbreviations and definitions

%	Percentage
[^{3}H]	Tritium, hydrogen-3
µg	Microgram
ul	Microlitre
µm	Micrometer
µM	Micromolar
ATCC	American Type Culture Collection
BMMCs	Bone marrow derived murine mast cells
BRDU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CAC	Colitis associated cancer
CAE	Chloroacetate esterase
CFSE	Carboxyfluorescein succinimidyl ester
CM	Conditioned medium
CPM	Counts per minute
CRC	Colorectal Cancer
CSF	Colony-stimulating factor
CSF1R	Colony-stimulating factor 1 receptor
DAPI	4',6-diamidino-2-phenylindole
DEN	Diethylnitroseamine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNP	2,4-Dinitrophenol

DSS	Dextran sulfate sodium
EHS	Engelbreth-Holm-Swarm
et al.	Latin “*et alii*”, - “and others”
FACS	Fluorescence activated cell sorter/ flow cytometry
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
GPCRs	G protein-coupled receptors
GMMCs	Gut derived murine mast cells
H&E	Hematoxylin and Eosin
HEPES	*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*
HMGB1	High mobility group box protein 1
M	Molar
MC	Mast cell
MCP	Mast cell progenitors
mg	Milligram
ml	Milliliter
mM	Millimolar
MMP	Matrix metalloprotease
ng	Nanogram
NSAIDs	Non-steroidal anti-inflammatory drugs
pAKT	Phosphorylated AKT
pAKT-T308	Phosphorylated AKT at Threonine 308 residue
pAKT-S473	Phosphorylated AKT at Serine 473 residue
PDGF	Platelet-derived growth factor

PB	Peripheral blood
PBMC	Peripheral Blood mononuclear cells
PBS	Phosphate buffered saline
PI3K	Phosphoinositol 3-Kinase
PI3Kγ	Phosphoinositide 3-kinase-γ
PNAG	4-nitrophenyl N- acetyl-β-D-glucosaminide
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute medium
RTKs	Receptor tyrosine kinases
SAA3	Serumamyloid A3
SCF	Stem cell factor
TAMs	Tumor-Associated Macrophages
TGF	Transforming growth factor
TILs	Tumor Infiltrating Leukocytes
TLR/IL1Rs	Toll-like/ IL-1 receptors
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
U	Units
UC	Ulcerative Colitis
VEGF	Vascular endothelial growth factor
X	Times

1.
Introduction

1. Introduction

1.1 Basics of cancer development

Cancer is the outcome of outgrowth of a clonal population of transformed cells from tissue(Hanahan & Weinberg, 2000). Carcinogenesis is a process of cancer development that could be modeled and characterized in various ways. One way to describe this process is to characterize the essential features of both cancer cells and tumors called as the “hallmarks” of cancer(Hanahan & Weinberg, 2000). Carcinogenesis requires the acquisition of six fundamental properties: insensitivity to anti-proliferative, signals, self-sufficient proliferation, unlimited replicative potential, evasion of apoptosis, persistent vascularization, and tissue invasion and metastasis needed for malignancy (**Figure 1**)(Hanahan & Weinberg, 2000).

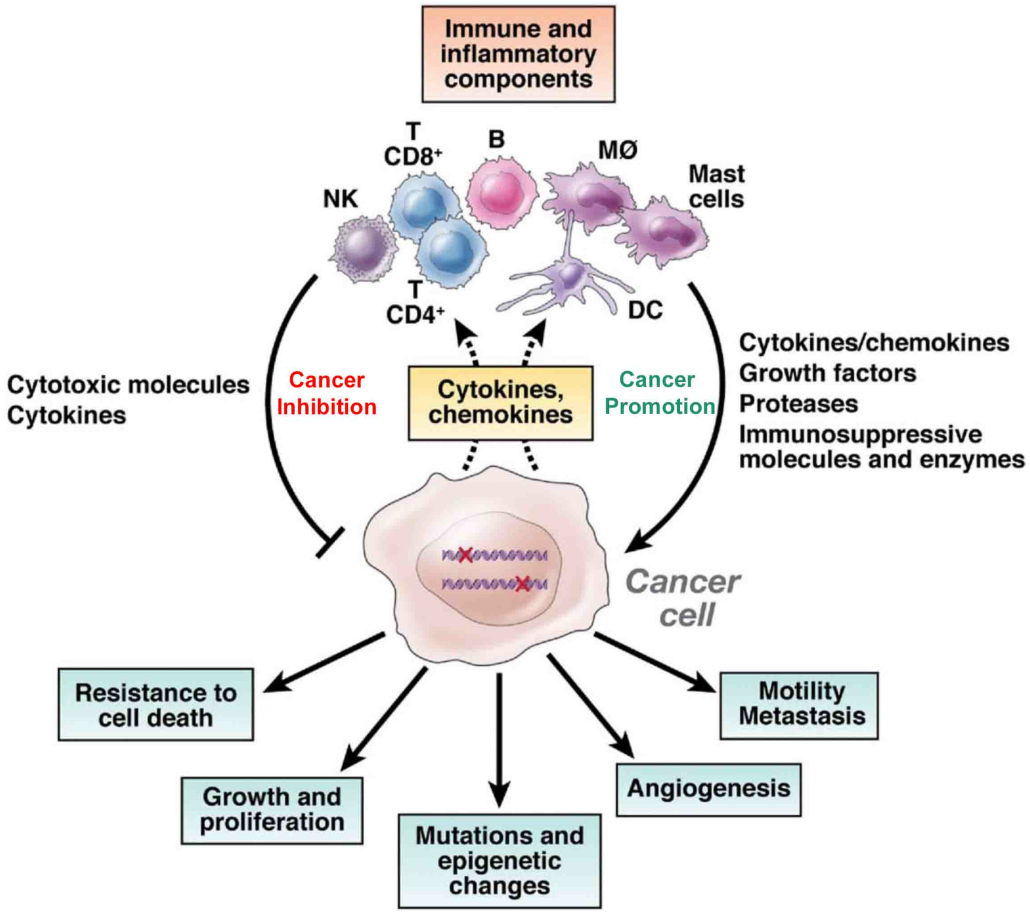

Figure 1. Immunosurveillance, inflammation and mechanism of colorectal cancer progression (CRC) and effector functions of CRC. [Modified and adapted from(Terzic et al, 2010)]

In another way carcinogenesis can be described with regard to a step-wise development, functionally grouped into three phases: initiation, promotion, and progression (Kinzler & Vogelstein, 1996). Initiation is a process characterized by genomic changes within the “cancer cell,” like gene deletion, point mutations, amplification and chromosomal rearrangements that lead to irreversible cellular changes(Kinzler & Vogelstein, 1996). Further tumor development is dependent on survival and clonal expansion of these “initiated” cells. Progression of cancer encompasses a substantial growth in size and either growth-related or mutually exclusive metastasis(Kinzler & Vogelstein, 1996).

Accumulation of genetic lesions in cells is an essential step in the development of the cancer. These events eventhough are required for initiation but may also play a role in the promotion or progression of tumor development (Kinzler & Vogelstein, 1996). These genomic events include the inactivation of tumor suppressor genes or/and activation of cellular proto-oncogenes that act in a “cancer-cell intrinsic” manner bestowing these cells with neoplastic properties(Terzic et al, 2010). However, eventhough these cell autonomous properties are required for tumorigenesis, they are not sufficient. Scientific studies over the last two decades have ratified the concept that carcinogenesis and malignancy is the result of processes involving both the cancer cells themselves and non-cancer cells(Terzic et al, 2010). A clear example of this is reported by the requirement of neo-angiogenesis for tumor growth and progression, and thus the contribution of the vascular endothelial cells is vital(Folkman, 2002).

1.2 Colorectal cancer (CRC)

Every year more than 1 million new cases of colorectal cancer (CRC) are diagnosed worldwide each year(Tenesa & Dunlop, 2009). Globally CRC is the 3rd most common malignancy and 4th most common cause of cancer mortality(Tenesa & Dunlop, 2009). Despite important advances in detection, surgery and chemotherapy CRC is the 2nd most common cause of cancer deaths in the United States and other developed countries(Jemal et al, 2009; Jemal et al, 2008). Colorectal cancer is caused by mutations that drive epithelial transformation through series of steps starting with pre-neoplasia to an intermediary dysplastic stage and finally invasive cancer (Muto et al, 1975; Powell et al, 1992). While inactivation of tumor suppressor genes and oncogenic mutations are necessary they are not sufficient for cancer progression (Kinzler & Vogelstein, 1998). However, only about 20% of CRC cases have a familial basis (Rustgi, 2007); some are associated with well-defined syndromes, such as familial adenomatous polyposis and hereditary non-polyposis colorectal cancer. Surprisingly, the largest fraction of CRC cases has been linked to environmental etiology rather than heritable genetic changes (Rustgi, 2007). Risk factors include specific intestinal commensals and pathogens, environmental and food-borne mutagens and chronic intestinal inflammation that precede tumor development(Rustgi, 2007).

1.2.1 Colitis-associated cancer (CAC) and colorectal cancer (CRC)

Colitis-associated cancer (CAC) is an inflammatory bowel disease (IBD) associated CRC subtype, has high mortality, and is difficult to treat(Feagins et al, 2009). Within 30 years of the onset of IBD more than 20% of IBD patients develop CAC, and more than 50% of them die from CAC(Lakatos & Lakatos, 2008). Inspite of immune-mediated mechanistic link between IBD and CAC(Atreya & Neurath, 2008; Greten et al, 2004), CAC shares lot of similarities with other types of CRC that develop without any signs of persistent inflammatory disease. Non-inflammatory CRC and CAC both share some of the essential

stages of carcinogenesis that includes formation of aberrant crypt foci, dysplasia, adenomas, polyps, and carcinomas(Terzic et al, 2010). However, for CAC somewhat different pathogenic sequences have been proposed. The sequences include injury-dysplasia, carcinoma and chronic inflammation that arise without the formation of well-defined adenoma. Nonetheless, Wnt, β-catenin, K-ras, p53, transforming growth factor (TGF)-β, and the DNA mismatch repair (MMR) proteins, are common genetic and signaling pathways that are altered in sporadic CRC and CAC(Terzic et al, 2010), however the time points at which inactivation of p53 and *Adenomatous Polyposis Coli* (*APC*) and activation of K-Ras takes place can differ between CRC and CAC(Lakatos & Lakatos, 2008; Sheng et al, 1998). Moreover, it has been reported in animal models that carcinogenesis in sporadic CRC and CAC is equally influenced by the gut microflora(Khazaie et al, 2012; Terzic et al, 2010). Intriguingly, even colorectal tumors that are not associated with clinically detectable Inflammatory bowel disease (IBD) develop robust inflammatory cell infiltration and pronounced expression of proinflammatory cytokines(Atreya et al, 2008; Atreya & Neurath, 2008; Clevers, 2004; Waldner & Neurath, 2008).

1.2.2 Cancer and inflammation

A link between the development of cancer and inflammation has been known for long(Balkwill & Mantovani, 2001; Coussens & Werb, 2002). Observing inflammation including leukocyte infiltration at tumors infected with microbes or at sites of chronic irritation is well known(Khazaie et al, 2012; Rakoff-Nahoum, 2006). However Virchow in 1863 first time reported the presence of leukocytes in cancer and the link of inflammation to cancer. Further evidence for the role of inflammation has come from the use of non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of spontaneous tumor formation in people with familial adenomatous polyposis (FAP)(Ulrich et al, 2006). Also, wound repair or inflammatory gene expression often correlate negatively with cancer stage and prognosis(Chang et al, 2004; Galon et al, 2006; Wang et al, 2006). Thus,

inflammation and cancer are linked by epidemiology, histopathology, inflammatory profiles, and the efficacy of anti-inflammatory drugs in prophylaxis.

Interestingly, several reports suggest that inflammatory and immune systems may inhibit the development of cancer (**Figure 1**). In tumor immunosurveillance, the host may develop a mechanism to perceive and eliminate transformed cells(Rakoff-Nahoum, 2006). Also, adaptive immune recognition of tumor-associated and specific antigens may be an important means by which the immune system controls the development of cancer(Smyth et al, 2006). However, it seems that the net effect of the inflammation is to positively affect tumor development. The relationship between cancer and inflammation is complex and cannot be reduced to one grand theory.

1.3 Inflammation can cause cancer

It is well established that long-standing inflammation secondary to chronic infection or irritation predisposes to cancer(Cheon et al, 2011; Gounaris et al, 2009; Gounaris et al, 2007; Gounaris et al, 2008; Khazaie et al, 2011; Strouch et al, 2010). The chronic inflammation associated with infection and irritation may lead to environments that foster genomic lesions and tumor initiation. One effector mechanism by which the host resists microbial infection is the production of free radicals such as reactive oxygen intermediated (ROI), hydroxyl radical (OH•) and superoxide (O_2-•) and reactive nitrogen intermediates (RNI), nitric oxide (NO•) and peroxynitrite (ONOO-)(Hussain et al, 2003). Primarily thought to be anti-microbial, these molecules are formed due to the activities of host enzymes such as NADPH oxidase, myeloperoxidase and nitric oxide that are regulated by inflammatory signaling pathways. Importantly, ROI and RNI lead to oxidative damage and nitration of DNA bases that increases the risk of DNA mutations(Hussain et al, 2003) . Cells have intrinsic mechanisms by which they avoid unregulated proliferation or the accumulation of DNA mutations. These mechanisms include but do not limit to tumor suppressor pathways that mediate cell cycle arrest, DNA repair, apoptosis and senescence. In the condition of DNA

damage or oncogenic activation, cells either repair their DNA and prevent mutations or initiated cells will undergo cell death(Rakoff-Nahoum, 2006). In the scenario of massive cell death as seen in infection or non-infectious tissue injury, lost cells gets repopulated by the expansion of other cells, often undifferentiated precursor cells such as tissue stem cells(Rakoff-Nahoum, 2006). There are two pre-requisites for this: Some cells amongst the dying population must survive the injury, and then these cells must expand to maintain cell numbers for a proper functioning tissue(Rakoff-Nahoum, 2006). Many inflammatory pathways play an important role to mediate these two prerequisites of tissue repair(Chen et al, 2003; Wang et al, 2005). In a addition to its physiologic role in mediating tissue repair or as a strategy in host defense to infection, the inflammatory response may play an important role in providing survival and proliferative signals to initiated cells, thereby leading to tumor promotion(Rakoff-Nahoum, 2006).

Direct evidence for a link between tumorigenesis and either host defense or tissue repair has been reported in numerous studies. (Rakoff-Nahoum, 2006). The Wnt/β-catenin pathway plays a vital role in both the maintenance of the steady-state proliferative compartment and tumorigenesis of tissues(Beachy et al, 2004). Molecules like COX-1 and -2, which are involved in the synthesis of prostaglandins that mediate the tissue repair process in the alimentary tract (Brown et al, 2007; Houchen et al, 2000; Morteau et al, 2000) play pivotal roles in tumor development at these sites(Chulada et al, 2000; Oshima et al, 1996). Moreover, key supportive evidence to support the role of these processes has come from studies showing that dedicated tissue injury and wound supports cell survival, tumor growth and neoplastic progression(Rakoff-Nahoum, 2006). Injection of Rous sarcoma virus (RSV) into chickens leads to the tumor growth at the site of injection, moreover sarcomas may form at other sites of the chicken if that site is wounded(Dolberg et al, 1985). The development of these wound-related tumors can be attenuated by glucocorticoids and may be mediated by the actions of transforming growth factor-β (TGF-β) and fibroblast growth factors (FGFs) (Martins-Green et al, 1994; Sieweke et al, 1989; Sieweke et al, 1990).

Tumor growth is enhanced in wounded limbs by the experimental induction of paracrine factors such as TGF-β and bFGF in wound fluid, in a B16 melanoma adoptive transfer study(Hofer et al, 1998).

Studies investigating the role of NF-κB (transcription factor central to the induction of inflammation) in tumorigenesis has provided some more detailed focus on the role of inflammation in tumor promotion. Greten et al. used a model of azoxymethane (AOM) induced colitis associated cancer (CAC), which develops multiple rounds of inflammation and leukocyte tissue infiltration caused by colonic epithelial cell toxin, dextran sulfate sodium (DSS) administration(Greten et al, 2004). This mouse model system makes it very clear that chronic inflammation augments tumorigenesis, as when one dose of AOM is given without DSS cycling, no tumors arise in the mice(Rakoff-Nahoum, 2006). It was observed that inactivation of the classical NF-κB pathway in colonic epithelial cells by conditional deletion of the IκB kinase β (IKKβ) protein resulted in a substantial decrease in the frequency of visible tumors, indicating the one of the mechanisms of tumorigenesis(Greten et al, 2004). Importantly, NF-κ signaling in epithelial cells was necessary for the inhibition of apoptosis in short time after administration of one round of AOM and DSS, perhaps by the induction of anti-apoptotic factors like Bcl-X_L(Rakoff-Nahoum, 2006). Thus after intestinal epithelial injury (DSS) and the addition of a mutagen (AOM), NF-κB provides a survival signal to the initiated cells. Importantly, IKKd of AOM and DSS, perhaps by the induction of anti-apoptoepithelial cell survival as a protection against both infectious and non-infectious injuries (Chae et al, 2006; Chen et al, 2003; Egan et al, 2004) and host defense pathways in the intestinal epithelium(Elewaut et al, 1999). In another study, a similar role for NF-κB in survival of initiated cells was reported in Mdr2-deficient mice a chronic inflammation model of spontaneous hepatocellular carcinoma,(Pikarsky et al, 2004). In this model, NF-κB activation was restricted by selectively expressing super-repressor of degradation of IκB in hepatic epithelial cells. Increase in the number of apoptotic hepatocytes was

found that is correlated with a decreased frequency of tumors compared to Mdr2-/- mice with degradable IκB (Pikarsky et al, 2004).

Tumor promotion depends upon not only the survival of initiated cells, but also their exponential expansion. Various inflammatory mediators such as eicanosids, cytokines and chemokines are capable of stimulating the proliferation of both untransformed and tumor cells(Balkwill & Mantovani, 2001). Mice that lack in TNF have fewer skin tumors upon administration of the phorbol ester TPA and the mutagen DMBA(Moore et al, 1999). NF-κB activation in myeloid cells has been reported to play a critical role in the production of TNF as a inflammatory mediator of tumor growth in both the AOM/DSS model of CAC(Greten et al, 2004) and mutagen-induced hepatocellular carcinoma upon administration of diethylnitroseamine (DEN)(Maeda et al, 2005). In both of these models, myeloid cells that were defective in activating NF-κB signaling via the classical pathway, there was impaired production of TNF and other inflammatory mediators, proliferation of dysplastic epithelium, and a reduction in both the frequency and size of tumors compared to the WT mice(Maeda et al, 2005).

1.4 Mast cells in cancer

1.4.1 Mast cell subsets and tissue distribution

Mast cells (MC) are tissue-resident sentinel cells. MC progenitors (MCP) have been suggested to branch off very early from hematopoietic stem cells (Chen et al, 2005a) or alternatively to differentiate late in the myeloid lineage from the granulocyte monocyte progenitor and have a common precursor with basophils(Arinobu et al, 2009). At least two distinct subpopulations of rodent MC have been identified based on morphologic characteristics, tissue localization, and protease content(Befus et al, 1982; Enerback, 1966; Enerback et al, 1986; Metcalfe et al, 1997). Mucosal MC can be distinguished from connective tissue MC by expression of chymase instead of tryptase and for lower expression of heparin in the secretory granules. Human MC are also divided into two types

depending on the expression of tryptase, chymase, and other proteases in their granules(Irani et al, 1986). MC that contains only tryptase are referred to at MCT and typically colocalizes with T cells in the respiratory and intestinal mucosa. MC that contain tryptase, chymase, and other proteases, such as carboxypeptidase A and cathepsin G, are referred to as MCTC and are found in connective tissues, including skin, submucosa of the gastrointestinal tract, breast parenchyma, myocardium, lymph nodes, conjunctiva, and synovium. In mice, mature MC are rarely present outside the connective tissues. In the intestine, isolated MC are detected in the mid-crypt region along with epithelial stem cells. Affinity of mature MC to stem cells is also highlighted by their localization in the vicinity of hair follicle stem cells and their involvement in regulating the transformation from resting (telogen) follicle to active hair growth (anagen)(Arck et al, 2001). Other than these exceptions, MC normally migrate and reside in tissue as progenitors(Hallgren & Gurish, 2007). Both the intestine mucosa and hair follicles are rich sources of MCP(Gounaris et al, 2007; Kumamoto et al, 2003). Committed but undifferentiated MCP reside within the lymph hematopoietic system comprising the bone marrow, spleen, peripheral blood, mesenteric lymph node, and gut mucosa. These progenitors differentiate into chymase-expressing mature MC upon challenge(Chen et al, 2005a; Kasugai et al, 1995; Rodewald et al, 1996). Primary MC expanded *ex vivo* have characteristics of both connective and mucosal MC irrespective of the tissue source. Extensive work has demonstrated plasticity of MC subsets in tissues(Gurish et al, 1995), showing that subtype classifications are not rigid and may be shifting within the tumor microenvironment. For example, MC that mediate immunosuppression in mice in tolerant allografts have been suggested to be distinct from other MC(Lu et al, 2006). Different subsets of MC infiltrate tumors at different stages of tumor progression. In benign adenomatous polyps of polyposis mouse strictly intraepithelial chymase-expressing MC are found(Gounaris et al, 2007), while MC infiltrating invasive carcinomas in mice are predominantly found in the invasive borders of tumors, as well as in tumor stroma, the muscularis mucosa and submucosa, and typically express tryptase(Maltby et al, 2009).

1.4.3 Mast cell arsenal of effector molecules

MC produce three categories of effector molecules. One category includes those effector molecules, which are stored in granules such as serotonin, histamine, heparin, tryptase, and chymase(Khazaie et al, 2011). Another includes those that are synthesized *de novo* upon cell stimulation such as lipid mediators (PAF), prostaglandins (PDG2), and leukotrienes (LTB4, LTD4). Lastly, a large variety of cytokines that are Th1-associated (IFN-γ, IL-2, IL-3, GM-CSF, and TNF-α), Th2-associated (IL-4, IL-5, IL-6, IL-10, IL-13, IL-33, and GM-CSF), or TH17-biased (TGF-β, IL-6, IL-1β, and TNF-α), chemokines and angiogenic factors including vascular endothelial growth factor(Boesiger et al, 1998) and tryptase(Blair et al, 1997) as well as proteases including tryptases, chymases, cathepsins, and carboxypeptidase(Khazaie et al, 2011). Release of mediators by MC occurs either within minutes of activation (immediate acute) or over hours (delayed) depending on whether these are pre-made and stored in granules for immediate release or require de novo synthesis. IL-1β, IL6, and TNF-α released by MC are proinflammatory cytokines that can generate TH17 cells, inactivate Treg, or otherwise render them pro-inflammatory. MC are a source of preformed TNF-α(Gordon & Galli, 1990), which also works as an autocrine MC stimulatory factor, and its elimination negatively impacts MC density in the gut(Gounaris et al, 2007). MC release TNF-α after incubation with bacteria, providing a potent chemotactic factor for neutrophils(Echtenacher et al, 1996; Malaviya et al, 1996; Mannel et al, 1996). The MC-deficient W-sh mice have chronic intestinal inflammation while TNF-α-deficient mice have increased mortality in the “cecal ligation and puncture” model compared with wild-type mice(Maurer et al, 1998). Other MC-derived products that contribute to the influx of neutrophils and control microbes, include leukotriene B4 (LTB4), human tryptase βI, macrophage inflammatory protein (MIP)-1α (CCL3), MIP-1β, MIP-2, monocyte chemoattractant protein-1, RANTES (CCL5), and IL- 8 (CXCL8)(Feger et al, 2002). MC can produce large quantities of IL-1β(Bochner et al, 1990) that may be processed by MC chymase or potentially by caspase-1 in an Nlrp3 inflammasome-dependent manner(Nigrovic

et al, 2010). It is also known that MC can induce production of IL-1β by macrophages, at least in the pathogenesis of rheumatoid arthritis(Nigrovic et al, 2010). IL-1β has a key role in chronic inflammatory reactions that help tumors flourish(Cook et al, 2010; Mantovani et al, 2008). IL-1β is also an important angiogenesis mediator regulating the synthesis of angiogenesis factors. A previous study has shown that human MC produce angiogenesis factor IL-8, when stimulated with IL-1β(Kim et al, 2010). It is tempting to speculate a connection between NLRP3 inflammasome activation in MC and cancer since NLPR3 and IL-1β are associated with environmental silica and asbestos carcinogenesis(Dostert et al, 2008). Of the long list of cytokines that can be released by MC, IL-10, and TGF-β deserve particular attention due to their role as immune-suppressive mediators that also generate induced Treg (iTreg). (Depinay et al, 2006). Secretion of IL-10 by MC has been implicated in down-regulation of antigen-specific immune responses by mosquito bites(Depinay et al, 2006). However, MC also respond to IL-10 differentially depending on the cellular source and level of activity. IL-10 can inhibit MC degranulation by suppressing MC IgE receptor expression and signaling (Kennedy Norton et al, 2008)while blocking antibodies to IL-10 can hinder antigen-induced recruitment of MCP to the lungs of C57BL/6 mice(Jones et al, 2010). In the mouse, at least five different chymases (mMCP-1, mMCP-2, MMCP-3, MMCP-4, and MMCP-5) and three different granule-associated tryptases (mMCP-6, mMCP-7, mMMP-11/transmembrane tryptase) have been described(Huang et al, 1998). C57BL/6 mice are defective in mMCP7 leaving them with mMCP6 as the major tryptase(Ghildyal et al, 1994). Expression of proteases in mouse MC is strictly related to the type of MC. Thus, mucosal MC express mMCP-1 and mMCP-2, whereas connective tissues MC express mMCP-3, mMCP-4 mMCP- 5, mMCP-6, mMCP-7, and carboxypeptidase(Miller & Pemberton, 2002; Stevens et al, 1994). MC have significant cyclooxygenase and lipoxygenase activity and generate inflammatory lipid metabolites of arachidonic acid(Khazaie et al, 2011). In mice, the major cyclooxygenase products of MC are prostanglandin-D2 (PGD2) and prostaglandin E2, and the major lipooxygenase products are LTC4, LTD4, and

LTE3 (Galli et al, 2005)LTB4 a MC product of 5-lypoxygenase is a chemoattractant for MC progenitors (Weller et al, 2005) and was recently shown to be a potent polyposis promoting factor in mice(Cheon et al, 2011). Human MC also produce LTB4, although in much smaller quantities than PGD2 or LCT4. MC are a source of platelet activating factor, and platelets are known to augment the growth and dissemination of primary tumors by promoting angiogenesis, immune evasion, and tumor extravasation(Jain et al, 2010). Release of certain mediators by MC requires degranulation (Khazaie et al, 2011. Degranulation is responsible for release of proteases as well as powerful anticoagulants such as heparin, chymase, and tryptase(Khazaie et al, 2011. Mechanisms of MC degranulation are best described in the context of the development of immediate hypersensitivity(Khazaie et al, 2011. The cross-linking of FcεRI, the high affinity IgE receptor, by allergen- or tumor-specific immunoglobulin IgE on MC is the primary trigger for the rapid release of their granules by exocytosis(Blank & Rivera, 2004). PI3K plays a key role in MC biology including degranulation (**Figure 2**) (Kim et al, 2008b).

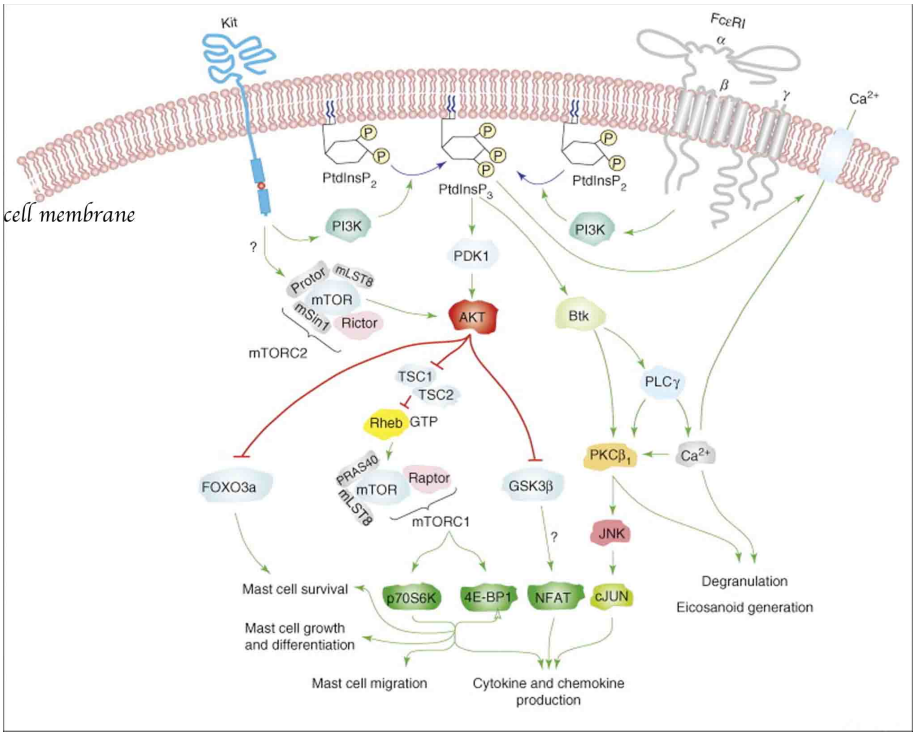

Figure 2. Downstream targets of phosphoinositide-3-kinase (PI3K) in

activated mast cells [Modified and adapted from (Kim et al, 2008b)]

MC treated with LY294002 (PI3K inhibitor) or inhibition of PI3K by over-expression of the dominant negative inhibitor Δp85 leads to a significant decline in MC degranulation via antigen-induced Ca2+ signals(Ching et al, 2001). MC may also be activated by "alternative," IgE -independent pathways, such as aggregation of low-affinity FcγRIII IgG receptors by IgG/antigen complexes, c-Kit, and pattern recognition Toll-like receptor mechanisms, activation of the complement receptors (C3aR, C5aR, CR2, CR4) by exposure to chemokines, anaphylatoxins C3a and C5a, fragments of fibrinogen, and fibronectin (Johnson et al, 1975; Marshall, 2004; Prodeus et al, 1997; Wojtecka-Lukasik & Maslinski, 1992). A recent study suggests that the release of microparticles from activated T cells induces MC degranulation and release of cytokines via the MAPK pathway independent of IgE(Shefler et al, 2010). These alternative pathways are thought to work through vesicle-mediated degranulation which involves small aliquots of granule-associated material that detach from the granule membrane for selective paracrine or endocrine transport to the cell exterior(Crivellato et al, 2010; Dvorak, 2005). This degranulation pattern has frequently been observed in MC infiltrating areas of chronic inflammation or tumors (Dvorak & Kissell, 1991; Dvorak et al, 1991). The alternative mechanism appears to be responsible for MC release of tumor-promoting cytokines and lipid mediators, particularly in early stages of cancer initiation such as in benign adenomatous polyps where degranulation of MC is not a major event(Gounaris et al, 2007).

1.4.4 Human studies show correlation between mast cells and cancer progression

A number of studies have documented correlations between the presence of MC and tumor development(Coussens et al, 1999; Imada et al, 2000; Ribatti et al, 2000; Ribatti et al, 2003; Takanami et al, 2000; Terada & Matsunaga, 2000; Toth-Jakatics et al, 2000). MC infiltration in tumor is an independent prognostic factor and predictor of poor outcome in prostate cancer(Nonomura et al, 2007)

and has been heralded as a novel prognostic marker(Johansson et al, 2010). Expression of c-Kit has been shown to predict recurrent disease and is suggested to be a marker of fibro-epithelial phyllodes tumors of the breast(Tan et al, 2005), but a recent report attributes this expression to the presence of infiltrating MC(Djordjevic & Hanna, 2008). High MC score is associated with unfavorable prognosis in patients with follicular lymphoma treated with immune-chemotherapy(Taskinen et al, 2008). Increased MC counts, tumor size, and lympho-vascular invasion are associated with an adverse prognosis in Merkel cell carcinomas(Beer et al, 2008). MC infiltration in Hodgkin lymphoma also demonstrated a poor prognosis associated with infiltration of CD30L-expressing MC(Molin et al, 2002). Intriguingly, this effect appears to occur independent of MC-mediated effects on angiogenesis(Glimelius et al, 2005), potentially via direct interaction between MC and Hodgkin and Reed–Sternberg cells expressing CD30. MC are etiologically associated with the formation of neurofibromas in human neurofibromatosis1 patients(Yang et al, 2006). Tumor promotion by MC is attributed to the release of mediators of angiogenesis and recruitment of macrophages, neutrophils, and eosinophils (**Figure 3**) (Maltby et al, 2009).

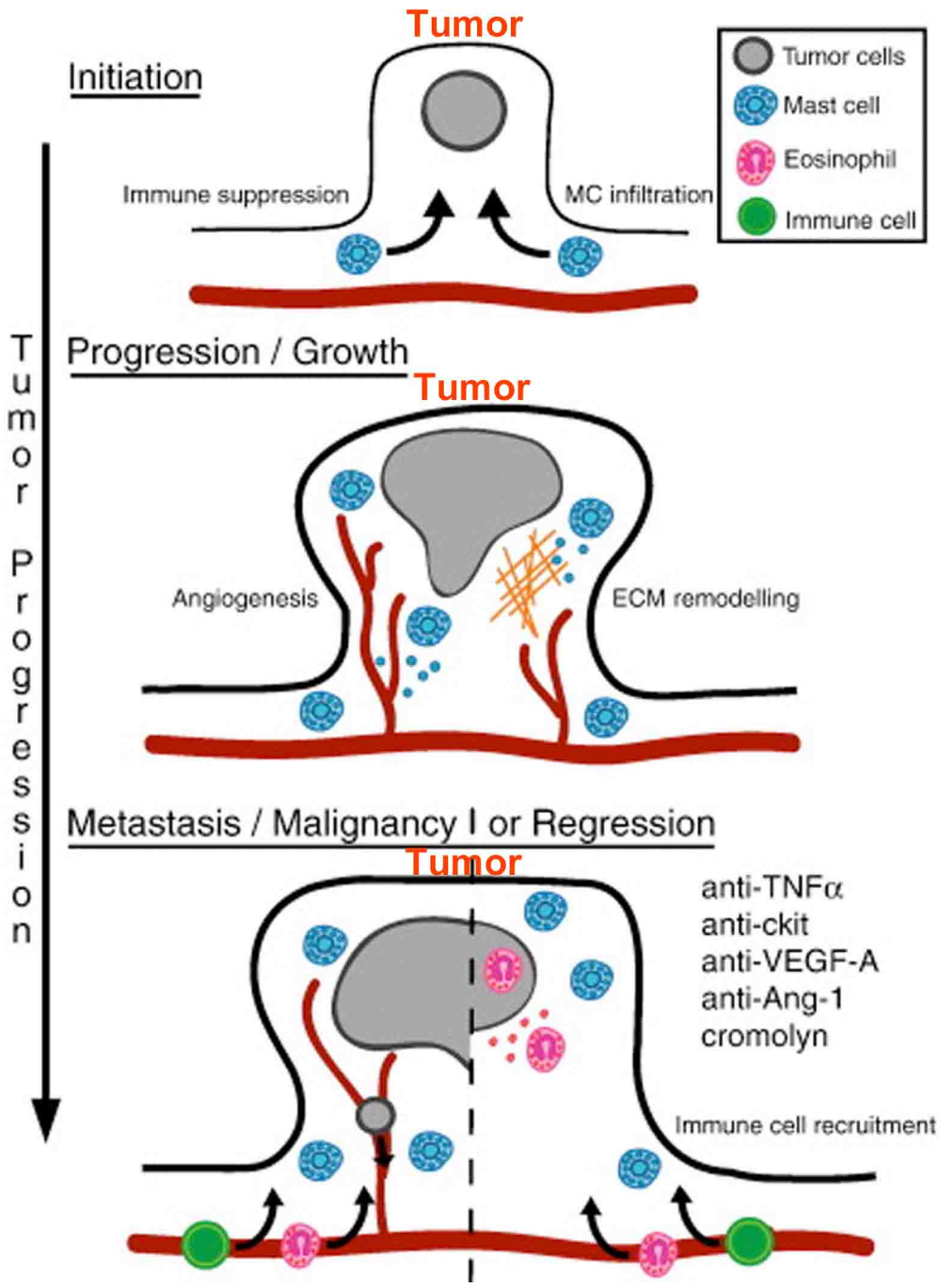

Figure 3. Schematic representation of mast cell functions at different stages in colonic polyp/tumor progression and formation [Modified and adapted from (Maltby et al, 2009)].

High MC density together with angiogenesis was predictive of poor clinical outcome in colorectal cancer(Acikalin et al, 2005; Gulubova & Vlaykova, 2009;

Yodavudh et al, 2008a), lung cancer(Imada et al, 2000), and pancreatic cancer(Strouch et al, 2010). Positive correlation between MC and microvessel densities was observed in colorectal cancer (Acikalin et al, 2005; Gulubova & Vlaykova, 2009; Yodavudh et al, 2008a)and lung cancer(Carlini et al, 2010; Ibaraki et al, 2005), supporting the involvement of MC in the tumor angiogenic process. MC tryptase can be detected in the peripheral blood of pancreatic cancer patients, presumably reflecting the abundance of tumor-infiltrating MC(Strouch et al, 2010). In hepatocellular carcinoma, higher peritumoral MC density was associated with worse clinical outcomes and shorter recurrence free survival, while higher density of MC was related to increased probability of early recurrence. Interestingly, peritumoral Treg were positively correlated with MC density and reversely related to clinical outcomes(Ju et al, 2009a; Ju et al, 2009b).

1.5 PI3K pathway in cancer

PI3Ks are the lipid kinases that phosphorylate the 3′-hydroxy group of PtdIns (phosphatidylinositol) and phosphoinositides (phosphorylated derivatives of PtdIns)(Vanhaesebroeck et al, 2010). The mammalian PI3Ks include eight enzymes that possess diverse roles in both vesicle trafficking and signal transduction. These enzymes are categorized into the categories known as class I, class II and class III, on the basis of their substrate preference and structure. However, only the class I PI3Ks have the ability to use PtdIns(4,5)P_2 as a substrate to generate the important second messenger PtdIns(3,4,5)P_3(So & Fruman, 2012). Certain proteins that contain a PH (pleckstrin homology) domain can specifically bind Ptdins(3,4,5)P_3 and be recruited to membranes where PI3K is active(Lemmon, 2008). Thus class I PI3K acts as a signaling hub at the plasma membrane to change the lipid composition in a way that links transmembrane receptors to the organization of multiprotein complexes, also known as signalosomes(Fruman et al, 2000). The composition of these signalosomes and the specific PH domain-containing PI3K effector proteins that are recruited to these assemblies varies according to the receptor that is engaged in this event(So & Fruman, 2012). In most cells, the serine/threonine

kinase Akt [also known as PKB (protein kinase B)] is the key PI3K effector molecule and Akt phosphorylation is used as a common readout of PI3K activation(Fayard et al, 2010). There are two amino acid residues in Akt that are phosphorylated in a PI3K-dependent manner; Ser^{473} by TORC [TOR (target of rapamycin) complex with rictor (rapamycin-insensitive companion of mammalian TOR) and other proteins] 2 and Thr^{308} by PDK-1 (phosphoinositide-dependent kinase-1)(Fayard et al, 2010).

The phosphatidylinositol-3_-kinase (PI3K) signaling cascade is involved in the regulation of various key cellular processes that are necessary for tumorigenesis including glucose metabolism, protein synthesis, cell survival, cell growth and proliferation, cell repair, cell migration, and angiogenesis (Katso et al, 2001). PI3K Signaling is regulated in several ways, hormones (estrogen, thyroid hormones), including growth factors (EGF, IGF1, FGF), vitamins, integrins, intracellular calcium, and the ras-dependent MAPK pathway(Saif & Chu, 2010). The PI3 kinase superfamily is composed of 12 members, and PI3K is made-up of two subunits: a 110-kDa catalytic subunit and an 85-kDaadaptor subunit. Upon cellular activation, the p85 subunit is recruited at the intracellular part of the growth factor receptor(Saif & Chu, 2010). Followed by dimerization with the p110 subunit, which results in full enzymatic activity of PI3K and subsequent generation of PIP3, a lipid “second messenger” that possess the capacity of binding and activating proteins with PH domains, localizing them to the cell membrane(Saif & Chu, 2010). Phospholipid phosphatases, such as the phosphatase and tensin homologue PTEN and the inositol 5_phosphatase-2 SHIP2PI3K inversely regulate PI3K the level of PIP3. The mechanism of this regulation of PI3K is via dephosphorylation of PIP3 into its inactive PIP2 form(Katso et al, 2001). Moreover, signaling via the PI3K pathway is controlled by cross-talk with other extracellular signals and pathways, including hormones (estrogen, thyroid hormones), vitamins, integrins, intracellular calcium, and the ras-dependent MAPK pathway(Katso et al, 2001).

The PI3K signaling pathway is constitutively activated in various malignancies

including CRC, prostate cancer, breast cancer, hematologic malignancies, glioblastoma multiforme, and lung cancer(Vivanco & Sawyers, 2002). This signaling pathway is activated by various mechanisms that include, activating mutations of PIK3CA, which is the gene encoding catalytic subunit of PI3K, gain-of-function mutations of oncogenes encoding positive regulators of PI3K (HER2, EGFR, and RAS, c-Src), loss-of-function mutations affecting negative regulators of PI3K such as PTEN (ie, loss of PTEN expression/function), amplification/overexpression of receptor tyrosine kinases, and mutations of genes encoding downstream effectors of the PI3K signaling cascade (eg, PDK-1, Akt/PKB, RPS6KB1) (Saif & Chu, 2010). Akt plays a vital role in the gene transcription by NF-kB pathway, regulation of apoptosis and cell cycle progression by inhibitory phosphorylation of Cdk inhibitors such as p21WAF1/CIP1 and p27KIP1(Saif & Chu, 2010). It is known that cancer cells express high levels of activated Akt, and phosphorylation of Akt at S473 was significantly associated with poor prognosis in several types of cancers(Downward, 2004). Alterations in PI3K levels have been identified in CRC, gastric cancer, breast cancer, and ovarian cancer, while *Akt* amplification of *Akt1, Akt2*, and *Akt3* have been found in breast, gastric, ovarian, pancreatic, and prostate cancers, respectively. There is increasing evidence suggesting that activation of various components of the PI3K/Akt pathway has prognostic importance in various malignancies(Saif & Chu, 2010). In addition, activation of PI3K has been identified as a potential mechanism by which cancer resist to chemotherapy, hormonal therapy, radiation therapy, and to various therapies targeting certain signaling pathways, such as trastuzumab and lapatinib(Saif & Chu, 2010). In all together, the frequent activation of the PI3K/Akt pathway in tumor cells and its potential role as a determinant of cellular drug resistance has made several individual components of this pathway attractive therapeutic targets for drug development(LoPiccolo et al, 2007).

However tumor is a complex system that contains but is not restricted to tumor cells. In addition to tumor cells, inflammatory cells, blood vessels and connective

tissue form a supplementary network to form the tumor. Whenever PI3K is studied, it is studied in a complete tumor inclusive of this supplementary network, however less is known in context specifically with the PI3K activity in the inflammatory component of the tumor(LoPiccolo et al, 2007).

1.6. PI3K in mast cells

Receptor-mediated mast cell growth, differentiation, homing to their target tissues, activation and survival are all controlled, to varying degrees, by PI3K-driven pathways, and PI3K deficient mice are devoid of mast cells (Cho et al, 2008). Mast cells express the class 1A p85α, p85β and p50α regulatory subunit isoforms(Tkaczyk et al, 2003) and (Lu-Kuo et al, 2000) in addition to all three class 1A PI3K catalytic subunit isoforms, p110α, p110β, and p110δ and the class 1B p110γ catalytic subunit (Ali et al, 2004; Okkenhaug et al, 2007). As Kit and the FcεRI of MC initiate their signaling processes through the activation of tyrosine kinases, either intrinsically or by recruitment of cytosolic kinases(Gilfillan & Tkaczyk, 2006; Roskoski, 2005), they use class 1A PI3Ks to mediate subsequent downstream signaling events, whereas GPCRs, such as those for adenosine, prostaglandin (PG)E_2, sphingosine 1 phosphate (S1P) and complement component C3a, mediate their responses via class 1B PI3K (Wymann et al, 2003) . The PI3K inhibitors, wortmannin and LY294002, have been reported in various studies to inhibit antigen-mediated degranulation and cytokine production in both rodent and human mast cells(Kim et al, 2008a; Okayama et al, 2003; Tkaczyk et al, 2003). Ironically, in some reports at least in human mast cells, these compounds fail to completely inhibit degranulation suggesting that although PI3K is essential for optimal degranulation of mast cells, PI3K-independent pathways might also regulate this response(Kim et al, 2008b). Studies using mouse bone marrow–derived mast cells (BMMCs) expressing a kinase-inactive mutant isoform of the p110δ catalytic subunit have demonstrated that p110δ is the major isoform responsible for antigen-mediated degranulation and cytokine production in mast cells(Ali et al, 2004; Ali et al, 2008). This

conclusion is further endorsed by the ability of the selective p110δ inhibitor, IC87114, to inhibit antigen-mediated mast cell activation and by its ability to inhibit the enhancement of antigen-mediated degranulation by stem cell factor (SCF)(Ali et al, 2004). By contrast, mast cells that are derived from the bone marrow of p85α and p85β knockout mice show normal antigen-mediated calcium flux and degranulation (Lu-Kuo et al, 2000; Tkaczyk et al, 2003), suggesting that the p110 catalytic subunit may use alternative regulatory subunits for its interaction with phosphorylated Gab2(Kim et al, 2008b).

In addition to its role in mast cell mediator release, PI3K is also vital for mast cell chemotaxis, adhesion and homeostasis. This has been evidenced by the ability of wortmannin and LY294002 to effectively attenuate SCF-mediated cell migration, adhesion to fibronectin-coated plates, proliferation and survival in human and mouse mast cell cultures (Kim et al, 2008a). In addition, reduction in the levels of mast cells is observed in the peritoneal cavity, but not dorsal skin, of mice expressing a mutation in the PI3K binding site on Kit (Kissel et al, 2000). As with degranulation, mast cell adhesion, chemotaxis and homeostasis seem to be mediated by specific PI3K isoforms. In bone marrow derived murine mast cells (BMMCs) inactive for p110δ, there is a dramatic defect in SCF-mediated mast cell adhesion and chemotaxis compared with the responses observed in wild-type mast cells (Ali et al, 2004). In addition, the ability of SCF to promote mast cell growth is significantly reduced in cells that express defective p110δ(Tkaczyk et al, 2003). Moreover, these attenuated responses are similarly observed in wild-type mouse mast cells incubated with the p110δ-selective PI3K inhibitor, IC87114, but not in cells incubated with the p110γ inhibitor, AS-252424(Ali et al, 2004; Ali et al, 2008). *In vivo*, there is a loss of gastrointestinal and peritoneal mast cells in p85α-deficient mice (Fukao et al, 2002) and reduction in the number of mast cells in the dermis of ear, but not in the back skin, of p110δ inactive mutant mice compared with wild-type mice(Ali et al, 2004).

1.7. PI3K in myeloid derived inflammatory cell population

In primary macrophages stimulated with the tyrosine kinase ligand colony-stimulating factor 1 (CSF1), all class IA PI3K isoforms get involved in the regulation of Rac1, whereas p110δ selectively controls the activities of Akt, RhoA and PTEN, in addition to controlling proliferation and chemotaxis (Schmid et al, 2011; Vanhaesebroeck et al, 2010). The prominent role of p110δ in these cells is indicative of being the main PI3K isoform that is recruited to the activated CSF1 receptor (CSF1R) (Schmid et al, 2011; Vanhaesebroeck et al, 2010). In immortalized BAC1.2F5 macrophages, however, the CSF1R also engages p110α, which takes up a more prominent role in CSF1R signaling, in processes including Akt phosphorylation and regulation of DNA synthesis(Schmid et al, 2011; Vanhaesebroeck et al, 2010). Cell migration, however, remains dependent mainly on p110δ. In other immortalized macrophage cell lines, such as IC-21 and J774.2, p110α is also found to be more prominently involved in CSF1-induced Akt phosphorylation, at the expense of p110δ(Schmid et al, 2011; Vanhaesebroeck et al, 2010). These data show that PI3K isoforms can be differentially monitored in distinct cellular contexts, with the dominant role of the p110δ isoform in Akt phosphorylation and proliferation being lost upon cell immortalization (Papakonstanti et al, 2008; Vanhaesebroeck et al, 2010).

In addition, a range of chemo- attractants activating G protein-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and Toll-like/ IL-1 receptors (TLR/IL1Rs) initiate tumor inflammation by activating the PI3K in $Gr1^{+}CD11b^{+}$ myeloid cells (Schmid et al, 2011). Tumor derived chemoattractants stimulating myeloid cell RTKs, TLR/IL1Rs, and GPCRs activate a single PI3-kinase isoform, p110g, and a single integrin, a4b1, to promote myeloid cell recruitment to tumors and tumor progression(Schmid et al, 2011). Myeloid cell p110g is unexpectedly activated by RTKs and TLR/IL1Rs via Ras and p87, refuting current dogma that p110g is activated only by GPCRs. Moreover PI3Kg is a single convergent point controlling tumor inflammation and progression. Selective inhibitors of p110g

could thus serve as therapeutics to suppress tumor malignancy by blocking diverse pathway promoting tumor inflammation (Schmid et al, 2011).

Animal modeling has shown that inhibition of PI3K is protective in experimental colitis (Brown et al, 2010; Gonzalez-Garcia et al, 2010; Lee et al, 2010; Strouch et al, 2010). Knowledge of the PI3K activity in pre-neoplastic tissue and tumor microenvironment is therefore important for understanding mechanisms of carcinogenesis and mode of action of PI3K targeting drugs that are currently being tested for clinical treatment of cancer (Chappell et al, 2011).

1.8 Role of LY294002 as a PI3K inhibitor

LY294002 is a chemical inhibitor of PI3K that has been used alone or in combination with chemotherapeutic agent to control tumor cell growth *in vitro* and in experimental animal models (Balkwill & Mantovani, 2001; Bernstein et al, 2001; Ching et al, 2001; Erez & Coussens, 2011; Heinemann et al, 2000; Makitie et al, 2001; Meng et al, 2006; Ng et al, 2001; Philp et al, 2001; Vlahos et al, 1994a; Workman, 2004). LY294002 competitively inhibits ATP binding to the catalytic subunit of PI 3-kinases and does not inhibit PI4-kinase, DAG-kinase, PKC, PKA, MAPK, S6 kinase, EGFR or c-src tyrosine kinases and rabbit kidney ATPase (Fruman et al, 1998; Fruman et al, 1999; Garlich et al, 2008; Rameh & Cantley, 1999; Scharenberg et al, 1998; Vlahos et al, 1995; Vlahos et al, 1994b). However, LY294002 is too insoluble for clinical investigation as a drug, although a prodrug derivative, SF1126 (**Figure 4**) has now entered human clinical trials as a pan-PI3K inhibitor, targeting cell growth, proliferation and angiogenesis(Garlich et al, 2008).

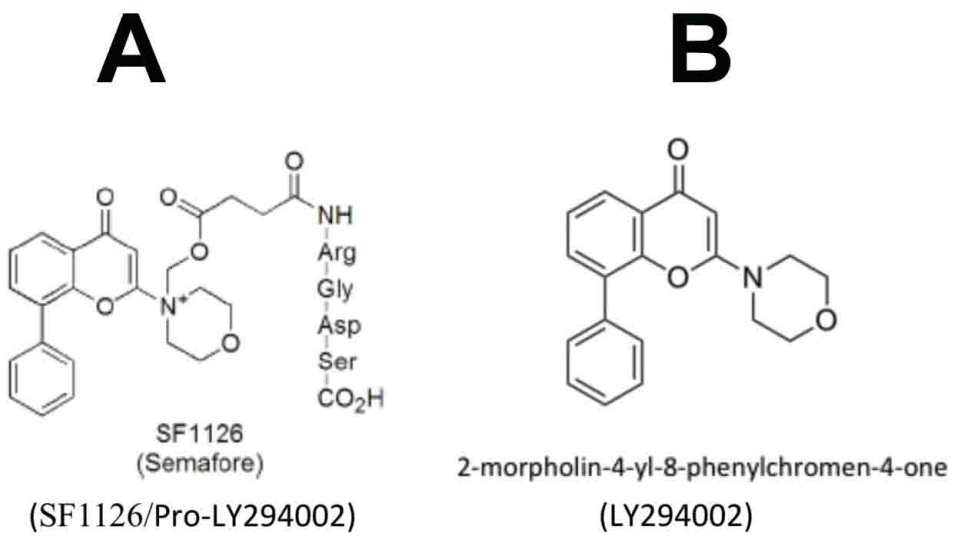

Figure 4. Chemical structure of (A) LY294002 and (B) pro-drug of LY294002 (SF1126), [Modified and adapted from (Garlich et al, 2008)]

LY294002 can directly affect cancer cells proliferation, invasion and metastasis (Lewis & Pollard, 2006).

1.9 Aims of the study

The aim of this project was to test the role of PI3K/AKT in the inflammatory subsets and investigate whether their tumor promoting properties are dependent of PI3K/AKT.

For these studies a chemical PI3K inhibitor LY294002 was used, an isoform of LY294002 called as SF1126 a pan-PI3K inhibitor is in human clinical trials. The potential efficacy of this approach was tested in *ex vivo* and *in vivo* experimental models of colon cancer.

Specific aims were

i) To investigate the pattern and status of PI3K/AKT activity and inflammatory cells (mainly mast cells and macrophages) in normal human colon, colitis without dysplasia, colitis induced dysplasia and ulcerative colitis induced invasive cancer.

ii) ii) To investigate the mechanistic role of PI3K/AKT in inflammatory cells in crosstalk with the tumor epithelium in context with the tumor promoting properties.

iii) iii) To investigate *in vivo* role of PI3K/AKT in inflammatory cells in context with tumor promotion and invasion in an experimental colitis associated cancer mouse model.

2.

Materials and Methods

2 Materials and Methods

2.1 Equipment and consumables

Equipment and consumables	Source
Analytic balance scale	Chemlab, USA
Cell Culture Flasks	BD Biosciences, USA
Centrifuge (high speed)	Lavei Centrifuge, USA
Centrifuge/Falcon tubes (15 and 50 ml)	BD Biosciences USA
CO2 incubator	Thermoscientific, USA
Coverslips	Tedpella, USA
Cryostat (CM 3050)	Leica Microsystems, USA
Cryotubes	Thermoscientific, USA
FACS Canto	BD Biosciences, USA
Fluorescence Microscope	Zeiss, USA
Gel documentation system	Bio-Rad, USA
Gloves	VWR, USA
Hemacytometer glasses	Fisher Scientific, USA
Ice machine	Fisher Scientific, USA
Laminar flow hood	Microflow, Airclean, USA
Light microscope	Leica, USA
Magnetic stirring hot plate	Biomega, USA
Microcentrifuge tubes (1,5 ml)	USAScientific, USA
Microscope slides	Thermofisher, USA
Microwave	Scientificamerican, USA
Neubauer hemacytometer	Sigma, USA
Parafilm M	SPI, USA
Paraffin embedding cassettes	Leica microsystems, USA
Pasteur pipettes	Sigma-Aldrich, USA
pH-meter	LIS, USA
Pipette tips (20 µL, 200 µL, 1000 µL)	USA Scientific, USA
Pipettes Gilson	Middleton, USA

Scintillation counter	LKB RackBeta, USA
Shaker (Unimax)	Heidolph, USA
Spectrophotometer	Labomed, USA
Stericup-Filter (0,22 µm)	Millipore, USA
Thermomixer	Eppendorf, USA
Tissue-tek based molds	Sakura, USA
TissueGnostics highthroughput imaging system	TissueGnostics, USA
Vortexer Gennie-2	LabX, USA
Waterbath ISOTEMP	Fisher Scientific, USA
Weighing paper	VWR, USA

2.2 Media, supplements and reagents for cell culture

Media, supplements and reagents for cell culture	**Source**
BSA (Bovine Serum Albumin)	Sigma, USA
DMSO (Dimethylsulfoxide, >99%)	Sigma-Aldrich, USA
DMEM (Dulbecco's Modified Eagle Medium, high glucose	Invitrogen, USA
Dulbecco's phosphate buffered saline	Invitrogen, USA
FBS (Fetal Bovine Serum)	Sigma,USA
HBSS, Hank's buffered salt solution (10x)	Sigma, USA
HEPES	Cellgro, USA
Gentamicin-Sulphate	Cellgro, USA
Glutamax	Cellgro, USA
McCoy's 5A medium	Sigma-Aldrich, USA

MEM non-essential amino acids	Cellgro, USA
PBS (Phosphate buffered saline)	Gibco/Invitrogen, USA
Penicillin-Streptomycin	Cellgro, USA
RPMI1640 Medium	Cellgro, USA
Sodium chloride	Sigma-Aldrich, USA
Sodium Pyruvate	CellGro, USA
Stempro	Lifetechnologies, USA
Trypanblue	Cellgro, USA
Trypsin-EDTA	Cellgro, USA

2.3 Cytokines

Cytokines	**Source**
Human Stem cell factor (SCF) for cell culture	Sigma-Aldrich, USA
Murine IL-3	Cell Signaling, USA
Murine SCF	Millipore, USA

2.4 Kits

DAB Kit	DAKO, USA
Naphthol AS-D Chloroacetate (Specific Esterase) Kit	Sigma-Aldrich, USA

2.5 Invitro assay sytems

Invitro assay sytems	**Source**
Chemo TX sytems	Neuroprobe, USA
LS column	Miltenyi Biotech, USA

Matrigel based-Invasion assay plate (24 well/12 insert)	BD Biocoat, USA

2.6 Chemicals and Biochemical

Chemicals and Biochemical	**Source**
Ammonium hydroxide	Sigma-Aldrich, USA
Antibody diluent	Dako, USA
Biotin-16-dUTP	Roche Diagnostic, USA
Boric acid	Sigma-Aldrich, USA
Bromodeoxyuridine (BRDU)	Sigma-Aldrich, USA
BSA (Bovine Serum Albumin)	Sigma-Aldrich, USA
Calcium Chloride ($CaCl_2$)	
Carboxyfluorescein succinimidyl ester (CFSE)	Sigma-Aldrich, USA
Cobalt chloride, Hexahydrate	Sigma-Aldrich, USA
Collagenase type IV	Worthington Biochemicals, USA
Dabco	Sigma-Aldrich, USA
DNase	Sigma-Aldrich, USA
DAPI (4',6-Diamidino-2-phenylindol Dihydrochlorid)	Invitrogen, USA
D-Glucose	Sigma-Aldrich, USA
Dinitrophenol-bovine serum albumin (DNP-BSA)	Sigma-Aldrich, USA
Dinitrophenol-human serum albumin (DNP-HSA)	Sigma-Aldrich, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA
Eosin Y	Sigma-Aldrich, USA
Ethanol 100%	Fisherscientific, USA
Ficoll-paque	GE-Healthcare, USA

Glacial acetic acid	Sigma-Aldrich, USA
Glycerin	Sigma-Aldrich, USA
Glycerol	Sigma-Aldrich, USA
Glycine	Sigma-Aldrich, USA
Heparin	StemCell Technologies Inc Canada
Hematoxylin solution, (Mayer's)	Sigma-Aldrich, USA
Hematoxylin solution, (Harris's II)	Sigma-Aldrich, USA
Hematoxylin solution, (Gill's II)	Sigma-Aldrich, USA
Hyaluronidase	Sigma-Aldrich, USA
Hydrochloric acid	Sigma-Aldrich, USA
Isopropanol	Sigma-Aldrich, USA
Lithium carbonate	Sigma-Aldrich, USA
LY294002	Sigma-Aldrich, USA
Magnesium Chloride ($MgCl_2$)	Sigma-Aldrich, USA
Mercaptoethanol	Sigma-Aldrich, USA
Methanol	Sigma-Aldrich, USA
NP-40	Sigma-Aldrich, USA
O.C.T. embedding medium for cryo-sections	Tissue-Tek, USA
Paraffin wax	Sigma-Aldrich, USA
Percoll	Sigma-Aldrich, USA
Phloxine B	Sigma-Aldrich, USA
Phosphotase inhibitor I and II cocktail	Sigma-Aldrich, USA
Piroxicam	Sigma-Aldrich, USA
Polyvinyl alcohol	Sigma-Aldrich, USA
PNAG (mM 4-nitrophenyl N- acetyl-β-D-glucosaminide)	Sigma-Aldrich, USA
Potassium Phosphate (K2HPO4)	Sigma-Aldrich, USA
Potassium Chloride (KCl)	Sigma-Aldrich, USA
Protease Inhibitor	Sigma-Aldrich, USA

Sodium Bicarbonate (NaHCO3)	Sigma-Aldrich, USA
Sodium Carbonate (Na2CO3)	Sigma-Aldrich, USA
Sodium Cacodylate	Sigma-Aldrich, USA
Sodium Citrate, Trihydrate	Sigma-Aldrich, USA
Sodium Chloride (NaCl)	Sigma-Aldrich, USA
Sodium Dodecyl Sulphate	Sigma-Aldrich, USA
Sodium Phosphate (Na2HPO4)	Sigma-Aldrich, USA
Sodium Tetraborate	Sigma-Aldrich, USA
Target-retrieval solution	Dako, USA
Terminal Transferase (TdT)	Roche Diagnostic, USA
Thymidine ^{3}[H]	Amersham Biotech, USA
Toluidine Blue powder	Sigma-Aldrich, USA
Tris-HCl	Sigma-Aldrich, USA
Triton X-100	Sigma-Aldrich, USA
Wash Buffer 10X	Dako, USA
Xylene	FisherScientific, USA
Xylene-based mounting meium-Micromount	Leica microsystems, USA

2.7 Antibodies

2.7.1 Antibodies for FACS

Rat anti human -PE CD11b, BD Pharmingen, USA
Iso-type control -rat IgG2b K, BD Pharmingen, USA

2.7.2 Antibodies for immunoblottting

Rabbit anti- human pAKT^{T308}, Cell Signaling, USA
Rabbit anti- human pAKT^{S473}, Cell Signaling, USA
Rabbit anti- human total AKT, Cell Signaling, USA
Rabbit anti- human beta actin, Cell Signaling, USA
Goat Anti-rabbit HRP, Dako, USA

2.7.3 Antibodies for cell isolation

Biotinylated anti-human CD11b alpha M chain, BD Biosciences, USA
Streptavidin magnetic beads, Miltenyi Biotech, USA

2.7.4 Antibodies for immunohistochemistry/immunofluorescence

Rabbit anti- mouse/human pAKT^{T308}, Cell Signaling, USA
Mouse anti-human Tryptase, Neomarker-Labvision, USA
Mouse anti-human CD68, Santacruz Biotech, USA
Mouse anti-human Mac1, Santacruz Biotech, USA
Anti-BRDU, Abcam, USA
Goat anti-rabbit Alexafluor 488 (H+L), Life technologies, Invitrogen, USA
Goat anti-mouse Alexafluor 594 (H+L), Life technologies, Invitrogen, USA
Goat-anti Rabbit HRP, Dako, USA
Streptavidin-HRP, Dako, USA

2.2 Recipes

2.2.1.Harris' Hematoxylin and Eosin (H&E)

Acid Alcohol Solution (1%)

- Hydrochloric acid, 1 ml
- 70% ethanol, 50 ml
- Mix well.

Ammonia Water Solution (0.2%)

- Ammonium hydroxide (concentrated), 2 ml
- Distilled water , 1000 ml
- Mix well.

Lithium Carbonate Solution (Saturated):

- Lithium carbonate 1.54 g
- Distilled water 100 ml
- Mix well.

Eosin-Phloxine B Solution

Prepare the stock solutions first, and then create the working solution as needed.

Eosin Stock Solution

- Eosin Y, 1 g
- Distilled water, 100 ml
- Mix to dissolve.

Phloxine Stock Solution

- Phloxine B, 1 g
- Distilled water, 100 ml
- Mix to dissolve.

Eosin-Phloxine B Working Solution

- Eosin stock solution, 100 ml
- Phloxine stock solution, 10 ml
- Ethanol (95%), 780 ml
- Glacial acetic acid, 4 ml
- Mix well.

Procedure:

- Heat to dissolve. Add 50 ml of 10% alcoholic hematoxylin solution and heat to boil for 1 minute.
- Remove from heat and slowly add 2.5 g of mercuric oxide (red).
- Heat to the solution and until it becomes dark purple color.
- Cool the solution in cold-water bath and add 20 ml of glacial acetic acid (concentrated).
- Filter.

2.2.2 TUNEL

TdT Buffer Stock Solution (125mM Tris-HCl, 1M Sodium Cacodylate, 1.25mg/ml BSA, pH 6.6)

- Tris-HCl (MW 157.6), 1.97 g
- Sodium cacodylate, Trihydrate (MW 214.0), 21.4 g
- BSA, 0.125 g
- Distilled water, 100 ml

Adjusted to pH to 6.6 and aliquot were stored at –20 ºC.

Cobalt Chloride Stock Solution (25mM Cobalt Chloride in Distilled Water)

- Cobalt chloride, Hexahydrate (MW 237.9), 0.6 g
- Distilled water, 100 ml
- Mixed to dissolve.

Aliquot were stored at –20 ºC.

TdT Reaction Buffer (25mM Tris-HCl, 200 mM Sodium Cacodylate, 0.25 mg/ml BSA, 1mM Cobalt Chloride)

- TdT Buffer Stock Solution, 40 ul
- Cobalt Chloride Stock Solution, 8 ul
- Distilled water, 160 ul

Mixed well and stored at –20 ºC

TdT Storage Buffer (60mM K-phosphate, pH 7.2, 150mM KCl, 1mM 2-Mercaptoethanol, 0.5% Triton X-100, 50% glycerol)

To make the buffer:

- K2HPO4 (MW174.18), 1.05 g
- KCl (FW 74.55), 1.12 g
- Distilled water, 50 ml

Stir to dissolve and adjust pH 7.2 using concentrated HCl. Add 50 ml of glycerin (100% glycerol), 0.5 ml of Triton X-100, and 8 ul of 2-Mercaptoethanol (99% Solution). Store at –20 ºC

Enzyme Reagent

- Terminal Transferase (TdT) (Roche Diagnostic), 4 ul
- TdT Storage Buffer, 100 ul

Mix well and store at –20 ºC

Label Reagent:

- Biotin-16-dUTP (Roche Diagnostic), 4 ul
- TdT Reaction Buffer, 1 ml

Mix well and store at –20 ºC

TdT Reaction Mixture:

- Enzyme Reagent, 100 ul
- Label Reagent, 900 ul

Mix just before use. The remaining 100 ul of Label Solution can be used for negative control.

Stop Wash Buffer (300mM NaCl, 30mM Sodium Citrate)

- NaCl (MW 58.44), 1.75 g
- Sodium citrate, Trihydrate (MW294.11), 0.88 g
- Distilled water, 100 ml

Mix to dissolve and store at room temperature.

2.2.3 Gelvatol

Step I

100 ml solution of: 0.14M NaCl

0.01M KH2PO4/Na2HPO4, pH 7.2

To the 100 ml solution, slowly 25 g polyvinyl alcohol** was added while stirring (total volume will be more than 100ml)
Stirred over night
Adjusted pH to 7.2 the next day.

Step II
50ml Glycerol was added to the solution above and stir over night (at least 16 Hours)
Suspension was spinned to remove undissolved particles
Aliquots were made and stored airtight, at least 4 C or colder.

Store opened vials at RT.

Before use: Dabco (Sigma) 100mg/ml final concentration was added. Vortexed well, then spun down in microfuge for 10 min at full speed.

2.2.4 RIPA Buffer

- Tris-Cl [pH 7.5], 10mM
- NaCl, 500mM
- SDS, 0.1 %
- NP-40, 1%
- Sodium deoxycholate,, 1%
- EDTA, 2mM
- and protease, phosphatase I and II inhibitor cocktail, 1%

2.2.5 Tyrode buffer

- HEPES, 10 mM
- NaCl, 130 mM

- D-glucose, 6.2 mM
- KCl, 3.0 mM
- $CaCl_2$, 1.4 mM
- $MgCl_2$, 1.0 mM
- and BSA, 0.1%

2.2.5 Borate buffer

- Sodium tetraborate , 7.6 g
- Boric acid, 5.0 g

Dissolve in in 4 L of water and adjust the pH to 8.5, which required approximately 100 µl of 10.0 m NaOH/L buffer.

2.2.6 Carbonate buffer, 0.1 M, pH 9.0

- 1.06 g Na_2CO_3
- 0.840 g $NaHCO_3$
- Bring to 100 ml with distilled H_2O
- Filter sterilize through 0.22-µm nylon filter

2.2.7 MACS Buffer

- PBS pH 7.2,
- EDTA, 2 mM
- BSA, 0.5 %

2.3 Methods

2.3.1 Primary and established cell lines

2.3.1.1 LAD-2

LAD-2 cells are mature human leukemic cell lines. LAD-2 cells were gift from Cleaveland Clinic, USA. LAD-2 cells were grown in stempro medium (Sigma Aldrich) with 100 ng/ml Stem Cell Factor (SCF) at 37^{o}C and 5% CO_2.

2.3.1.2 HT-29

HT-29 is human colon adenocarcinoma cell line. Packaged HT-29 cells were obtained from American Type Culture Collection (Manassas, VA, USA). HT-29 cells were cultures in McCoy's 5A medium with 10% compliment-heat inactivated fetal bovine serum, 1% Penicillin-Streptomycin, 1% MEM non-essential amino acid, 1% sodium pyruvate and 1% Glutamax.

2.3.1.3 CT44

The tumor cell line CT44 was generated by transfecting CT26 cells (a cell line derived from a chemically induced murine colon carcinoma) with a fusion protein of influenza hemagglutinin and EGFP(Chen et al, 2005b) and was a gift from Dr. Weissleder Ralph.
CT44 were cultured in DMEM (Life Techonolgies, USA) with 10% compliment-heat inactivated fetal bovine serum, 1% Penicillin-Streptomycin, 1% MEM non-essential amino acid, 1% sodium pyruvate and 1% Glutamax.

2.3.1.4 Isolation and culture of gut derived mouse mast cells (GMMCs)

Primary cell culture of GMMCs was obtained by isolating mast cell progenitors from IL-10$^{-/-}$ mice colons. Mice colons were chopped using surgical blades and subjected to collagenase digestion in RPMI at 37^{o}C for 20 min. The supernatant was centrifuged at 1600 rpm for 10 min at 37^{o}C, pellet was mixed in 40 % Percoll

(made from 4 parts of 100% Percoll and 6 parts of 1X Hank's balanced salt solution) and underlayed with 60% Percoll (made from 6 parts of 100% Percoll and 4 parts of 1X Hank's balanced salt solution) and centrifuged at 2600 rpm for 30 min (brake-off) at 37°C. Interphase was collected and plated in 25-cm^2 flasks with complete RPMI 1640 complete (RPMI with 10% compliment-heat inactivated fetal bovine serum, 1% Penicillin-Streptomycin, 1% MEM non-essential amino acid and 1% Glutamax and 1% HEPES), 20 ng/mL IL-3 and 10 ng/mL stem cell factor *(*SCF) for 3 weeks. Purity of MC was checked using flow cytometry (**Figure 5**).

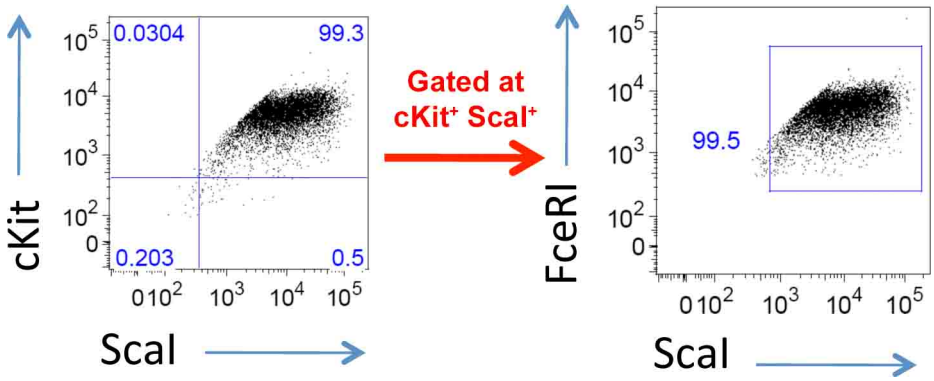

Figure 5. Purity checks of GMMCs gated at cKit⁺ScaI⁺FcerI⁺ cells. Figure depicts representative picture for GMMCs purity check.

2.3.1.5 Isolation and culture of Tumor infiltrating Leukocytes (TILs)

To obtain tumor infiltrating leukocytes surgical colon cancer tissue samples from 3 patients bearing UC associated colon cancer were washed with (DMEM with 0.5% penicillin/streptomycin, 10 µg/mL gentamycin sulfate), minced with surgical blades, digested using (750 U/mL type IV collagenase, Worthington Bio-chemical; 500 U/mg hyaluronidase, Sigma; 0.1 µg/mL DNase, Sigma) and subjected to Percoll gradient centrifugation (40–80%) in 15 ml falcon tubes. Tubes were centrifuged at 2600 rpm for 30 min at 37°C brake-off. Interphase was collected washed 3X with complete RPMI and centrifuged at 1600 rpm for 10 min at 37°C, pellet was resuspended finally in complete RPMI and incubated at 37°C.

2.3.2 Culture of established cell lines

Cells were cultured in an incubator at 37°C and 5% CO2. Media were supplemented, if not indicated differently, with 10% [v/v] FBS and 10% compliment-heat inactivated fetal bovine serum, 1% Penicillin-Streptomycin, 1% MEM non-essential amino acid and 1% Glutamax. Media were prewarmed at 37°C prior to use. FBS was heat-inactivated for compliments for 30 min at 56°C. For quantification and determination of the number of viable cells, cell suspension was diluted in 1:1 ration in Trypan blue solution (0.125%).
Cells were counted using Neubauer counting chamber and coverslip.

2.3.2.1 Passaging of adherent cell lines

Media from 70-90% confluent cell cultures were aspirated and cells were washed twice with 10 ml PBS. After aspirating PBS, 3 ml 1xTrypsin was added and cells were incubated at 37°C and 5% CO2 until they were completely detached. Trypsin was inactivated by addition of 10% FBS-supplemented culture medium. Cells were thoroughly resuspended followed by determination of cell concentration and split ratio.

2.3.2.2 Cryostorage of eukaryotic cell lines

Established cell lines were frozen at -80 °C short-term storage and at liquid nitrogen at 320 F for long-term storage. Cells were pelleted at 1600 rpm for 10 min. Aliquots of $3x10^6$-$8x10^6$ cells were resuspended in heat-inactivated FCS and 10% (v/v) DMSO in a total volume of 1 ml in cryotubes. To allow a constant decrease in the temperature at a rate of 1 °C per minute, cryotubes were placed in a freezing container filled with isopropanol. Subsequently, cryotubes were immediately transferred to the -80 °C freezer for overnight and then liquid nitrogen for infinity.

2.3.2.3 Thawing of cells

In order to avoid the toxic side effects of DMSO in freezing medium, thawing of

cells was performed as fast as possible. Cryotubes were placed in the waterbath at 37°C until a small piece of ice was still visible in the cryotube. Prewarmed culture medium was added drop by drop in the cryotube and then sucked in the pipette and subsequently cell suspension was transferred to 15 ml falcon tube in total of 10 ml of volume. After centrifugation at 1600 rpm for 10 min, supernatant was removed and pellet was carefully washed two times in the culture medium and finally resuspended in fresh culture medium in a new culture flask. Media were changed 24 h later, in order to remove the remnants of toxic dimethyl sulphoxide (DMSO) and dead cells. Cells were passaged after 2-3 days depending on cell growth rate.

2.3.2 Tissue and tumor specimens

Paraffin embedded specimens of normal, non-inflamed colon from 8 patients who had surgery for non-malignant lesions like colonic AVM or diverticular disease were used as control [normal group]. Additionally, surgical specimens from 12 UC patients with active colitis [colitis group], 7 UC patients with active colitis , dysplasia (dysplasia group) and 7 UC patients with colitis and invasive colorectal cancer (cancer group) were obtained from Rush University Medical Center, Chicago. All procedures were approved by Rush University Medical Center Institutional Review Boards.

2.3.3 Mice

IL-$10^{-/-}$ mice and C57LB6 mice were obtained from Jackson laboratories. Mice were maintained under specific pathogen-free conditions at Northwestern University Animal Care Facility, and Animal Care Usage Committee of Northwestern University approved all experiments. IL-$10^{-/-}$ mice (6 weeks old) were transferred to conventional housing and allowed 1 week to acclimate.

2.3.3.1 Piroxicam and LY294002 treatment

To synchronize colitis, 6-week-old *IL-$10^{-/-}$* mice were fed pellet-chow containing

piroxicam (Sigma) for 2 weeks; for first week 65mg/250g piroxicam was fed followed by 85 mg/250g the next week (Harlan Teklad custom diet). LY294002 (Sigma) were IP-injected at 50 mg/kg dissolved in 20% dimethyl sulfoxide (Sigma) every other day (**Figure 6**). Untreated mice received dimethyl sulfoxide.

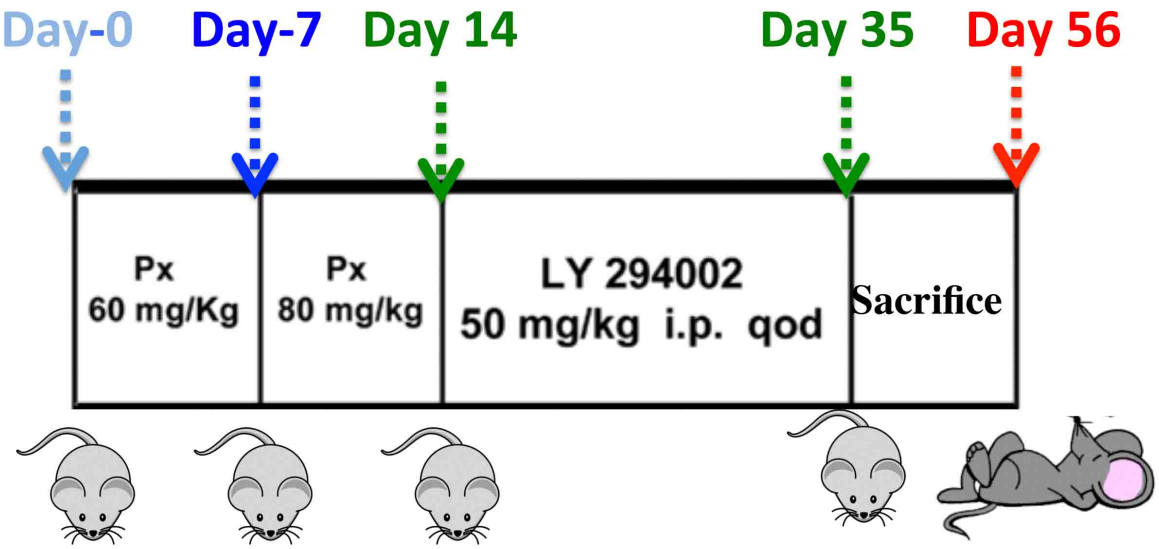

Figure 6. Timeline of colitis induction with piroxicam in *IL-10_/_* mice and LY294002 treatment [Modified and adapted from (Lee et al, 2010)].

2.3.3.2 Bromodeoxyuridine (BRDU) incorporation

Mice were injected intra-peritoneally with 1 mg of bromodeoxyuridine (BrdU; Sigma) 2 hours before death.

2.3.3.3 Fixing and embedding of mice colon

Colons, were collected, cleaned for feces using PBS, fillet-laid opened and fixed in 10% formalin for 12 hours, followed by rolling and placing in embedding-cassettes in 70% ethanol until paraffin embedding. Colons in embedding cassettes were dehydrated for paraffin embedding (water to paraffin) as per following steps:

- Colons were passed through 70% ethanol, 2 changes, 1h each

- Followed by, 80% ethanol, 2 changes, 1h each
- 95% ethanol, 2 changes, 1h each
- 100% ethanol, 3 changes, 1h each
- Xylene or substitute (i.e. Clear Rite 3), 3 changes, 1h each
- Paraffin wax (56-58ºC), 2 changes, 1.5h each
- and finally tissues were embedded into paraffin blocks

2.3.4 Chemical staining

2.3.4.1 Hematoxylin and Eosin staining (H&E staining)

4 µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water and stained in Harris hematoxylin solution for 8 minutes. Subsequently the sections were washed in running tap water for 5 minutes and placed in 1% acid alcohol for 30 seconds followed again by washing with tap water for 1 minute. Next, bluing was performed using 0.2% ammonia water or saturated lithium carbonate solution for 30 seconds to 1 minute. Sections were washed in running tap water for 5 minutes, rinsed in 95% alcohol for 10 dips and counterstain in eosin-phloxine solution for 30 seconds to 1 minute. Finally, sections were dehydrated through 95% alcohol with 2 changes of absolute alcohol for 5 minutes each, cleared in 2 changes of xylene for 5 minutes each and mounted with xylene based mounting medium.

2.3.4.2 Chloroacetate esterase staining

4 µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95%

alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water and sections were stained with Naphthol-AS-D chloroacetate for 20 min, washed in tap water for 5 min and counterstained with Hematoxylin Gills II for 30 seconds. Finally sections were washed in water and dehydrated through 95% alcohol with 2 changes of absolute alcohol for 5 minutes each, cleared in 2 changes of xylene for 5 minutes each and mounted with xylene based mounting medium.

2.3.4.3 Toluidine Blue Staining

4 µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water and sections were stained with Toluidine Blue working solution for 20 minutes, washed in tap water for 5 min. Finally sections were washed in water and dehydrated through 95% alcohol with 2 changes of absolute alcohol for 5 minutes each, cleared in 2 changes of xylene for 5 minutes each and mounted with xylene based mounting medium.

2.3.4.4 BRDU staining

4µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water. Sections were washed in 0.1M Phosphate Buffered Saline (PBS) (pH 7.4) with 1% Triton X100 (3x for 5 minutes). Sections were incubated in HCl (1N) for 10 minutes on ice to break open the DNA structure of the labeled cells. This was followed by HCl (2N) for 10 minutes at room temperature before moving them to incubator for 20 minutes at 37°C.

Immediately after the acid washes, Borate buffer (0.1M) is added to buffer the cells for 12 minutes at room temperature. Samples were then washed in 0.1M PBS (pH 7.4) with 1% TritonX100 (3x 5 minutes) at room temperature. Next, sections were incubated in 0.1M PBS (pH 7.4) + 1% TritonX100 + Glycine (1M) + 1% BSA (1hr) prior to incubating overnight at room temperature with anti-BrdU. Following the incubation overnight sections were washed in 0.1M PBS (pH 7.4) with 1% TritonX100 (3x 5min). Finally, sections were labeled with HRP labeled secondary antibodies, washed in PBS 2x for 5 minutes, counterstained with Hematoxylin Gill's II for 30 seconds, washed in water, dehydrated in 95% and 100% ethanol, cleared in xylene and mounted in xylene based mounting medium.

2.3.4.5 TUNEL staining

4 µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water. Sections were pretreated using proteinase K digestion method, followed by incubation of sections in 3% H_2O_2 in PBS for 10 minutes to block endogenous peroxidase activity, incubated in TdT Reaction Buffer for 10 minutes, followed by TdT Reaction Mixture for 1-2 hours at 37-40 °C in humidified chamber. Reaction was stopped using stop wash buffer for 10 minutes. Sections were incubated with Streptavidin-HRP in PBS for 20 minutes at room temperature, followed by DAB for 1-2 minutes, washed in running tap water for 5 minutes and counterstained with Gill's hematoxylin for 30 seconds. Sections were dehydrated through 95% ethanol for 5 minutes, 100% ethanol for 2x for 3 minutes, cleared in xylene for 2x for 5 minutes and mounted with xylene based mounting medium. Sections were washed in intermittent steps with PBS-Tween 20 for 3x for 2 minutes each.

2.3.5 Immunohistochemistry for pAKT or Tryptase staining

4 µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water and antigen retrieval was performed at de-cloaking chamber at 120^{o}C for 30 seconds and 90^{o}C for 10 seconds using Dako target-retrieval solution, peroxidase blocking was performed using Dako peroxidase block for 20 min and sections were additionally blocked using 1% bovine serum albumin (BSA). Rabbit anti-mouse/human pAKT or Mouse anti-human Tryptase was applied for overnight at 1:50 dilution (in Dako antibody-diluent) at 4^{o}C, next day washed with 2X with Dako wash buffer 5 minutes each and incubated with Dako anti-rabbit HRP or Dako anti-mouse HRP secondary antibodies for 1 hour at room temperature. Sections were washed 2X with Dako wash buffer 5 minutes each and DAB substrate was applied for 60 seconds and reaction was stopped in tap water for 5 minutes. Sections were finally counterstained using Gill's II hematoxylin, washed in water, dehydrated through 95% ethanol for 5 minutes, 100% ethanol for 2x for 3 minutes, cleared in xylene for 2x for 5 minutes and mounted with xylene based mounting medium.

2.3.6 Double Immunofluorescence

2.3.6.1 Double Immunofluorescence staining for CD68 and pAKT

4 µm thick paraffin sections were cut and placed on charged glass slides, subsequently air-dried, baked in hot-air oven at 50^{o}C for 30 min and deparaffinized in two changes of xylene for 10 minutes each. Next, sections were re-hydrated in 2 changes of absolute alcohol for 5 minutes each followed by 95% alcohol for 2 minutes and 70% alcohol for 2 minutes. Sections were washed briefly in distilled water and antigen retrieval was performed at de-cloaking chamber at 120^{o}C for 30 seconds and 90^{o}C for 10 seconds using Dako target-

retrieval solution, peroxidase blocking was performed using Dako peroxidase block for 20 min and sections were additionally blocked using 1% bovine serum albumin (BSA). Rabbit anti- human pAKT and Mouse anti-human CD68 was applied for overnight at 1:50 dilution (in Dako antibody-diluent) at 4^{o}C, next day washed with 2X with Dako wash buffer 5 minutes each and incubated at 1:150 dilutions with anti-rabbit Alexafluor488 and anti-mouse Alexafluor594 secondary antibodies for 1 hour at room temperature in dark. Sections were washed 2X with Dako wash buffer 5 minutes each incubated with 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes. Sections were finally washed in PBS 3x for 5 minute each and mounted with Gelvatol.

2.3.6.2 Double Immunofluorescence staining for Mac1 and pAKT

5 µm thick frozen sections embedded in OCT were cut, placed on glass charged slides and incubated in PBS for 10 minutes or until sections are free from OCT at room temperature. Sections were fixed in ice-cold methanol at -20^{o}C for 15 minutes, washed 2x in PBS at room temperature and blocked with 1%BSA.). Rabbit anti-human pAKT and Mouse anti-human Mac1 was applied for overnight at 1:50 dilution (in Dako antibody-diluent) at 4^{o}C, next day washed with 2X with Dako wash buffer 5 minutes each and incubated at 1:150 dilutions with anti-rabbit Alexafluor488 and anti-mouse Alexafluor594 secondary antibodies for 1 hour at room temperature in dark. Sections were washed 2X with Dako wash buffer 5 minutes each incubated with 4',6-diamidino-2-phenylindole (DAPI) for 15 minutes. Sections were finally washed in PBS 3x for 5 minute each and mounted with Gelvatol.

2.3.7 TissueGnostics

TissueGnostics Tissue/Cell High Throughput Imaging and Analysis System and semi-automated image acquisition microscope was used to acquire 200X magnification brightfield and fluorescence images throughout the section. Images were stitched in Adobe Photoshop Program and analyzed using ImageJ software. Total nuclei count was calculated using particle analysis software for

either hematoxylin or DAPI positive nuclei.

2.3.8 Immunoblotting for MC and TILs

LAD-2 MC or tumor infiltrating leukocytes (TILs) either treated with carrier DMSO or 10 μM LY294002 for 1 hour at 37^{o}C were washed 3X in PBS for 5 minutes each and centrifuged at 1000 rpm for 10 minutes, pellet was used for immunobloting. Whole-cell extracts were prepared in RIPA buffer. Proteins (30ug) were separated by SDS-PAGE, transferred following standard protocols. Immunoreactive proteins were detected with antibodies to phospho-AKT T308, phospho-AKT S473, total AKT (Cell Signaling) and beta-actin (Sigma) using the HRP-conjugated secondary antibodies and SuperSignal chemiluminescent reagent (Thermo Scientific).

2.3.9 ß- hexososaminidase release (mast cell degranulation) assay

For degranulation assays GMMCs were stimulated overnight using mouse anti-DNP IgE 1ug/ml concentration and LAD-2 MC were stimulated overnight using human anti-DNP 1ug/ml concentration. Next day cells were harvested, excess IgE was washed with Tyrode buffer and treated for 120 minutes either with 10 μM LY294002 or the carrier and subsequently challenged with DNP-BSA or DNP-HSA from Sigma at 100ng/ml for 30 min. The supernatant was collected and stored at 4^{o}C the pellet was lysed with 0.1% TritonX. The 20 μl of supernatant or pellet lysate were incubated with 1 mM 4-nitrophenyl N- acetyl-β-D-glucosaminide (PNAG) for 60 minutes at 37^{o}C and reaction was stopped with 200 μl carbonate buffer (0.1 M, pH 10). ß-hexosaminidase release in the supernatant was measured at 405 absorbance and interpreted as the % of total cellular (lysate + supernatant) ß-hexosaminidase.

2.3.10 Conditioned medium

For the production of conditioned medium, 2x10^{6}/ml LAD-2 MC or GMMCs or 1.5x10^{6}/750 μl TILs were treated with carrier DMSO or 10 μM LY294002 for 1

hour washed 4x with serum-free medium and kept in fresh culture for 1 week (LAD-2 MC or GMMCs) and 72 hours (TILs) at 37^{o}C and 5% CO_2. The conditioned medium was removed, filtered using 0.22-µm filters, and used for medium transfer experiments.

2.3.11 Macrophage Migration assay

2.3.11.1 Isolation of CD11b from blood of healthy donors

Peripheral blood from healthy donors was collected in heparinized vials. Blood was transferred in the 15 ml falcon tubes. Ficoll-Paque from GE Healthcare was underlayed and tubes were centrifuged at 1600 rpm for 20 minutes with break-off at 37^{o}C. Interphase of mononuclear cells was collected, washed 2X in PBS with 1% BSA. Mononuclear cells were incubated at 1:50 of anti-human biotinylated anti-CD11b, alpha M chain (BD Biosciences) on ice for 15 minutes. Cells were washed 3x in PBS with 1% BSA and centrifuged at 1600 rpm for 10 minutes. Pellet of mononuclear cells was brought up in streptavidin magnetic beads (Miltenyi Biotech) and incubated for 10 minutes in ice. Finally, cells were washed 3x in PBS with 1% BSA and centrifuged at 1600 rpm for 10 minutes and finally resuspended in MACS buffer. LS column (Miltenyi Biotic) was pre-wetted with MACS buffer 3x before passage of cells, cells in MACS buffer were passed through LS column, finally column was washed 3x with MACS buffer to remove any unbound cells from the column. Attached $CD11b^{+}$ cells were collected in MACS buffer and checked for the purity using flow cytometry.

2.3.11.2 Migration assay

For migration assay, $CD11b^{+}$ cells were resuspended in serum-free RPMI at 10^{6}/ml concentration in 22.5 µl and seeded in triplicates on top well of 5 micron uncoated 96-well Chemo TX system (Neuro Probe, Gaithersburg, MD). The bottom well was loaded with 29 µl of Stempro medium with 100 ng/ml SCF, or LAD-2 mast cell conditioned medium without or with 10µm LY294002, or

conditioned medium obtained from LAD-2 mast cells that were pretreated with 10 μM LY294002. After 3 hours of incubation at 37°C, the migrated $CD11b^+$ cells were counted with trypan blue on hemocytometer. Assays were performed in triplicates and repeated three times with different healthy donors.

2.3.12 Colon cancer epithelial cell proliferation assay

2.3.12.1 Cell harvestaion

HT-29 cells or CT44 cells were allowed to grow to the confluency of 50-60% in 75-cm^2 cell culture flasks. Cells were washed 2x with 10 ml PBS pre-warmed at 37°C in water bath, PBS was aspirated, 3 ml of 1x Trypsin was added cells to the cells and flask was incubated at 37°C until cells wells trypsinized and detached completely. 10 ml of complete McCoy's 5A medium or complete DMEM was added to stop the action of trypsin on the respective cell lines and cell-number was counted using 10 μl of the cell suspension and 10 μl of the Trypan blue dye at (1:1 ratio) in the Neubauer's Chamber. Cells were transferred to a 15 ml falcon tube and centrifuged for 1600 rpm for 10 minutes. Pellet was resuspended and washed 3x using 10 ml of McCoy's 5A or complete DMEM and subsequent centrifugation at 1600 rpm for 10 minutes at 37°C.

2.3.12.2 Epithelial-cell proliferation assay

Pellet obtained from cell harvestation was finally resuspended in the McCoy's 5A or complete DMEM, such that final concentrations of cells were 1 x 10^4/ 100 μl. HT-29 cells were seeded in triplicates in 100 μl per well in a sterile 96 well flat-bottom plate for 24 hours at 37°C and 5% CO_2. For HT-29 proliferation assay studies, next day 100 μl of either fresh complete McCoy's 5A or Stempro with 100 ng/ml SCF or LAD-2 mast cell conditioned medium or LAD-2 mast cell conditioned medium with 10 μM LY294002 or conditioned medium obtained after

10 µM LY294002 treatment of LAD-2 mast cells were added and incubated for 24, 48 and 72 hours at 37^{o}C and 5% CO_2, separate 96 well plates were used for each time-point analysis. For CT44 epithelial proliferation assay, next day either 100 µl of conditioned medium obtained from carrier DMSO or 10 µM LY294002 treated GMMCs was added and incubated for 24, 48 and 72 hours at 37^{o}C and 5% CO_2, separate 96 well plates were used for each time-point analysis.0.5mCurie of [^{3}H] Thymidine was added to each well and incubated for 6 hours after 24, 48 and 72 hours time points. HT-29 and CT44 cell proliferation was measured using a scintillation counter (LKB RackBeta; Wallac).

2.3.13 Colon cancer epithelial cell invasion assay

Cells were harvested as described in cell harvestation section of colon cancer epithelial cell proliferation assay.

Matrigel-based invasion assay plates were kept at -20^{o}C and were taken out only few hours before actual assay under sterile conditions in a Laminar-flow hood and were allowed to gain the room temperature. Inserts and invasion assay bottom chambers were added with 500 µl of pre-warmed, sterile serum-free respective medium (McCoy's 5A or DMEM) for 2 hours.

2.3.13.1 Invasion assay for the study of the role of PI3K/AKT in LAD-2 MC based colon cancer epithelial invasion

Pellet for HT-29 cells obtained from cell harvestation was resuspended in serum-free McCoy's 5A medium such that the final concentrations of cells in the cell suspension was 6x10^{4} cells/ml. Next, 500 µl of serum-free McCoy's 5A was aspirated from the invasion assay bottom chambers and inserts. To the bottom chamber 750 µl of the Stempro medium with 100 ng/ml SCF, or LAD-2 mast cell conditioned medium without or with 10µm LY294002, or conditioned medium obtained from LAD-2 mast cells that were pretreated with 10 µM LY294002 were added. 3x10^{4} colon cancer epithelial cells HT-29 were seeded in triplicates in 500 µl per well of serum-free McCoy's 5A in the top wells (inserts) in the 24 well (12)

insert invasion assay plate. After 48 hours of incubation at 37°C and 5% CO_2 the invasion assay was stopped. Medium from the inserts was aspirated and non-invaded cells were removed; the inserts were passed through 2X methanol for 5 minutes each for fixing the invaded cells in the membrane and subsequently staining with Toluidine blue working solution for 5 minutes. Invading cells were counted using a brightfield microscope.

2.3.13.2 Invasion assay for the study of the role of PI3K/AKT in TILs based colon cancer epithelial invasion

Invasion assay for TILS was performed in two different experimental setus. In setup 1, after warming the invasion assay plate and pre-wetting for 2 hours with serum-free DMEM, 3 x 10^4 CFSE labeled HT-29 cells were seeded in triplicates in the top wells (inserts) of invasion assay plate and in the lower chamber conditioned medium from TILs was added. In experimental setup 2, CFSE labeled 3 x 10^4 HT-29 cells were seeded either alone, or in 1:1 ratio with TILs untreated or treated with 10 μM LY294002 in the top wells for co-culture, the bottom wells were filled with complete McCoy's 5A. After incubating the invasion assay plate for 48 hours at 37°C and 5% CO2, tumor invasion was manually recorded and quantified as described in **2.3.13.1**.

2.3.13.3 Invasion assay for the study of the role of PI3K/AKT in GMMC based mouse colon cancer epithelial invasion

3 x 10^4 CT44 cells were seeded in top wells in serum free DMEM in triplicates, in bottom wells conditioned medium obtained from LY294002 or carrier DMSO pre-treated primary mouse mast cells (GMMCs) medium was added in the invasion assay chamber (BD biocoat). Remaining assay was performed as described in **2.3.13.1.**

2.3.14 Statistical analysis

All experiments were repeated 3 times and at least 10 mice were used in each

group. Comparison of groups was assessed using the Student *t* test. *P* values lower than 0.05 were considered statistically significant.

3.

Results

3 Results

Stromal interactions that sustain chronic inflammation and predispose to cancer are poorly understood. Experimental models of colitis implicate the PI3K pathway in activation of gut enterocytes and tissue remodeling. While mouse models are important for gaining mechanistic insights into diseases that affect us, validation of the findings in humans remains the only way to evaluate their clinical relevance. Here, in this study it is shown that in contrast to mouse models of colitis by far the greatest fraction of PI3K active cells are tissue infiltrating pro-inflammatory cells. A potent inhibitor of PI3K that is currently in clinical use, in combination with *ex vivo* assays and animal modeling was used in this study to elucidate the contribution of PI3K activity towards recruitment of inflammatory cells and predisposition to cancer. These findings point to the stromal interactions as the prime site of action of PI3K inhibitors in prevention and therapy of inflammation induced colon cancer.

3.1: Characterization and selection of the paraffin embedded colonic tissue specimens from patients

To understand the spatial distribution and kinetics of inflammation and PI3K activity *in situ* during progression from colitis to cancer, human surgical specimens were separated in four groups according to their histopathological and clinical findings namely 1) no colitis no dysplasia (designated “normal” in this study), 2) ulcerative colitis without dysplasia (colitis), 3) ulcerative colitis with dysplasia (dysplasia) and 4) ulcerative colitis with invasive colorectal cancer (cancer)(**Figure 7A** and **Table 1-4**) and the study was distributed in mucosal and submucosal findings (**Figure 7B and 7C**). For mucosal tissue data was analyzed from tissue above muscularis mucosa and extended to lumen, that included epithelium, lamina propria and mucularis mucosa. Tissue underneath muscularis mucosa was considered for the submucosal tissue analysis **(Figure 7)**.

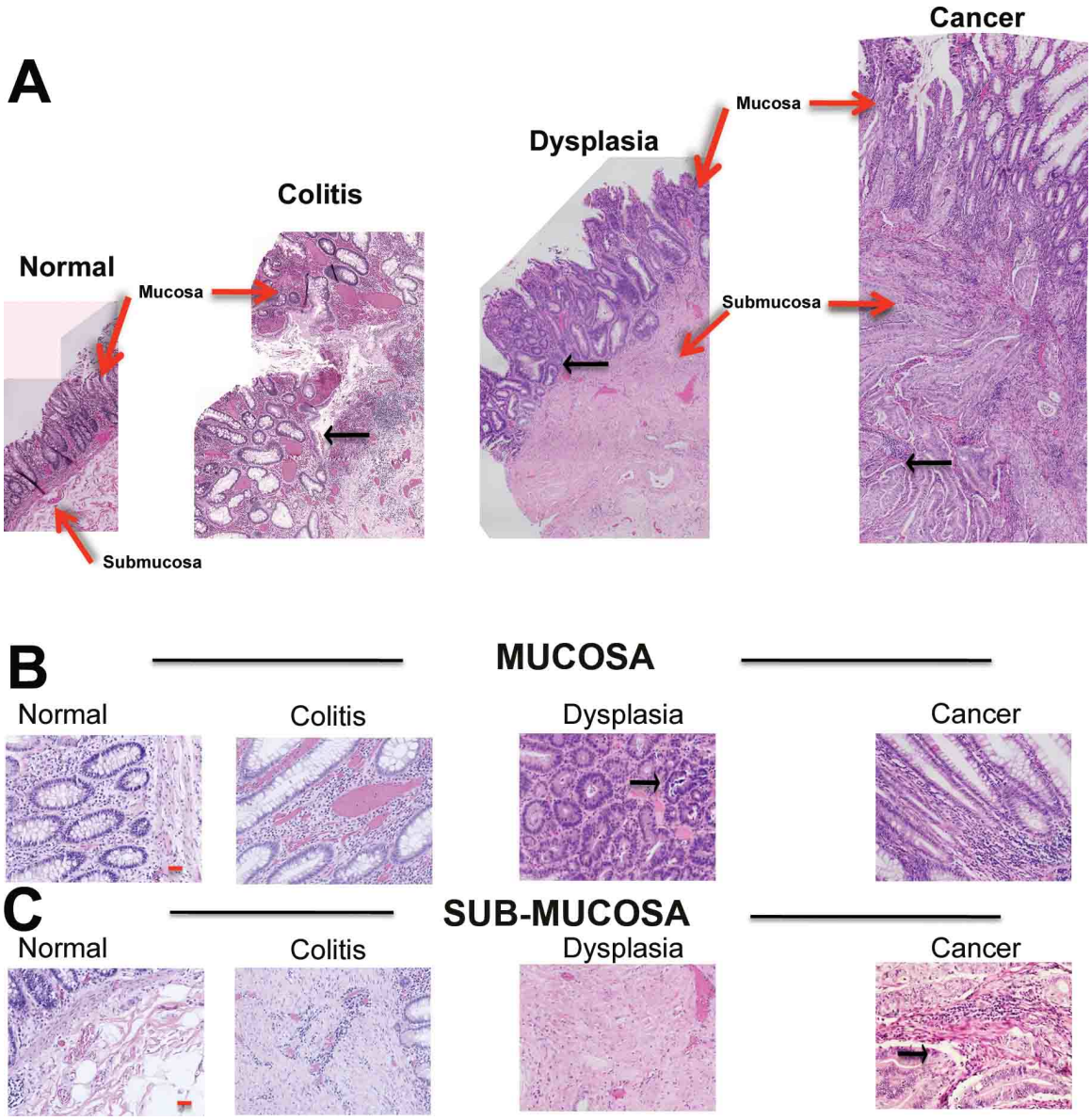

Figure 7. Categorization of patient tissue specimens using Haematoxylin and Eosin staining. (A) Tissuegnostic acquired stitched images of Normal, Colitis, Dysplasia and Invasive cancer. (B) H&E staining of mucosal tissue of Normal, Colitis (black arrow indicates ulcer), Dysplasia (black arrow indicates dysplastic crypts) and Invasive cancer. (C) H&E staining of submucosal tissue of Normal, Colitis, Dysplasia and Invasive cancer (black arrow indicates invaded crypts). Red arrows show mucosal and submucosal tissue. Red scale bar = 50 μm.

In total 8 normal, 11 UC without Dysplasia, 7 UC with Dysplasia and 8 UC with invasive Cancer patient specimen were used in this study.

	Sex	Mean Age	Race	Disease Course (≥ 5 years)	PanColitis	Active Colitis
Controls	4/8	49.8 (34-73)	4/8	NA	NA	NA
UC without Dysplasia	4/11	39.2 (19-66)	6/11	7/11	10/11	8/11
UC with Dysplasia	4/7	53.4 (25-75)	6/7	3/7	6/7	6/7
UC with CRC	6/7	51.4 (37-71)	6/7	5/7	7/7	4/7

Table.1 Patient Cohorts. Sex is defined as ratio of male over total. Age is defined as age at time of biopsy with range. Race is listed as Caucasian race versus other. Disease course is defined as a diagnosed presence ≥ 5 years. "Active Colitis" is defined at time of biopsy as equal to or greater to mild colitis but does not include "quiescent" or inactive colitis. Controls defined as no dysplasia and no evidence of colitis. NA=not applicable

3.1.1 Ulcerative colitis patients without dysplasia used in the study

In total 11 patient samples were used for immunohistochemical analysis in this study.

Sex	Race	Age	Disease Course (years)	Disease Extent	Severity of Colitis
F	AA	40	5	Left	S
F	C	58	2	Pan	S
F	AA	33	2	Pan	S
F	C	26	3	Pan	M
F	C	21	7	Pan	M
M	C	28	>10	Pan	M
F	AA	66	>10	Pan	Q
M	C	62	>5	Pan	Q
M	H	44	3	Pan	Q
F	C	19	5	Pan	M
M	H	62	5	Pan	S

Table 2. Ulcerative Colitis Patients Absent Colonic Dysplasia. F=female, M=Male, C=Caucasian, AA=African American, H=Hispanic. Patients' Age at time of biopsy is listed. Colitis extent is defined as either "pan" colitis when extends proximally past the splenic flexure. Severity of colitis is defined as Q=quiescent, MI=mild, M=moderate, S=severe.

3.1.2 Ulcerative Colitis patients with Dysplasia used in this study

In total 7 patient samples were used for immunohistochemical analysis in this study.

Dysplasia	Sex	Race	Age	Disease Course (years)	Disease Extent	Severity of Colitis
Low	M	C	74	2	Pan	M
Low	F	H	54	>20	Pan	M
High	F	C	75	7	Pan	M
Low	M	C	46	NA	Pan	MI
Low	F	C	25	9	Pan	NA
Low	M	C	52	3	Left	M
High	M	C	48	NA	Pan	M

Table 3. Ulcerative Colitis patients with Dysplasia. Patients' degree of dysplasia is defined as high or low grade dysplasia based on the maximum degree of dysplasia identified. F=female, M=Male. C=Caucasian, H=Hispanic. Age at time of colectomy is listed. Colitis extent is defined as either "pan" colitis when extends proximally past the splenic flexure. Severity of colitis is defined as Q=quiescent, MI=mild, M=moderate, S=severe.

3.1.3 Ulcerative Colitis patients with invasive cancer used in this study

In total 7 patient samples were used for immunohistochemical analysis in this study.

Cancer	Stage	Tumor differentiation	Sex	Race	Age	Length of Disease Course (years)	Disease Extent	Severity of Colitis
Cancer 1	T2N0	M	M	C	71	>10	pan	Q
Cancer 2	T3N0	P	F	C	45	2	pan	M
Cancer 3	T2N0	M	M	AA	63	1	pan	M
Cancer 4	T3N0	M	M	C	53	>10	pan	Q
Cancer 5	T3N2	W	M	C	48	>10	pan	M
Cancer 6	T3N1	M	M	C	43	>10	pan	S
Cancer 7	T3N0	P	M	C	37	>10	pan	MI

Table 4. Patients diagnosed with ulcerative colitis and associated colorectal cancers. All cancers come from specimens with evidence of prior colitis in the location of the neoplasia at time of colectomy. Tumor differentiation is defined as either W=well differentiated, M=moderately differentiated or p=poorly differentiated. Race defined as AA= African American, C=Caucasian. Age is defined as age at time of colectomy specimen. Disease extent= defined as pan=pancolitis extending proximally from the splenic flexure. Severity of colitis is defined as Q=quiescent, Mi=mild, M=moderate, S=severe.

3.2 Immunohistological study of human patient tissue specimens

3.2.1 Bone marrow derived pAKT positive cells progressively increase in colitis, dysplasia, and colon cancer

Paraffin embedded tissue specimens from the patients **(Table 1-4)** were cut in 4 µm thick sections and were stained using antibodies against pAKT. TissueGnostics high-throughput Imaging microscope was used to acquire pictures at 200X magnification of the entire tissue section. Images were stitched using Adobe Photoshop Program and mucosal and submucosal tissue-data analysis was performed using ImageJ software on 50 fields of vision. $pAKT^+$ cells were detected by immunohistology in mucosa (**Figure 8A)** and sub-mucosa (**Figure 8B**).

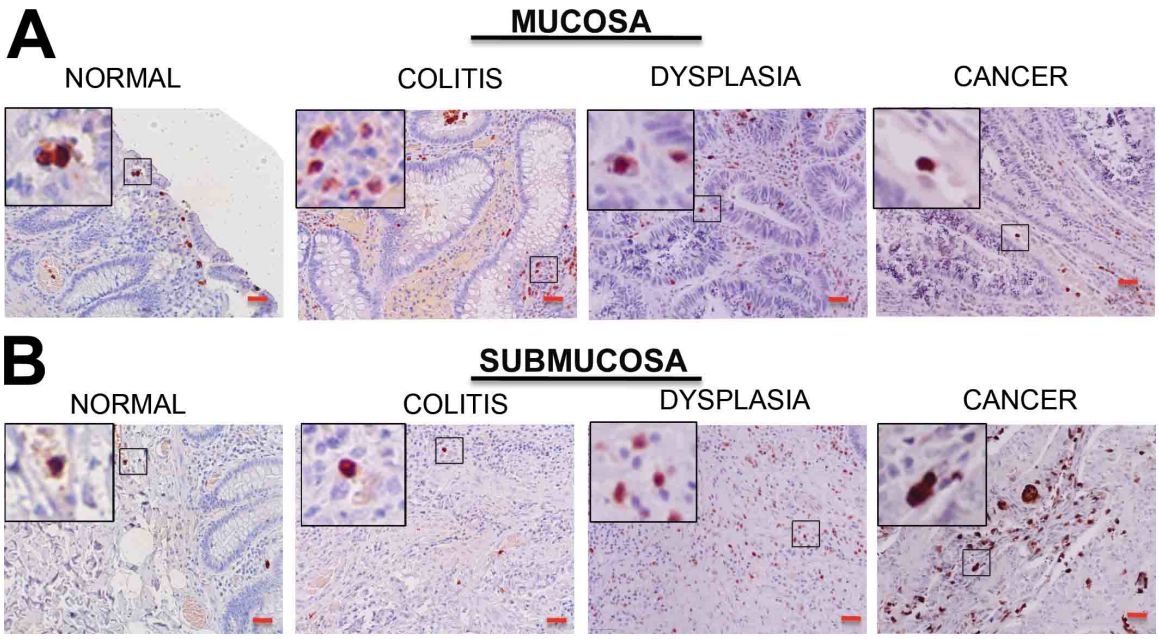

Figure 8. **$pAKT^+$ macrophages progressively infiltrate the colonic submucosa with progression to colitis and cancer.** Representative immunohistochemical staining depicting $pAKT^+$ (brown) cells in healthy and diseased human colonic (A) mucosa and (B) submucosa. Red scale bar = 50 µm.

The mean frequencies of epithelial pAKT$^+$ cells in mucosa did not show significant differences when comparing normal (0.59 ± 0.23) to colitis (0.74 ± 0.13) to dysplasia (0.69 ± 0.13) and to invasive cancer (1.10 ± 0.17)(**Figure 9A**). The frequency of stromal pAKT$^+$ cells infiltrating the mucosa in all cases outnumbered pAKT$^+$ epithelial cells (**compare Figure 9A and 9B**). Significant increases in pAKT$^+$ cells were detected in the stroma of the mucosa when progressed from "normal" (2.33 ± 0.65) to colitis tissue (6.83 ± 1.12, **P*<0.05), but thereafter plateaued (**Figure 9B**) with no significant differences from colitis to dysplasia (5.81 ± 1.27) to invasive cancer (8.27 ± 1.48). By contrast the frequency of pAKT$^+$ cells were steadily and significantly increased in the submucosa with each transition from "normal" (2.33 ± 0.65), to colitis (6.56 ± 0.80, **P*<0.05), to dysplasia (16.19 ± 4.70, **P*<0.05) and finally to cancer (37.87 ± 7.39, **P*<0.05), with cancer having the highest density of pAKT$^+$ cells (**Figure 9C**).

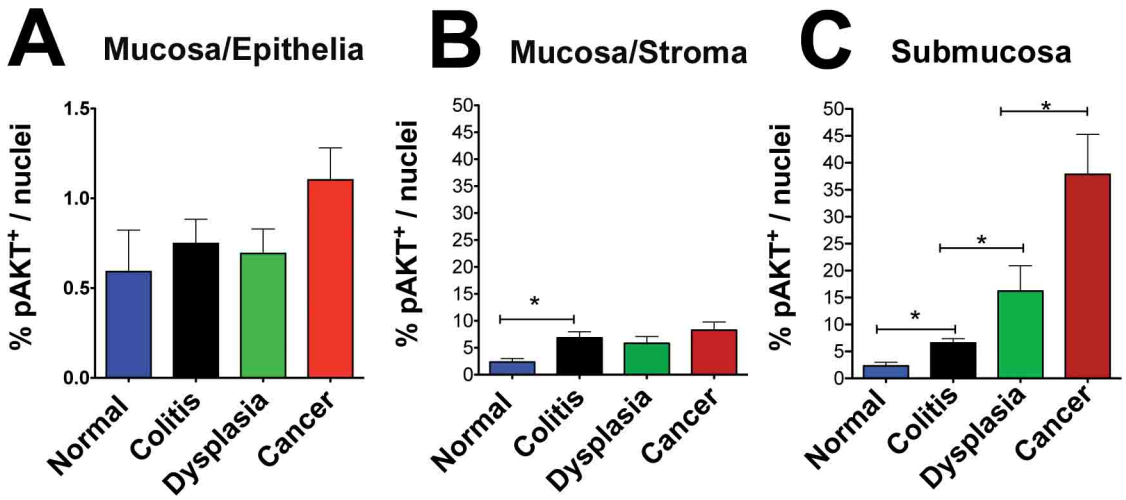

Figure 9. Quantification of pAKT$^+$ cells in colonic mucosa and submucosa with progression to colitis and cancer. Bar graphs shows quantification of % mean ± standard error of positively stained cells of

pAKT per total nuclei in (A) mucosal crypt-epithelium (B) mucosal stroma and (C) submucosa of normal and diseased human colonic tissue. * $P < 0.05$ represents the result of Student *t* test.

3.2.2 Mast cell frequencies increase with progression to colitis and then cancer

MCs are sentinel cells that are activated early in the process of intestinal carcinogenesis, and contribute to cancer initiation (Khazaie et al, 2011). In mouse models of cancer, MCs orchestrate further inflammatory reactions by mobilizing tumor associate macrophages (TAMs) (Cheon et al, 2011; Gounaris et al, 2007; Gounaris et al, 2008) It is known for long that MC recruit TAMs in human pancreatic cancer (Brown et al, 2010). To relate tissue MC densities to mobilization of TAMs in colitis progression to cancer paraffin embedded tissues were stained for MC-tryptase (**Figure 10A and 10B**). Images from 50 fields of vision were recorded for quantification by Tissuegnostics high-throughput imaging microscope for each mucosa and submucosa. MCs were detected in mucosa (**Figure 10A**) and sub-mucosa (**Figure 10B**), of samples with colitis, dysplasia, and cancer.

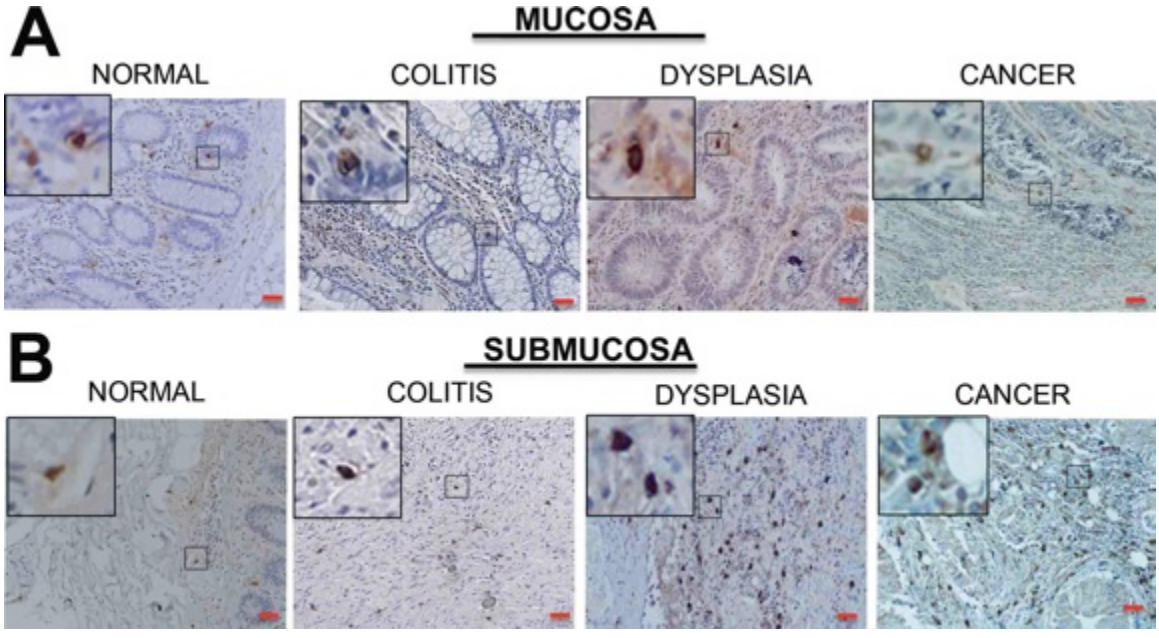

Figure 10 Tryptase$^+$ mast cell densities progressively increase in the colonic submucosa with progression to colitis and cancer. Representative immunohistochemical staining depicting tryptase (brown) positive mast cells in healthy and diseased human colonic (A) mucosa and (B) submucosa Red scale bar = 50 μm

Relative densities and sub-tissue distributions of MC mirrored that of pAKT$^+$ cells. Thus, MC frequencies in mucosa increased significantly from normal colon (4.75 ± 0.56) to colitis (14.17 ± 1.82, *P<0.05), but did not increase further as the disease progressed from colitis to invasive cancer (colitis: 14.17 ± 1.82; dysplasia: 15.54 ± 3.07; invasive cancer: 19.44 ± 3.74) (**Figure 11A**). In contrast, mean MC frequencies in sub-mucosa increased steadily as the disease progressed from normal (6.34 ± 0.99) to colitis (12.35 ± 1.86, *P<0.05), to dysplasia (33.54 ± 8.55, *P<0.05) and to invasive cancer (59.99 ± 7.09, *P<0.05) (**Figure 11B**).

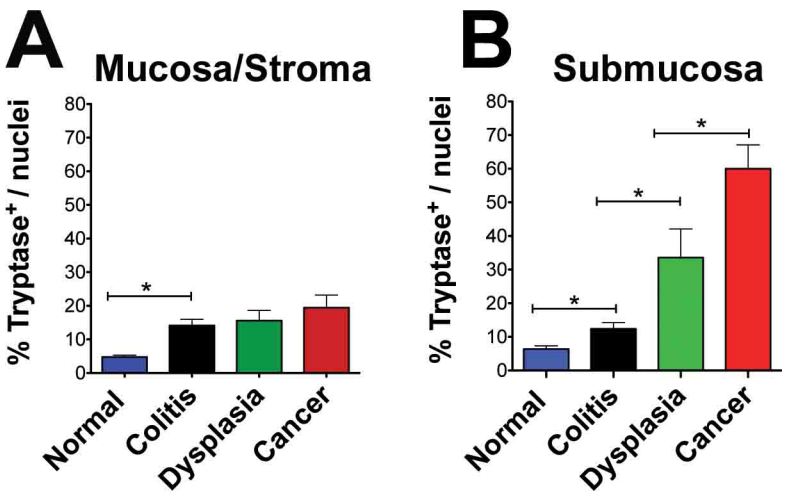

Figure 11. Quantification of tryptase positive cells in colonic mucosa and submucosa with progression to colitis and cancer. Bar graphs show quantification of % mean ± standard error of positively stained cells of human mast cell tryptase per total nuclei in (A) mucosa and (B)

submucosa of normal and diseased human colonic tissue. * $P < 0.05$ represents the result of Student t test.

3.2.3 Tumor Associated Macrophages frequencies increase with progression to colitis and then cancer

MC are known to mobilize TAMs, and both critically contribute to tumor growth and invasion (Erreni et al, 2011; Khazaie et al, 2011; Maltby et al, 2009).To identify TAMs in tissue samples histologic sections were stained with antibodies to pan-macrophage antigen CD11b (Mac1) (Arnaout, 1990) and CD68(Heinemann et al, 2000; Strobl et al, 1995). Based on mouse modeling it has been suggested that chemo- attractants, growth factors, and pathogen associated molecular patterns initiate tumor inflammation by activating PI3-kinase in $CD11b^+$ myeloid cells (Schmid et al, 2011). To test this notion, double immunofluorescence staining for pAKT and macrophage markers was performed (**Figure 12**). The data was acquired and analyzed as described above for pAKT and Tryptase. There was abundant co-localization of pAKT with CD68 (**Figure 12A and 12B**).

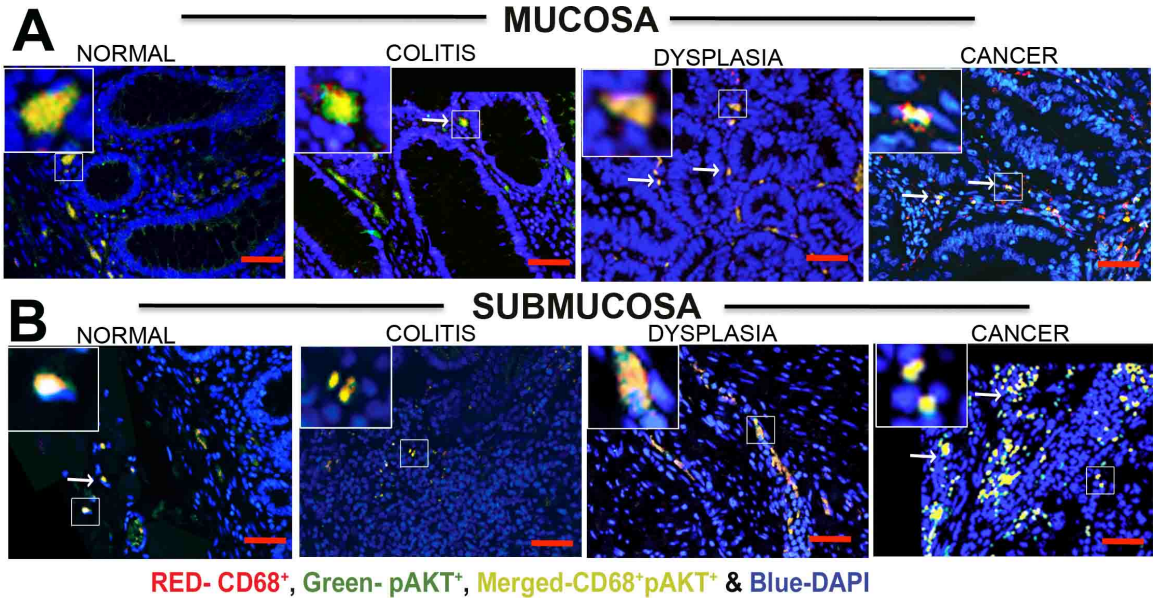

Figure 12. $CD68^+$ $pAKT^+$ macrophages progressively infiltrate the colonic

submucosa with progression to colitis and cancer. Representative double-immunofluorescence staining depicting CD68 (Red), $pAKT^+$ (Green), DAPI (blue) and $CD68^+$ $pAKT^+$ (Yellow) cells in healthy and diseased human colonic (A) mucosa and (B) submucosa. Red scale bar = 50 μm.

Densities of $pAKT^+$ $CD68^+$ TAMs were found in identical pattern with the densities of $pAKT^+$ cells (**Figure 13**) in the mucosa when disease progressed from normal (2.33 ± 0.56) to colitis (4.77 ± 0.96), dysplasia (5.81 ± 1.27) and cancer (6.27 ± 1.14) (**Figure 13A**). Interestingly, the densities of $pAKT^+$ $CD68^+$ TAMs increased steadily from normal tissue (1.56 ± 0.44) to colitis (4.83 ± 0.67), dysplasia (12.58 ± 3.83) and cancer (34.54 ± 4.56) in the submucosa (**Figure 13B**), showing the same pattern as $pAKT^+$ cells. However, the recruitment of TAMs progressively in the disease was not restricted to $pAKT^+$ $CD68^+$ TAMs, but total $CD68^+$ cell densities (inclusive of all pAKT±) also increased progressively in the submucosa from normal (2.13 ± 0.45), colitis (6.18 ± 0.84), dysplasia (18.81 ± 4.23) to cancer (38.62 ± 4.51) (data not shown). In addition, the data was validated using total Mac1+ and Mac1+ pAKT+ cell densities in the disease progression, where similar pattern of recruitment to the total $CD68^+$ and $CD68^+pAKT^+$ cell frequencies was observed (data not shown).

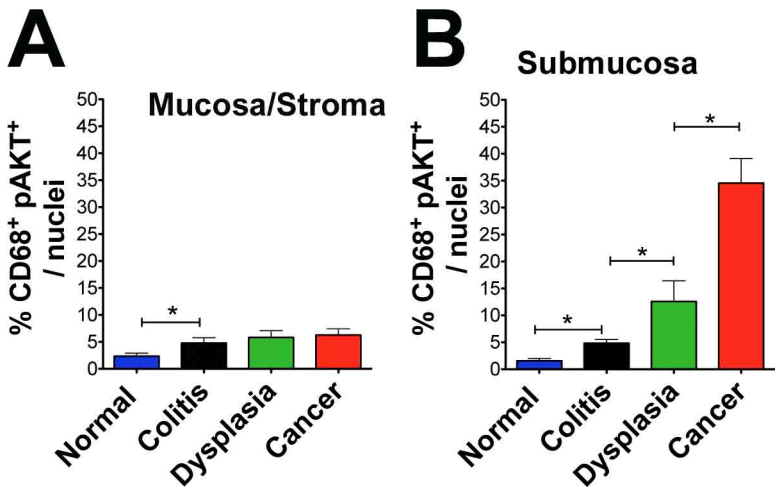

Figure 13. Quantification of $CD68^+$ $pAKT^+$ macrophages in colonic mucosa and submucosa with progression to colitis and cancer. Bar

graphs show quantification of % mean ± standard error of positively stained cells of human $CD68^+$ $pAKT^+$ macrophages per total nuclei in (A) mucosa and (B) submucosa of normal and diseased human colonic tissue. * $P < 0.05$ represents the result of Student *t* test.

3.3 Role of PI3K/AKT in mast cells and macrophage migration

3.3.1 LAD-2 mast cells posses phospho-AKT T308, phospho-AKT S473

PI3K Is extremely necessary for mast cell biology, survival and function (Kim et al, 2008b). To ensure presence of PI3K/AKT and inhibition of PI3K activity by LY294002, first MC extracts were prepared for analysis of phospho-proteins. Proteins separated by gel electrophoresis and transferred to membrane were reacted with antibodies to phospho-AKT T308, phospho-AKT S473, total AKT (Cell Signaling) and beta-actin (Sigma). This analysis showed that pre-incubation of LAD-2 MC for 1 hour with 10 mM of LY294002 hindered T308-phosphorylation of AKT by 1.74 ± 0.15 fold and S473 by 4.01 ± 0.38 fold. (**Figure 14A and 14B**).

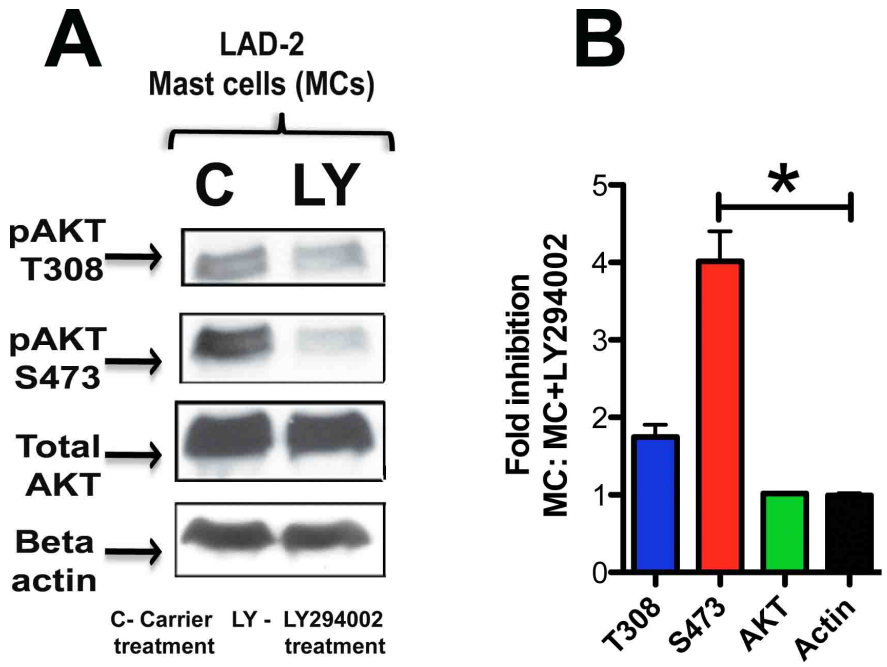

Figure 14. LY294002 attenuates pAKT levels in LAD-2 mast cells. A)

Immunoblot of total and phosphorylated AKT (Threonie/T308) and (Serine/S473) in LAD-2 mast cells untreated and treated with 10 μM LY294002 or T308, S473, AKT and beta actin. Bar graphs indicate quantitation of (B) the ratio of LAD-2 MC band intensity with LAD-2 MC treated with 10μM LY294002. * $P < 0.05$ represents the result of Student *t* test.

3.3.2 MC regulate macrophage migration via PI3K signaling

MC orchestrate secondary inflammatory reactions by recruiting other bone marrow derived cells (Khazaie et al, 2011; Maltby et al, 2009), including TAMs that critically contribute to CRC progression (Erreni et al, 2011). The hypothesis was to test if PI3K activity in MC is needed for their chemotactic potential. To test this hypothesis, LAD-2 human mast cell line was used. LAD-2 MC were pre-incubated in the presence or absence of LY294002, washed three times and added back into culture to collect conditioned medium. The chemotactic activity of conditioned media was then tested by measuring migration of $CD11b^+$ macrophages freshly prepared from human peripheral blood mononuclear cells (PBMC) through Chemo TX system.

To assay migration of macrophages the Chemo TX 5-μm pore size migration assay system was used. $CD11b^+$ macrophages were isolated from the freshly drawn blood from healthy donors using anti-human CD11b-biotinylated antibody (BD Pharmingen) and columns (Miltenyi Biotech). Isolated CD11b+ cells were loaded in the top chamber and the conditioned mediums from LAD-2 mast cells were loaded in the bottom chamber. There was a significant migration of the $CD11b^+$ macrophages to the bottom chamber as compared to similar setups where regular non-conditioned medium was used for comparison (*P<0.05) (**Figure 15A, 15B and 15F**). Pre-treatment of LAD-2 MC with 10 mM LY294002 abrogated the bioactivity of the conditioned medium in this assay and thus $CD11b^+$ macrophage migration (*P<0.05, **Figure 15C, 15D and 15F).** Cd11b+ cells used in this study were at least 99% pure (**Figure 15E**).

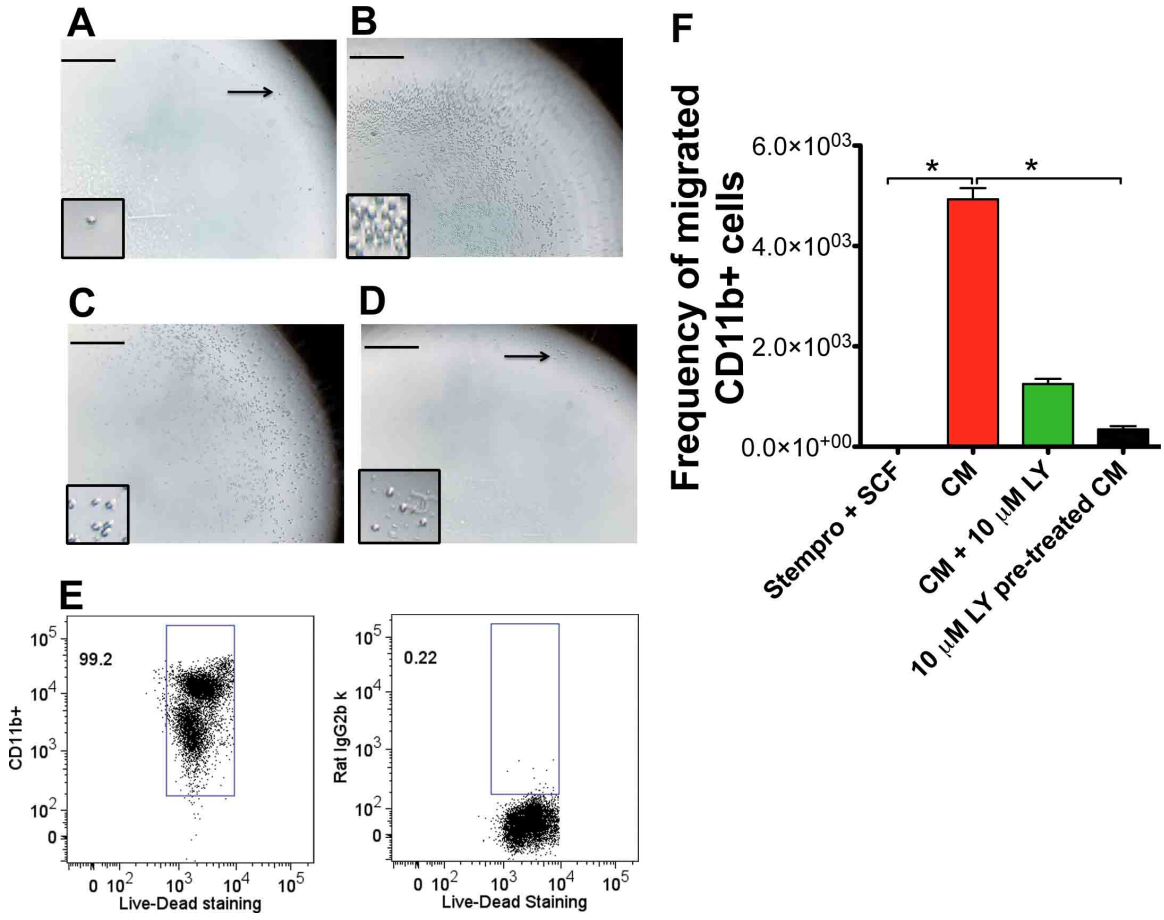

Figure 15. LY294002 treatment attenuates mast cells associated macrophage migration. Representative images show the migration of CD11b macrophages in response to (A) Stempro + SCF (control/ LAD-2 base growth medium), (B) CM (LAD-2 conditioned medium), (C) CM + 10 μM LY294002 (CM with 10 μM LY294002) and (D) 10 μM LY pretreated CM (CM obtained after treatment of LAD-2 cells with 10 μM LY294002). (E) Purity of $CD11b^+$ cells obtained after using either biotinylated Rat ant-human CD11b or biotinylated Rat IgG2bK (F) Quantification expressed in mean ± standard error of CD11b migration in response to conditions mentioned in (A-D) . Black arrows indicate migrated $CD11b^+$ cells. Black scale bar = 20 μm. * $P < 0.05$ represents the result of Student *t* test.

3.4 PI3K activity in MC is indispensable for MC biological functions and MC dependent-tumor promoting properties

3.4.1 PI3K/AKT is essential for the MC degranulation

PI3K activity is essential for differentiation of MC, as well as their long-term survival and function (Kim et al, 2008b). Mature MCs produce various biologically active mediators, which are released either by secretion or by degranulation. In particular it has been reported that in mouse models of cancer, inhibiting MC degranulation abrogates tumor-promoting properties of MC (Soucek et al, 2007). Hence, next important investigation was to test the impact of different concentrations (5 and 10 μM) of LY294002 on MC degranulation, for this purpose human LAD-2 MC were used. Treatment of LAD-2 MC with LY294002 inhibited degranulation, the ß-hexososaminidase release (%) in carrier-treated/control (72.38 ± 5.78) was reduced after 5 μM LY294002 (47.76 ± 6.43, **P*<0.05) and 10 μM LY294002 treatment (39.82± 4.39, **P*<0.05) (**Figure 16**).

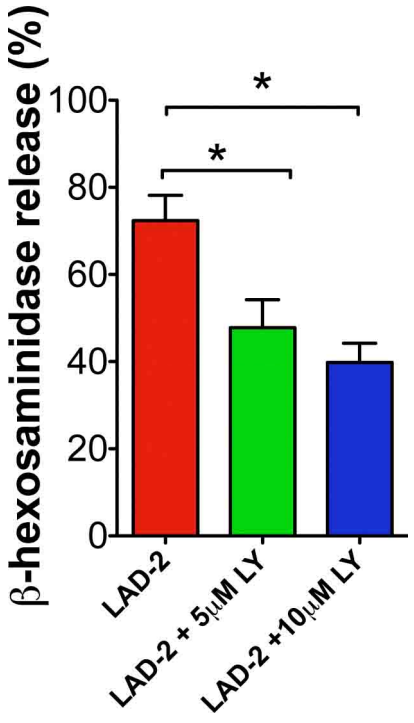

Figure 16. LY294002 treatment attenuates LAD-2 human mast cell degranulation. Figure depicts % ß-hexosaminidase release from LAD-2

mast cells before and after treatment of 5 or 10 μM LY294002. * $P < 0.05$ represents the result of Student *t* test.

3.4.2 PI3K/AKT is essential for the MC dependent tumor proliferation and tumor invasion

MC produce array of cytokines, chemokines and various growth factors, that plays an important role in the survival of tumor epithelium and cancer progression(Maltby et al, 2009). Hence next question was to study whether the soluble factors produced by MC enhance the proliferation and invasion of epithelial cancer cells, and whether it is governed by PI3K/AKT in mast cells (Brown et al, 2010). Also, the role of PI3K/AKT in MC in context with the proliferative response of HT-29 colon cancer cells was studied using human LAD-2 MC (Kirshenbaum et al, 2003).

3.4.2.1 PI3K/AKT is essential for the MC dependent ex vivo HT-29 cell proliferation

LAD-2 cell conditioned medium enhanced the rate of proliferation of HT-29 cells progressively at 24 (**Figure 17A**), 48 (**Figure 17B**) and 72 hours (**Figure 17C**) (*P<0.05). Next investigation was to test the effect of inhibition of PI3K by treating LAD-2 cells with 10 μM LY294002 and preparing conditioned medium from washed cells. Pre-incubation with LY294002 significantly reduced the ability of the LAD2 conditioned medium to stimulate proliferation of HT-29 cells, measured at three separate time-points (*P<0.05, **Figure 17**). Furthermore, direct effect of LY294002 treatment on the proliferative responses of HT-29 cells was studied. LY294002 was found to have direct inhibitory effects on the tumor cells. However, even in the presence of this inhibitor LAD2 conditioned medium elicited a significant proliferative response in the tumor cells (**Figure 17**). These observations suggest that PI3K activity in MC contributes to tumor proliferation and its inhibition by LY294002 is a critical event in suppression of tumor growth.

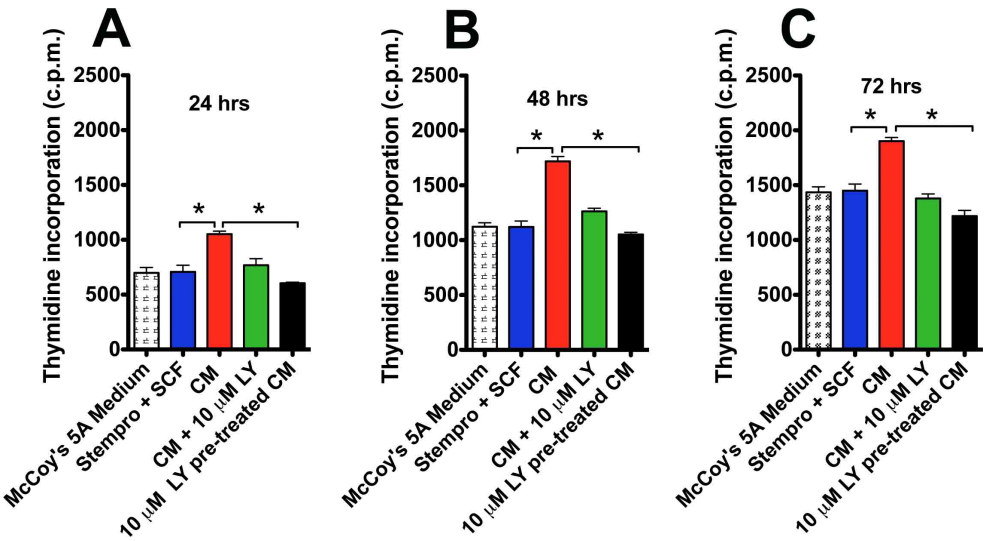

Figure 17. LY294002 treatment attenuates mast cells associated HT-29 tumor proliferation. Figure depicts quantitation expressed as mean ± standard error HT-29 proliferation (A) post 24 hrs, (B) post 48 hrs and (C) post 72 hrs in response to the treatment of McCoy's 5A (negative control), Stempro + SCF (internal control for LAD-2 CM), CM (LAD-2 CM), CM+ 10 μM LY294002 (CM with 10 μM LY294002) and 10 μM LY pretreated CM (CM obtained after treatment of LAD-2 cells with 10 μM LY294002). * $P < 0.05$ represents the result of Student *t* test.

3.4.2.2 PI3K/AKT is essential for the MC dependent ex vivo HT-29 tumor invasion

After establishing the role of PI3K/AKT in MC dependent HT-29 proliferation, next goal was to investigate whether PI3K activity and phosphorylation of AKT in MC contribute to tumor invasion. To test this hypothesis, *in vitro* invasion assays were performed with the HT-29 colon cancer cells in the presence or absence of LAD-2 conditioned medium. Since, LY294002 treated LAD2-CM attenuates HT-29 proliferation by 40% at 48 hours, the HT-29 invaded cell count was normalized (reduced the cell number by 40% in Control/Stempto+SCF and LAD-2CM groups for analysis and graphical representation). There was a significant increase in mean HT-29 cell invasion/well in Matrigel in response to LAD-2 MC conditioned medium (64.80 ± 6.92, *P<0.05) in comparison with the control

(11.40 ± 1.03 **P<0.05)* (**Figure 18A and 18B**). Invasion was attenuated when the conditioned medium was obtained from LAD-2 MC that had been previously treated with 10 μM LY294002 as described above (17.67 ± 1.45, **P*<0.05, **Figure 17A and 17B**). As with the proliferation response, LY294002 had direct inhibitory effect on the tumor cells. However, even in the presence of this inhibitor LAD2 conditioned medium elicited a significant invasion response in the tumor cells (38.67 ± 4.91, **P<0.05*, **Figure 18A and 18B**). These observations strongly suggest that MC promote tumor invasion and that this property of MC is PI3K dependent. Thus, release of tumor promoting agents by MC and potential contribution of MC to tumor growth and invasion were blocked by LY294002.

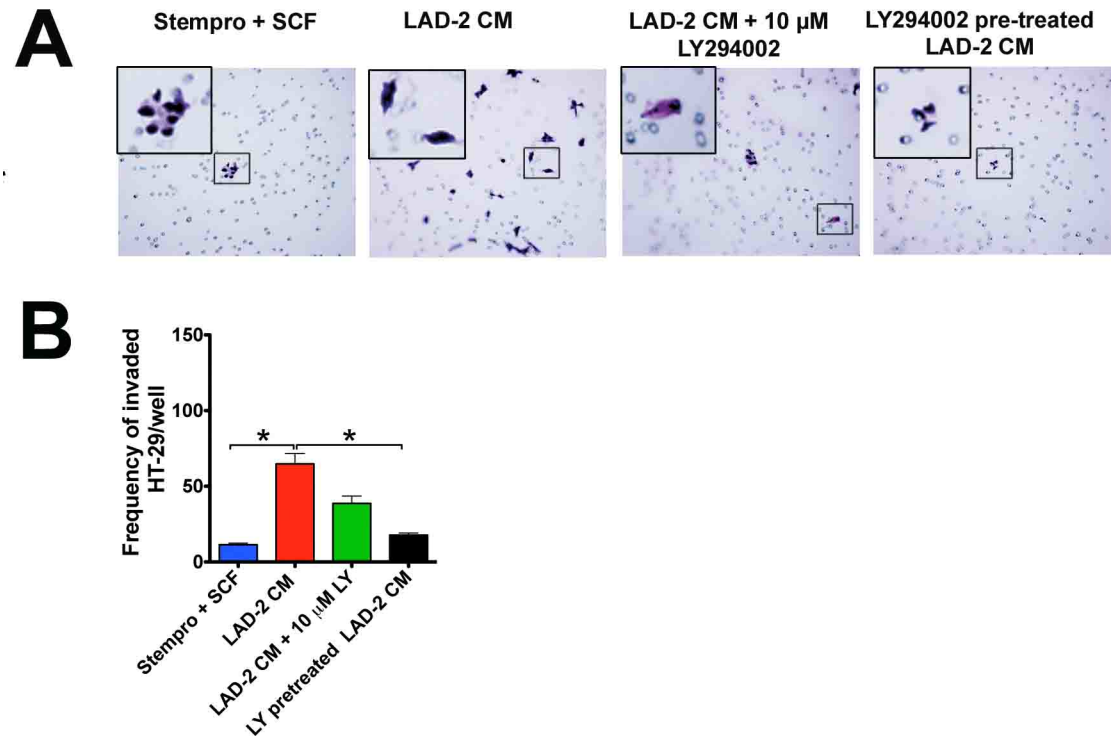

Figure 18. LY294002 treatment attenuates mast cells associated HT-29 tumor invasion. (A) Representative images of HT-29 invasion study in response to Stempro + SCF (internal control for LAD-2 CM), LAD-2 CM, CM + 10 μM LY294002 (LAD-2 CM with 10 μM LY294002) and 10 μM LY pretreated CM (CM obtained after treatment of LAD-2 cells with 10 μM

LY294002). Invaded cells can be seen as blue cells. (B) Quantification of mean ± standard error invaded HT-29 cells per well or chamber in response to conditions mentioned in (A). * $P < 0.05$ represents the result of Student *t* test.

3.4.2.3 PI3K/AKT in TILs promote ex vivo HT-29 tumor invasion

Next goal was to study the role of PI3K/AKT in tumor infiltrating leukocytes (TILs) that forms the tumor-stroma and investigate the link with tumor invasion. For this study, TILs were isolated from the freshly obtained ulcerative colitis associated cancer samples. TILs were checked for phosphorylation at Threonine 308 residue (pAKT-T308) and Serine 473 residue of AKT (pAKT-S473), 10μM LY294002 treatment significantly attenuated pAKT-T308 1.62 ± 0.05 fold and pAKT-S473 3.53 ± 0.17 fold in comparison with the carrier treated pAKT-T308 and pAKT-S473 (**P<0.05*, **Figure 19A and 19B**).

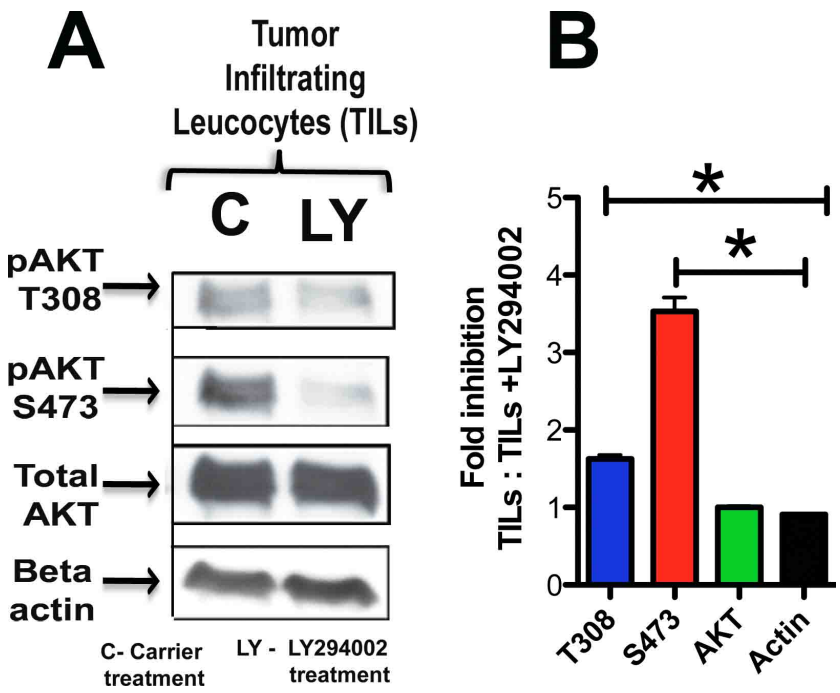

Figure 19. LY294002 attenuates pAKT levels in TILs. A) Immunoblot of total and phosphorylated AKT (Threonine/T308) and (Serine/S473) in TILs untreated and treated with 10 μM LY294002. Bar graphs indicate quantitation of (B) the ratio of TILs band intensity with TILs treated with 10 μM LY294002 for T308, S473, AKT and beta actin. * $P < 0.05$ represents the result of Student *t* test.

Next, tumor-infiltrating leukocytes (TILs) isolated from CRC tumors were measured for the ability to promote tumor cell invasion. By pretreating the TILs with LY294002 the dependence of the tumor invasion promoting activity on PI3K was tested. To address this question, TILs were isolated from fresh surgical specimens derived from tumors of colitis associated colon cancer patients. Two different setups were used. In the first setup tumor cells were added in top chamber and conditioned medium from the TILs (carrier or 10 μM LY294002 pretreated) in the bottom chamber, and in the second setup the TILs were co-cultured with the tumor cells. After normalization of HT-29 cell counts in Stempro+SCF, TILs CM and HT-29+TILs study groups, mean invasion of HT-29 cells into matrigel was significantly enhanced by the TILs in both experimental setups (76.80 ± 5.67 for setup-1 and 133.80 ± 7.99 for setup-2, *P<0.05). Pretreatment of the TILs with 10 μM LY294002 significantly inhibited tumor invasion relative to treatment with CM from carrier-treated TILs or co-culture with carrier-treated TILs respectively (26.67 ± 6.93 for setup-1 and 57.00 ± 4.72 for setup-2, *P<0.05, **Figure 20A, 20B and 20C**). These results are compatible with those obtained with MC conditional medium and demonstrate that the PI3K inhibitor LY294002 inhibits production of mediators of tumor invasion by TILs, that include TAMs and MCs.

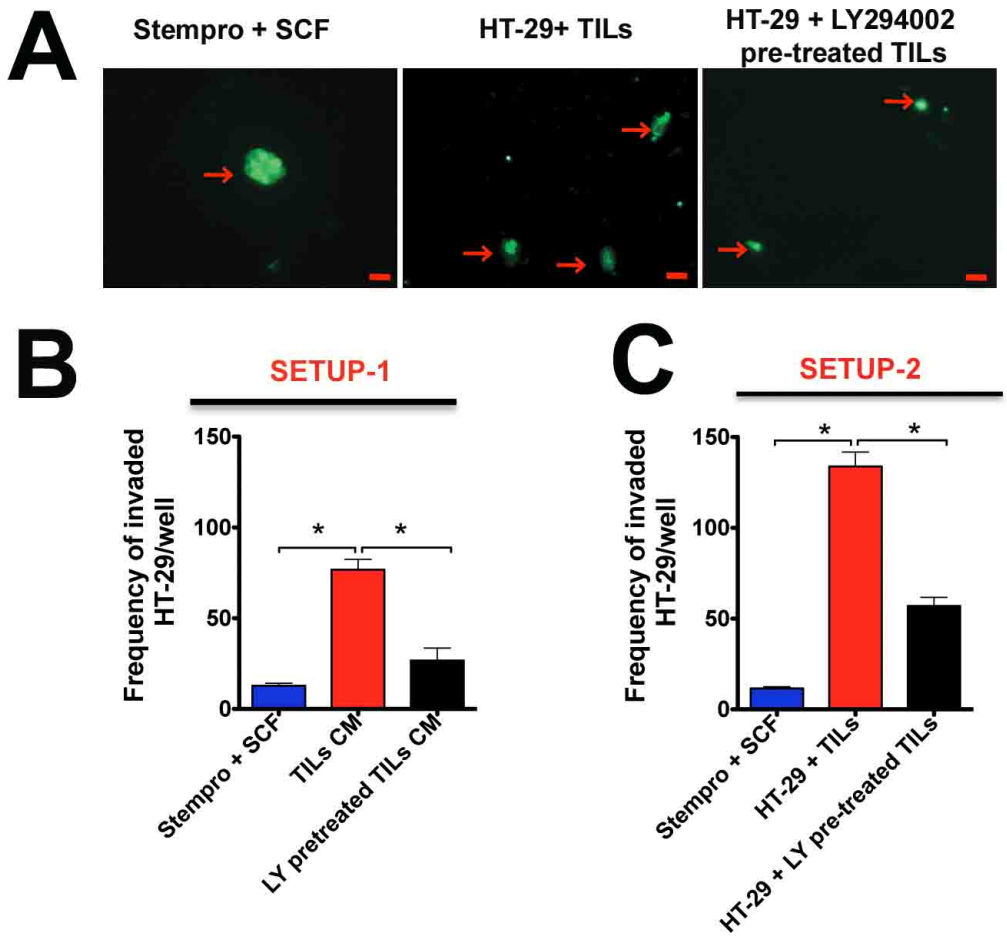

Figure 20. LY294002 treatment attenuates TILs associated HT-29 tumor invasion. (A) Representative images of CFSE labeled HT-29 invasion study in response to tumor infiltrating leukocytes (TILs) isolated from UC associated cancer patients. Red arrows show invaded HT-29. (B) Quantitation of mean ± standard error invaded HT-29 cells per well in the experimental setup-1 (HT-29 in top chamber and CM from TILs with or without 10 μM LY294002 pretreatment in bottom well) & (K) Quantitation of mean ± standard error invaded HT-29 cells per well in the experimental setup-2 (HT-29 in 1:1 coculture with TILs with or without 10μM LY294002 pretreatment in top chamber). Red scale bar = 50 μm, * $P < 0.05$ represents the result of Student *t* test.

3.5 Pi3k/Akt inhibitor LY294002 attenuates mast cells, colitis, and cancer development in the IL-10$^{-/-}$ Piroxicam mouse model

To further validate *in vitro observations* and to see if they also have central roles in progression of inflammation to cancer *in the in vivo* system, cancer prone colitis mice were treated with LY294002. IL-10$^{-/-}$ mice when treated with Piroxicam develop colitis with ulcers, followed by invasive cancer by day 56 (mean invasive lesions 2.30 ± 0.26, **Figure 21A and 21B)** (Lee et al, 2010). LY294002 treatment reduced the incidence of invasive cancer in this model (0.100 ± 0.10, **P*<0.05, **Figure 21A and 21B**).

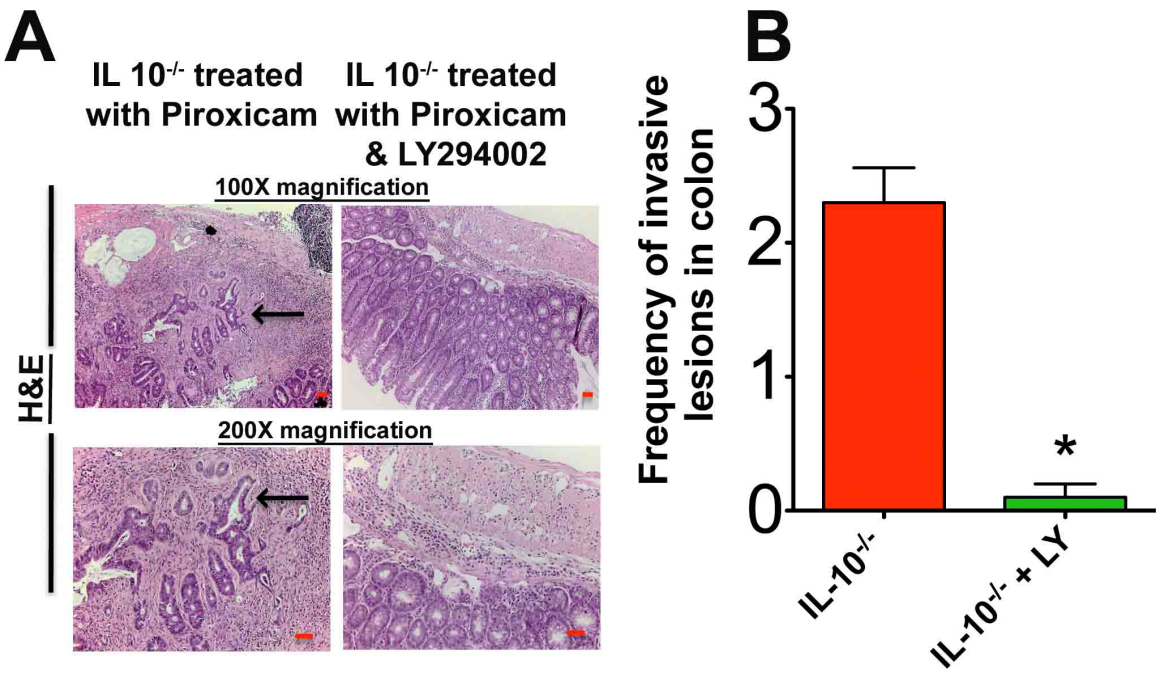

Figure 21. LY294002 attenuates development of cancer in IL-10$^{-/-}$ mice treated with Piroxicam. (A) Histological evaluation of IL-10$^{-/-}$ and IL-10$^{-/-}$ mice treated with LY294002. H & E staining at 100X & 200X magnification of IL-10$^{-/-}$ mice 56 days post Piroxicam ± LY294002 treatment; black arrow indicates invaded colonic crypts. (B) Graphical representation of the mean frequencies ± error of invasive lesions in IL-10$^{-/-}$ (10$^{-/-}$ treated with Piroxicam) and IL-10$^{-/-}$ mice+ LY (10$^{-/-}$ treated

with Piroxicam and LY294002. Red scale bar = 50 μm, * $P < 0.05$ represents the result of Student *t* test.

Also, LY294002 treatment reduced the frequency of Bromodeoxyuridine (BrdU) positive cells (*$P<0.05$, **Figure 22A and 22E**), increased apoptosis as measured by TUNEL (*$P<0.05$, **Figure 22C and 22F**), and reduced the pAKT levels within the crypt-epithelium (*$P<0.05$, **Figure 22B and 22G**) and stroma (*$P<0.05$, **Figure 22D and 22H**).

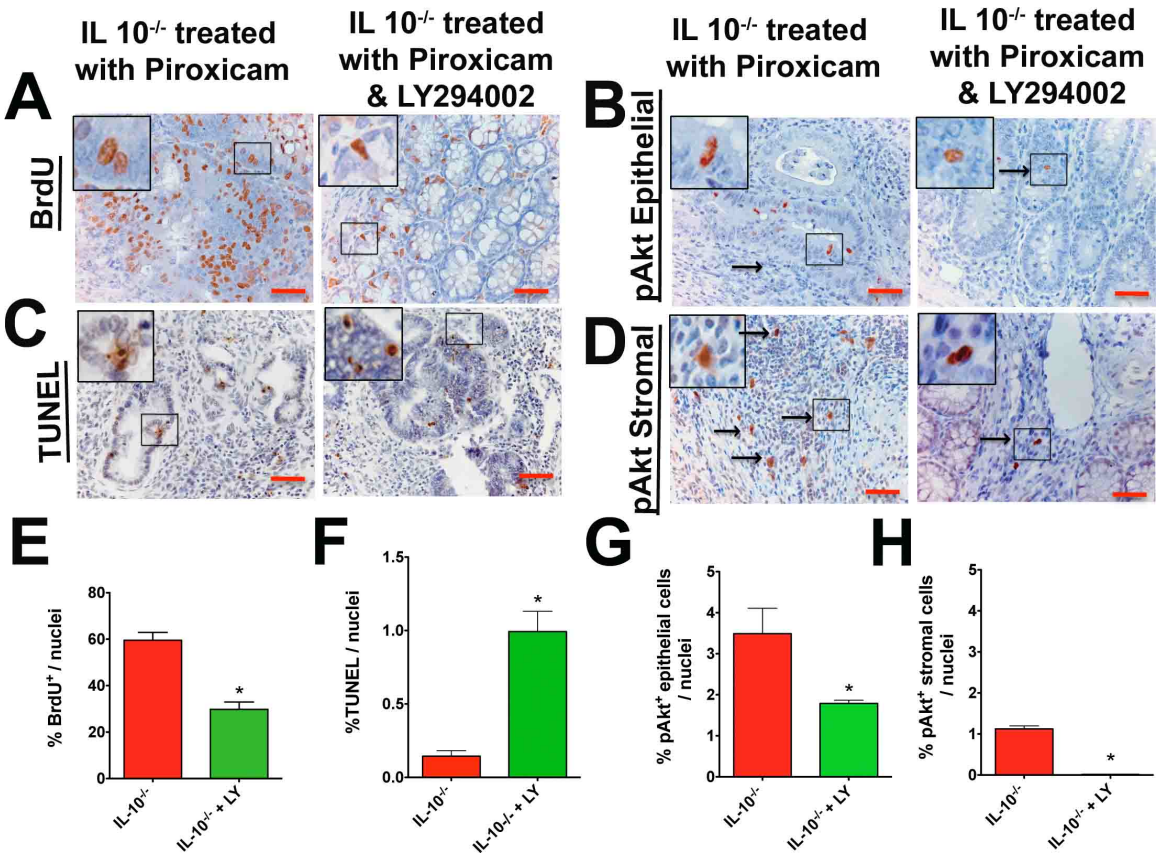

Figure 22. LY294002 attenuates epithelial proliferation, induces apoptosis and restricts pAkt levels in IL-10$^{-/-}$ mice treated with Piroxicam. Histological evaluation of (A) BrdU staining in colon of IL-10$^{-/-}$ or IL-10$^{-/-}$ mice treated with LY294002 (note brown staining) (B) TUNEL staining on colon of IL-10$^{-/-}$ or IL-10$^{-/-}$ treated with LY294002 (C) pAkt

staining in colonic mucosal epithelium of IL-10$^{-/-}$ or IL-10$^{-/-}$ mice treated with LY294002. (D) pAkt staining in colonic stroma of IL-10$^{-/-}$ or IL-10$^{-/-}$ mice treated with LY294002. Graphical representation of the mean frequencies ± error of (E) % BRDU^{+}, (F) %TUNEL^{+}, (G) %pAkt^{+} epithelial and (H) %pAkt^{+} stromal cells per total nuclei in the colon of untreated and LY294002 treated Il-10$^{-/-}$ mice. Black arrows indicate pAkt^{+} cells. Red scale bar = 50 μm, * $P < 0.05$ represents the result of Student *t* test.

In earlier studies a causative role for focal mastocytosis and pre-neoplasia in the mouse intestine has been reported (Gounaris et al, 2007; Khazaie et al, 2011). Thus, next goal was to investigate the effect of pi3k/akt inhibition on the frequencies of mast cells in the cancer. Chloroacetate esterase (CAE) staining was used to study the *in vivo* impact of LY294002 on MCs infiltrating the gut tissue. CAE is a cytochemical staining that stains MCs and granulocytes (Lichtman & Segel, 2005). LY294002 treatment inhibited the mean frequencies of tissue infiltrating CAE^{+} cell (0.262 ± 0.06) in comparison with control untreated mice (0.98 ± 0.09, *P<0.05, **Figure 23A and 23B**).

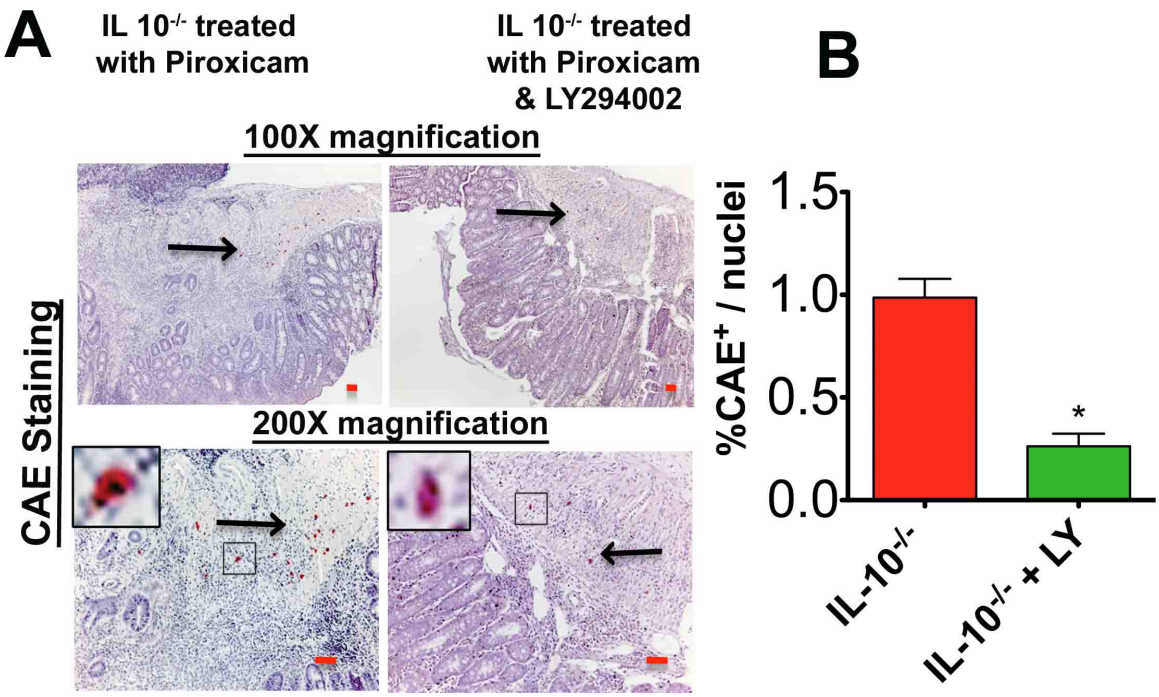

Figure 23. LY294002 attenuates the frequency of CAE positive cells in IL-10$^{-/-}$ mice *in vivo*. (A) Representative images of CAE staining at 100X & 200X magnification respectively of IL-10$^{-/-}$ mice 56 days post Piroxicam ± LY294002 treatment; black arrows indicate pink CAE positive cells. (B) Quantification of % mean ± standard error CAE^{+} cells per total nuclei in LY294002 untreated and treated IL-10$^{-/-}$ mice. Red scale bar in = 50 µm, * $P < 0.05$ represents the result of Student *t* test.

Moreover, the effect LY294002 treatment on the *in situ* degranulating mast cells using Toluidine Blue staining was studied. Frequency of *in situ* degranulating MC (purple MCs), found predominantly in the sub-mucosa (site of invasion) was significantly attenuated (% mean 30.12 ± 2.98) in comparison with the non-LY294002 treated mice (85.02 ± 1.57, **P*<0.05, **Figure 24A and 24B**).

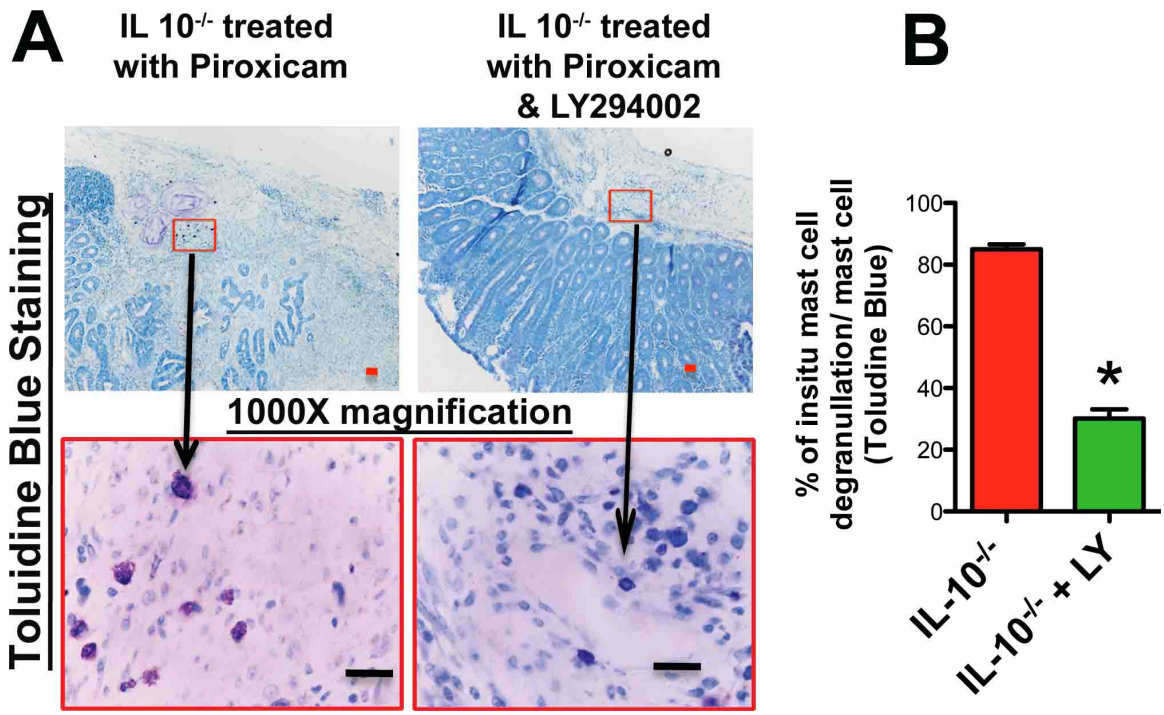

Figure 24. LY294002 attenuates in situ mast cell degranulation in IL-10$^{-/-}$ mice (A) Representative images of toluidine blue staining at 100X &

1000X magnification respectively of IL-10$^{-/-}$ mice 56 days post Piroxicam ± LY294002 treatment, black arrow indicates magnified, purple degranulating or blue non-degranulating mast cells. (B) Quantitation of % mean ± standard error in vivo mast cell degranulation per total mast cells in the colon of the LY294002 untreated or treated IL-10$^{-/-}$ mice (% in situ degranulation = total purple mast cells X 100 / total mast cells in LY294002 untreated or treated IL-10$^{-/-}$). Red scale bar in = 50 μm, Black scale bar = 20 μm, * $P < 0.05$ represents the result of Student *t* test.

3.6 Pi3k/Akt inhibitor LY294002 attenuates mast cell induced tumor proliferation and invasion

3.6.1 Pi3k/Akt inhibitor LY294002 inhibits mouse MC degranulation

In vitro assays were performed to validate inhibition of degranulation of the gut derived primary mouse mast cells by LY294002. The ß-hexososaminidase release (%) in carrier-treated GMMCs (33.75 ± 0.49) dropped after 5 μM LY294002 (11.28 ± 0.47, *P<0.05) and 10 μM LY294002 (6.86 ± 0.39, *P<0.05, **Figure 25**) treatment.

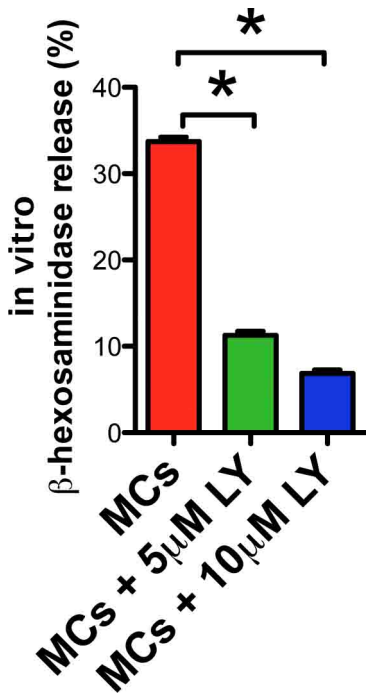

Figure 25. LY294002 attenuates *in vitro* murine primary mast cell degranulation in IL-10$^{-/-}$ mice. Figure depicts quantitation of % *in vitro* β-hexosaminidase release from GMMCs after treatment of carrier or 5 μM or 10 μM LY294002. * $P < 0.05$ represents the result of Student *t* test.

Next, pretreated conditioned medium (with 10 μM LY294002 or carrier) obtained from mouse mast cells to study the effect of PI3K inhibition on mouse MCs in context with CT44 mouse colon cancer proliferation and invasion.

3.6.2 Pi3k/Akt inhibitor LY294002 attenuates the rate of mouse MC induced CT44 proliferation

LY294002 pretreated conditioned medium significantly attenuated the mean CT44 cell proliferation counts (8580.00 ± 1009) in comparison to conditioned medium obtained from carrier-treated mouse MCs at 24 hours time points (12910.00 ± 678.20, *P<0.05, **Figure 26**). However, at 48 and 72 hours time points there was no significant difference between the two groups (data not shown).

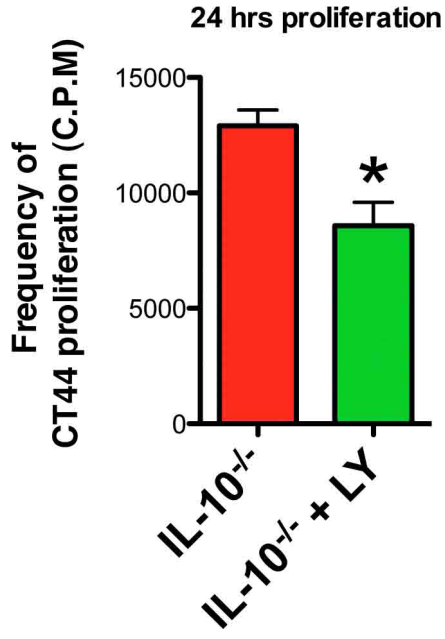

Figure 26. LY294002 attenuates *in vitro* murine mast cell associated CT44 tumor cell proliferation. Figure depicts quantitation of mean ± standard error CT44 mouse colon cancer proliferation at 24 hour time point in response to carrier or 10 μM LY294002 treated GMMC conditioned medium. * $P < 0.05$ represents the result of Student *t* test.

3.6.3 Pi3k/akt inhibitor LY294002 attenuates mouse MC induced CT44 invasion

Similarly, the mean CT44 cell invasion/well after normalization (CT44 invasion count number in carrier-treated study group only was normalized by reducing 33.53% since at 24 hours the CT44 proliferation was attenuated by 33.53%) was significantly attenuated by 10 μM LY294002 pretreated conditioned medium (303.70 ± 16.70) in comparison with conditioned medium from carried-pretreated mouse mast cells (516.70 ± 45.18, *P<0.05, **Figure 27A and 27B**).

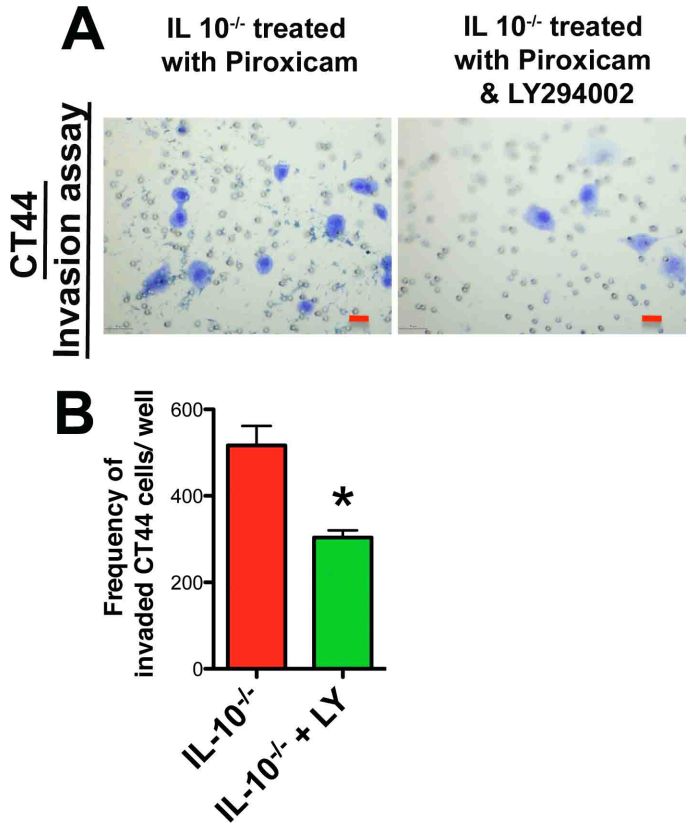

Figure 27. LY294002 attenuates *in vitro* murine mast cell associated CT44 tumor cell invasion. (A) Representative images of CT44 mouse colon cancer cell invasion in response to conditioned medium obtained either carrier or LY294002 treated GMMCs. (B) Quantification of mean ± standard error CT44 cell invasion/well in response to carrier or 10 μM LY294002 treated GMMC conditioned medium. * $P < 0.05$ represents the result of Student *t* test.

These observations show that PI3K and phosphorylation of AKT are critical for mast cell functions that promote cancer and LY294002 inhibits the cancer promotion by inhibiting these properties.

4.

Discussions

4. Discussions

It has been known for long that inflammation plays an important role in cancer development. Ulcerative colitis patients with prolonged chronic inflammation are at high risk to develop invasive colon cancer. PI3K pathway has been a subject of interest in cancer development for researchers globally, however less is known about the role played by PI3K/AKT in the inflammatory component for the tumor development. The over-all aim of this project was to study the change in the pattern of PI3K/AKT and inflammation as disease progress from colitis to cancer, followed by a detailed investigation of the role of PI3K/AKT in inflammation and whether PI3K/AKT in inflammation regulate the tumor progression and cancer development using human patient specimens, *in vitro* assays and *in vivo* experimental colitis associated cancer mouse model system.

4.1 Selection and characterization of patients based on histopathological study

Inflammation plays a pivotal role in initiation and progression of colon cancer (Khazaie et al, 2011; Terzic et al, 2010). Chronic inflammation in UC patients increases the risk of rapidly progressing CRC (Allavena et al, 2008; Bargen & Gage, 1960; Bouma & Strober, 2003; Cho et al, 2008; Crivellato et al, 2008; Dostert et al, 2008; Ekbom et al, 1990; Gounaris et al, 2008; Gupta et al, 2007; Gyde et al, 1988; Itzkowitz & Yio, 2004; Karlen et al, 1999; Kobayashi et al, 2006; Lakatos & Lakatos, 2008; Lemmon, 2008; Ransohoff, 1988; Taskinen et al, 2008; Ullman, 2003; Yodavudh et al, 2008a; Yodavudh et al, 2008b). Hence, based on histopathological study patient specimens were chosen and grouped into 4 categories i.e. normal, colitis, dysplasia and cancer. The objective here was to study the immunopathological changes happening in the colon, when the disease is progressing from normal to colitis, dysplasia and cancer. Previous studies in colitis have majorly been restricted to either comparison between

normal versus colitis, or colitis versus colitis associated cancer (Coussens et al, 1999; Imada et al, 2000; Johansson et al, 2010; Nonomura et al, 2007; Ribatti et al, 2000; Ribatti et al, 2003; Takanami et al, 2000; Terada & Matsunaga, 2000; Toth-Jakatics et al, 2000). For the first time a novel study pattern was decided where the pathological and immunological studies were correlated and studied from normal to inflammation-associated cancer development.

4.1.1 PI3K in human colonic specimen study

It is known that PI3K activity significantly rises in CRC and is associated with poor prognosis (Philp et al, 2001; Velho et al, 2005). Mouse models of UC have shown that PI3K activity is critical for progression to cancer (Brown et al, 2010; Gounaris et al, 2007; Jain et al, 2010; Kim et al, 2010; Lee et al, 2010; Nigrovic et al, 2010; Strouch et al, 2010). However, much of what is known is focused on the role of PI3K signaling in tumor cells; furthermore, the relevance to inflammation driven colon cancer in humans remains unclear (Brown et al, 2010; Cook et al, 2010; Gounaris et al, 2007; Jain et al, 2010; Kim et al, 2010; Lee et al, 2010; Nigrovic et al, 2010; Strouch et al, 2010).

Hence, pAKT levels were studied using immunohistochemical staining and were differentially characterized at histological sites of mucosa (epithelial vs. stromal) and submucosa in a novel investigation of chronic inflammation associated colonic dysplasia and invasive cancer. By *in situ* staining of human surgical specimens, the first evidence found was that PI3K/AKT activity is found predominantly in the stromal infiltrates and submucosal inflammatory cells in comparison with the epithelium. Moreover, the mucosal pAKT levels did not change with the progression of disease from colitis to dysplasia and cancer. However, the submucosal pAKT levels increased progressively from normal to colitis, dysplasia and cancer. Hence, next step was to investigate what subsets of inflammatory cell population display PI3K/pAKT. pAKT activity overlapped abundantly with $CD68^+$ macrophages and tumor infiltrating inflammatory cells. These $CD68^+pAKT^+$ macrophage cell population showed identical pattern of

infiltration as total pAKT cells. They progressively rose in the submucosal areas from normal to colitis, dysplasia and cancer. These observations are indicative of production of macrophage-chemotactic factors in the submucosal area of active inflammation. These macrophages are recruited in the submucosal area and with them they bring in high pAKT activity incrementally in the colitis, dysplastic and invasive cancer submucosal area. Progressive rise of $pAKT^+$ macrophages in the submucosal area with peak levels in the invasive cancer is indicative of highest levels of macrophage chemotactic factors being produced by the submucosa of invasive colon cancer. In a similar study of murine model for breast cancer, macrophages were recruited by the tumor cell-derived chemokine CCL5. When treated with the receptor antagonist met-CCL5, both the number of infiltrating macrophages and the size of the tumor were significantly reduced(Robinson et al, 2003). Moreover, TAMs are differentiated from monocytes via number of chemoattractants that are produced by tumor and stromal cells. For instance, tumor-derived chemokine CCL2, previously known as monocyte chemotactic protein (MCP), is critical for the recruitment of macrophages(Coussens & Werb, 2002; Graves et al, 1989). Tumor cells, fibroblasts, and macrophages produce CCL2, and high CCL2 levels are correlated with increased numbers of TAMs and a poor cancer prognosis (Siveen & Kuttan, 2009). Other chemokines, such as CCL3, CCL4, CCL5, CCL7, CCL8, CXCL12, and cytokines, including vascular endothelial growth factor (VEGF), IL-10 and, platelet-derived growth factor (PDGF) are also reported to promote macrophage recruitment (Allavena et al, 2008; Balkwill & Coussens, 2004; Murdoch et al, 2004; Solinas et al, 2009). In addition, another group of monocyte chemoattractants, called the alarmins, have been reported to promote the recruitment of monocytes and other myeloid cells(Coffelt & Scandurro, 2008). For example, the high mobility group box protein 1 (HMGB1), which is one of the molecules released by dying or apoptotic tumor cells, is found in the necrotic areas where TAMs preferentially reside. Other alarmins, such as S100A8, S100A9, serumamyloid A3 (SAA3), and fibronectin, have also been reported to attract $CD11b^+$ myeloid origin cells(Coffelt et al, 2009). In total, it can be concluded that macrophages are driven to the area

of active inflammation, predominantly at submucosa of colon where an array of macrophage chemotactic factors are produced.

4.1.2 Mast cells in human colonic specimen study

In addition to tumor cells, MC the sentinel cells have been known to produce huge array of chemottractants for macrophages(Cheon et al, 2011; Crivellato et al, 2008; Khazaie et al, 2011; Maltby et al, 2009). Hence, the presence of MC in the patient tissue specimens was investigated. MC frequencies were found in identical pattern with pAKT and $CD68^{+}$ macrophages. MC were found predominantly in the submucosa in a progressive pattern from normal to colitis, dysplasia and cancer. These results are in accordance with numerous observations and findings that report the increase in MC frequencies in cancer(Cheon et al, 2011; Crivellato et al, 2008; Khazaie et al, 2011; Maltby et al, 2009), but this study for the first time report the spatial distribution of MC in the mucosal and submucosal areas, where other study have not been accurate to point the area of predilection for inflammation and pAKT. Hence from above it can be understood that mast cells may recruit macrophages in the submucosal tumor microenvironment.

4.2 Mast cells recruit macrophages and promote tumor proliferation and invasion in PI3K/AKT dependent manner

4.2.1 PI3K/AKT in mast cells recruit macrophage migration

MC are found predominantly in submucosa of cancer in this study; are sentinel cells that proliferate on activation at the site of inflammation and are known to recruit macrophages(Cheon et al, 2011; Crivellato et al, 2008; Khazaie et al, 2011; Maltby et al, 2009), moreover PI3K is essential for the survival and biological functions of MC (Kim et al, 2008c), hence investigation was performed if MC associated macrophages recruitment is PI3K dependent? Conditioned medium obtained from MC promoted migration of healthy peripheral blood

derived macrophages. Next, addition of LY294002 to the conditioned medium attenuated macrophage migration. Moreover, the macrophage migration was attenuated significantly even further when the conditioned medium obtained after LY294002 pre-treated MC was used. These observations are first evidence for the role of PI3K/AKT signaling in the biology of MC in context with tumor promoting properties. Moreover, the soluble factors from MC are released in the tumor microenvironment in a PI3K dependent manner that recruits the macrophages and PI3K/AKT activity to the tumor. Addition of LY294002 to the conditioned medium has a direct effect on macrophage migration. Similar observations were reported for mouse and human macrophage migration using LY294002 (Baek et al, 2001). Moreover, soluble factors released by MC were tightly regulated by PI3K/AKT and blocking PI3K/AKT in MC attenuates the macrophage migration to the highest degree. Thus immunohistochemical-based spatial distribution study for pAKT, macrophages and MC was critical in understanding that MC might be at the central-axis of macrophage and PI3K/AKT recruitment to the tumor microenvironment and predominantly in the sub-musoca, which is the site of crypt-invasion in invasive cancers. Moreover, these *in vitro* studies results were pivotal in understanding that PI3K upregulation seen in colitis-associated invasive colon cancer is attributed to the macrophages and the tumor infiltrating leukocytes that are recruited predominantly in the colonic submucosa.

4.2.2 PI3K/AKT in mast cells regulate mast cell degranulation

Mast cells release various factors by secretion and degranulation in the tumor microenvironment(Khazaie et al, 2011; Maltby et al, 2009). In addition to secretion PI3K regulates the degranulation of MC(Kim et al, 2008b). A dose-dependent inhibition of the release of β-hexosaminidase (degranulation) was observed in the MC that were treated with LY294002. These observations are in agreement with various studies that have investigated the role of PI3K in mast cell biology(Kim et al, 2008b; Lam et al, 2008). The PI3K inhibitors, Wortmannin and LY294002, have been widely reported to inhibit antigen-mediated

degranulation and cytokine production in both human and rodent mast cells (Kim et al, 2008a; Okayama et al, 2003; Tkaczyk et al, 2003). Studies utilizing mouse bone marrow-derived mast cells (BMMCs) expressing a kinase-inactive mutant isoform of the p110δ catalytic subunit have shown that p110δ is the major isoform responsible for antigen-mediated degranulation and cytokine production in MC(Ali et al, 2004; Ali et al, 2008). This observation is further supported by the ability of the selective p110δ inhibitor, IC87114, to inhibit antigen-mediated MC activation and by its ability to inhibit the enhancement of antigen-mediated degranulation by stem cell factor (SCF)(Ali et al, 2004). By contrast, MC derived from the bone marrow of p85α and p85β knock out mice show normal antigen-mediated calcium flux and degranulation(Lu-Kuo et al, 2000; Tkaczyk et al, 2003), suggesting that the p110 catalytic subunit can utilize alternative regulatory subunits for its interaction with phosphorylated Gab2(Kim et al, 2008c) .

4.2.3 PI3K/AKT in mast cells promote tumor proliferation

MC are known to produce various growth factors and array of tumor promoting cytokines, chemokines and proteases that plays an vital role in tumor promotion(Maltby et al, 2009). MC release these growth factors in a PI3K/AKT dependent manner, hence the role of PI3K in MC dependent tumor proliferation and invasion was studied. MC conditioned medium that possess the soluble growth factors released by MC (Cheon et al, 2011; Strouch et al, 2010), when co-cultured with tumor cells promoted *ex vivo* tumor proliferation at 24, 48 and 72 hour time points. Whereas, when LY294002 was added to this conditioned medium for co-culture the rate of tumor proliferation was restricted. However, when LY294002 pre-treated MC conditioned medium was co-cultured with tumor cells, the rate of tumor proliferation was further significantly attenuated. These findings suggest that tumor proliferation was promoted via soluble factors released from MC that are regulated by PI3K. Moreover, reduction in the tumor proliferation rate by addition of LY294002 to the MC conditioned medium indicates that LY294002 targets the PI3K in the tumor cells directly and restricts their proliferation in the presence of active MC soluble factors. Finally, the

highest attenuation of tumor proliferation achieved with co-culture of LY294002 pre-treated MC conditioned medium indicates that PI3K/AKT in MC regulate the release of MC soluble factors that promote tumor growth. In addition, this blockage of MC soluble factor release is very potent and strongly attenuates the tumor proliferation in comparison with the study group where LY294002 is added to the MC (non LY294002 pre-treated) conditioned medium for coculture with tumor cells, which could be attributable to the absence of any active MC soluble tumor-promoting growth factors in the former group. Similar observations were reported with MC conditioned medium induced tumor proliferation in pancreatic cells lines Panc-1, HPDE and AsPC1(Strouch et al, 2010), where increase in pancreatic cell proliferation was found using LAD-2 MC conditioned medium by all three cell lines at 24, 48 and 72 hours time points in comparison with non-conditioned medium. Furthermore, in agreement to these observations another study demonstrated that $APC^{\Delta 468}$ and wild type mouse MC conditioned medium promoted *in vitro* colonic epithelial proliferation incrementally at 24 hours, 48 hours and 72 hours time point(Cheon et al, 2011). In a similar study aimed to investigate direct effect of LY294002 on cancer cells, LY294002 was found to be effective *in vitro* against a range of colon cancer cell lines including HT-29 and COLO-320 (Garman et al, 2008). Moreover, the tumor recurrence was significantly affected after LY294002 treatment (Garman et al, 2008). In another *in vitro* study LY294002 alone did not produce cytotoxic effects, however PI3K inhibition with LY294002 significantly radiosensitized the cervical cancer cells and showed significant time-dependent effects, increased apoptosis, and altered gene expression (Lee et al, 2006). In this study by Lee et al, no effect of LY294002 on tumor proliferation could be attributable to the different cancer lineages and acquired secondary mutations in the cervical cancer cell lines. However, in another study mixed results were seen on tumor growth using LY294002. In this study, LY294002 demonstrated a remarkable growth-inhibitory and apoptosis-inducing effect in these colon cancer cell lines, with reduced expression of phosphorylated Akt (Ser^{473}). However, there was a great discrepancy in between the sensitivity for LY294002 and the levels of expression

of pAKT. Although, the LoVo and Colo205 cells displayed high sensitivity to LY294002 with increased apoptosis, the DLD-1 and HCT15 cells did not exhibited rapid induction of apoptosis. The caspase-3 activity was significantly elevated in the LoVo cells but not in the DLD-1 cells. In the *in vivo* experiments using mouse xenografts, LY294002 administration was found effective in the suppression of tumor growth and induction of apoptosis, especially in the LoVo tumors, and therefore showed striking effectiveness in the mouse peritonitis carcinomatosa model.

4.2.4 PI3K/AKT in mast cells promote tumor invasion

In addition to tumor proliferation, tumor invasion is an important phenotype displayed by tumors, necessary for subsequent metastases. Tumor invasion and metastases constitute is a major problem in the treatment of carcinoma patients. About 30% of cancer patients with newly diagnosed solid tumors already have clinically detectable metastases. A metastatic colony is the result of a continuous process starting from the early growth of the primary tumor, and detachment of invasive tumor cells from the primary tumor leading to the colonization of other organs (Fidler *et al*. 1978, Fidler & Hart 1982, Weiss 1985, Fidler & Balch 1987). Tumor cell invasion is a complex process that involves genetic and cellular alterations leading to proteolysis and dispersion through three-dimensional biological barriers(Friedl & Wolf, 2003; Gehlsen et al, 1992; Mignatti & Rifkin, 1993; Stetler-Stevenson et al, 1993). Extracellular matrix (ECM) including the type I collagen, which is the most abundant component of the extracellular matrix (ECM) forms a significant barrier for tumor cell dissemination into the lymphatics, vasculature, and surrounding areas(Sabeh et al, 2004; Seiki, 2003). Thus, in most cases, collagen and ECM must be degraded in order for tumor cells to spread into surrounding anatomic structures and metastasize(Chambers et al, 2002). Cell migration, regulated by polarity and reorganization of the cellular cytoskeleton, is also an integral aspect of tumor cell invasion(Hanahan & Weinberg, 2000; Ridley et al, 2003). Dissecting the etiological factors governing the tumor cell migration and invasion is necessary because the latter, in

conjunction with metastasis, is a significant cause of morbidity and mortality in cancer patients (Sporn, 1996).

Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, which resembles the complex extracellular environment and ECM found in many tissues(Benton et al, 2009; Hughes et al, 2010). Mast cells are rich source of metalloproteases that contribute the majority of proteolytic components necessary to break the ECM for tumor invasiveness(Cassano et al, 2006; Theoharides & Conti, 2004). Mast cells can disturb normal stromal-epithelial communication and degrade matrix at sites of tumor invasion. Mast cells also generate and secrete IL-8, which can act as an angiogenic factor, as well as a tumor cell chemotactic factor and tumor mitogen(Moller et al, 1993). In fact, blocking of IL-8 by use of neutralizing antibodies reduces human non-small cell lung carcinoma progression in mice(Brew et al, 2000).

Using *in vitro* experiments with human derived cell lines and MC soluble factors containing conditioned medium, studies were performed to investigate the role of PI3K in MCs in matrigel based invasion assays. Conditioned medium obtained from MCs promoted tumor invasion and migration in comparison with the non-conditioned medium. Moreover, addition of LY294002 to MC conditioned medium attenuated tumor cell invasion. Furthermore, in line with the observations from *in vitro* assays discussed previously, conditioned medium obtained from MC pretreated with LY294002 attenuated the tumor invasion to the highest levels. In a similar study MC conditioned medium was reported to promote Pancreatic cancer cell invasion of Panc-1 and HDPE through the matrigel by upregulation of matrix metalloproease- 2 and -9(Strouch et al, 2010). In another group of study, in an attempt to investigate direct effect of PI3K/AKT attenuation via LY294002 on invasive potential of gastric cancer similar observations were found, where the *in vitro* invasion assay and the *in vivo* nude mice assay suggested that LY249002 had the potential to inhibit the invasion and metastasis of gastric cancer(Xing et al, 2009). This inhibition could be a result of the decrease in the expression of

MMP-2, MMP-9, MVD and VEGF together with the cytotoxicity towards the tumor MMP-2, MMP-9, MVD and VEGF together with the cytotoxicity towards the tumor cells, both induced by LY294002 (Xing et al, 2009). In another study invasion assays were performed with or without LY294002 (10 μM for 48 hours) and a reduction in the invasion of melanoma cell line WM35RhoC was observed (Ruth et al, 2006). Finally *in vitro* direct effect of LY294002 on tumor invasion was reported extensively in HT1080 human fibrosarcoma cells, MDA-MB-231 breast cancer cells and B16F10 melanoma cells(Yoon et al, 2006), furthermore MMP-9 based mechanism was reported as critical for these tumor invasions (Yoon et al, 2006).

4.2.5 PI3K/AKT in tumor infiltrating leukocytes promote tumor invasion

In addition to MC, the role of PI3K/AKT in tumor infiltrating leukocytes (TILs) were investigated for *in vitro* matrigel based colon cancer invasion. Once the tumor is vascularized, it is infiltrated by leukocytes, a phenomenon seen in all solid cancers. The first observation of TILs was done by Rudolf Virchow in 1863 (Dirkx et al, 2006)and was thought to be the result of chronic inflammation, which was already present before tumor development. However, now it is known that the presence of leukocytes is a consequence of an immune reaction to the tumor itself- first, innate, and later, specific immunity- as the immune system is able to recognize tumor-associated antigens(Brigati et al, 2002). In cancer patients, specific cytotoxic T lymphocytes recognizing tumor antigens have been reported, and the presence of these cells is associated with better prognosis. In addition, antibodies to tumor-associated antigens produced by B cells may also play a critical role in limiting tumor growth(Scanlan et al, 2001; Vaughan et al, 2004). Leukocyte infiltration in tumors is, therefore, often associated with better prognosis and overall better patient survival. However, the role of TILs in cancer progression and metastasis has been debated frequently. Although often considered to be associated with improved prognosis and leading to the enhanced survival in cancer patients, inflammatory cells have also been reported to assist the tumor's capabilities to progress, proliferate, and metastasize.

Tumor-associated macrophages (TAMs), for instance, have been shown to be symbiotically related to tumor cells: Tumor cells recruit TAMs and provide them with survival factors, and TAMs in turn produce a variety of angiogenic factors in response to the tumor microenvironment(Allavena et al, 2008; Solinas et al, 2009).

TILs were isolated from the freshly obtained surgical tissues of ulcerative colitis associated cancer. TILs were found to be high in pAKT and LY294002 treatment attenuated both Ser473 and Thr308 pAKT. *In vitro* tumor invasion studies were performed mimicking the *in vivo* phenotype as seen in the immunohistochemical studies and spatial distribution of the TAMs and pAKT positive inflammatory cells. TAMs from UC patients associated cancer patients promoted HT-29 invasion, either through their secreted soluble factors in the conditioned medium (experimental setup-1) or direct cell-cell contact (experimental setup-2). Indeed, when these cells were co-cultured invasion was even more pronounced (experimental setup-2). However, LY294002 treatment of Tumor Infiltrating Leukocytes reduced their ability to promote HT-29 invasion in both setups. In experimental setup-1 PI3K/AKT was found critical for the release of tumor invasion-promoting soluble factors from TILs. Whereas, in experimental septup-2 cell-cell contact between TILs and tumor cells promoted tumor invasion and PI3K/AKT was vital in TILs for regulating this cell-cell contact with tumor cells for subsequent tumor invasion. In agreement with these observations T-cell exosomes were found to promote lung cancer cell invasion(Cai et al, 2012), while in another study freshly isolated peripheral blood granulocytes and peritoneal macrophages promoted tumor invasion of T-47D (human ductal breast carcinoma) and SW620 (human colon carcinoma), interestingly when granulocytes and macrophages were used together a synergistic effect on tumor invasion was seen(Barbera-Guillem et al, 1999). Moreover, macrophages were reported to promote tumor cell invasion by producing EGF ligands that stimulate tumor cell motility, especially along collagen fibers that macrophages help to fabricate. Macrophages also induce the formation of blood new vessels that are

sheaved in collagen, and thus, the migration of tumor cells tends to be focused toward these newly formed vessels, thereby directing tumor cells to sites of intravasation (Pollard, 2008). The latter process in turn is enhanced by macrophages that align upon the outside of the blood vessels that are elaborated under the influence of macrophages within the malignant areas and provide portals of escape for the tumor cells. All of these macrophage-promoted activities of increased angiogenesis as well as enhanced tumor cell migration, invasion, and intravasation results in increased metastatic capacity of the tumor (Pollard, 2008). Few attempts have been made to coculture macrophages with the tumor cells in *in vitro* invasion assays and investigate the change in invasive potential of the tumor cell. The human breast cancer cell lines MCF-7, SK-BR-3 and the benign mammary epithelial cell line hTERT-HME1 were cocultured with macrophages(Hagemann et al, 2004). In agreement with results obtained from our current study, Hagemann et al, found that coculture enhanced invasiveness of the cancer cells on cell-cell contact, while hTERT-HME1, the benign counterpart remained non-invasive. Moreover, addition of the broad-spectrum matrix metalloprotease (MMP)-inhibitor FN 439, neutralizing MMP-9 or tumor necrosis factor-alpha (TNF-a) antibodies reduced invasiveness to basal levels(Hagemann et al, 2004). At basal levels, all cell lines produced low amounts of MMP-2, -3, -7 and -9, while basal MMP production by macrophages was significantly higher. Upon coculture, supernatant levels of MMPs -2, -3, -7 and -9 increased significantly, paralleled by an increase of MMP-2 activation. Co-culture of macrophages and hTERT-HME1 did not lead to MMP induction. In the cocultures, mRNAs for MMPs and TNF-a were significantly upregulated in macrophages, while the mRNA concentrations in the tumor cells remained unchanged. In conclusion, cell-cell contact of tumor cells with macrophages leads to enhancement in the invasiveness of the malignant cells due to TNF-a dependent MMP induction in the macrophages(Hagemann et al, 2004). In an extension to this study by Hageman et al, coculture of macrophages with ovarian or breast cancer cell lines led to TNF-α-dependent activation of JNK and NF-κB pathways in tumor cells, but not in benign immortalized epithelial cells. Tumor

cells with upregulation of JNK and NF-κB activity exhibited enhanced invasiveness. Blockage of the NF-κB pathway by TNF-α neutralizing Abs, an NF-κB inhibitor, RNAi to RelA, or overexpression of IκB attenuated tumor cell invasiveness(Hagemann et al, 2005). Blockade of JNK also significantly restricted invasiveness, but blockade of p38 MAPK or p42 MAPK had no effect(Hagemann et al, 2005). Cocultured tumor cells when screened for the expression genes associated with inflammation and invasion that also contained an AP-1 and NF-κB binding site, EMMPRIN and MIF were found to be upregulated in cocultured tumor cells in a JNK- and NF-κB-dependent manner(Hagemann et al, 2005). Knocking down either MIF or EMMPRIN by RNAi in the tumor cells significantly attenuated tumor cell invasiveness and matrix metalloprotease activity in the coculture supernatant. Hence, in conclusion TNF-α, via NF-κB, and JNK induces MIF and EMMPRIN in macrophage to tumor cell cell-cell contact cocultures, leading to increase in the invasive potential of the tumor cells(Hagemann et al, 2005).

4.3 PI3K/AKT inhibition attenuates *in vivo* cancer invasion, cancer-associated inflammation and cancer promoting properties in mouse model

Observations from *in vitro* studies were tested in the *in vivo* mouse model system. IL-$10^{-/-}$ mice treated with Piroxicam develop experimental colitis associated cancer by day 56 and were used to study the effect of LY294002 treatment/PI3K inhibition on colitis-associated cancer. In the IL10 deficient mouse model PI3K is required for induction of colitis, and its targeted genetic ablation (Brown et al, 2010; Cook et al, 2010; Gonzalez-Garcia et al, 2010; Gounaris et al, 2007; Jain et al, 2010; Kim et al, 2010; Lee et al, 2010; Nigrovic et al, 2010) or treatment of mice with the broad PI3K inhibitors Mesalamine (Brown et al, 2010) or LY294002 (Brown et al, 2010; Cook et al, 2010; Gounaris et al, 2007; Jain et al, 2010; Kim et al, 2010; Lee et al, 2010; Nigrovic et al, 2010) protect against colitis. PI3K was described to mediate proliferation and activation of Akt resulting in Crypt architectural changes that predispose to colitis (Brown et al, 2010; Cook et al, 2010; Gounaris et al, 2007; Jain et al, 2010; Kim et al, 2010;

Lee et al, 2010; Nigrovic et al, 2010). However, as described above using *in situ* staining of human colonic tissue sections that the relative frequency of detectable PI3K active cells in the stroma and submucosa is more than 10 fold above that in of epithelial cells and increases with colitis and cancer; by contrast, in the $IL10^{-/-}$ mouse colon this ratio is reversed. This difference between human and mouse tissue may have masked the significance of PI3K activity by tissue infiltrating cells in predisposition to colitis and progression to cancer. $IL\text{-}10^{-/-}$ that receives Piroxicam develops colitis followed by invasive lesions by day 56, however simultaneous LY294002 treatment attenuated the development of invasive lesion counts. Treatment of LY294002 inhibited epithelial and stromal pAkt counts, however LY294002 showed a striking attenuation of stromal pAkt in comparison with epithelial pAkt, reduction in the inflammatory scores with LY294002 treatment could be partially responsible for this observation. Moreover, epithelial proliferation was reduced and tumor apoptosis was increased with LY294002 treatment, which could be well correlated with the reduction in the invasive lesion counts. Furthermore, LY294002 treatment reduced granulocyte and mast cell frequencies and *in situ* mast cell degranulation. In agreement with these observations in a similar experimental setup in another study, LY294002 treatment reduced the frequency of dysplastic lesions, reduced epithelial proliferation, epithelial Akt and increased apoptosis (Lee et al, 2006). The critical role of phosphoinositide 3-kinase-γ (PI3Kγ) in inflammatory cell activation and recruitment makes it an attractive target for immunomodulatory therapy. A study with 5-Quinoxilin- 6-methylene-1,3-thiazolidine-2,4-dione (AS605240), a potent PI3K inhibitor, has been reported to ameliorate chronic inflammatory disorders including rheumatoid arthritis, systemic lupus erythematosus, and atherosclerosis and colitis(Peng et al, 2010). In this study therapeutic potential of AS605240 in mice with dextran sodium sulfate (DSS)- induced acute and chronic colitis were studied. AS605240 improved survival rate, disease activity index, and histological damage score in mice administered DSS in both preventive and therapeutic studies. AS605240 treatment also significantly inhibited the increase in myeloperoxidase levels, macrophage infiltration, and CD4 T-cell number in the

colon of DSS-fed mice (Peng et al, 2010). The DSS induced overproduction of colonic proinflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor-α, and interferon-γ was significantly suppressed in mice undergoing AS605240 therapy, whereas colonic anti-inflammatory cytokines such as IL-4 were up-regulated(Peng et al, 2010). The downregulation of the phospho-Akt level in immunological cells from the inflamed colon tissue and spleen of AS605240-treated mice was detected both by immunohistochemical analysis and Western blotting. These findings demonstrate that AS605240, another PI3K inhibitor, may represent a promising novel agent for the treatment of inflammatory bowel disease by suppressing leukocyte infiltration as well as by immunoregulating the imbalance between proinflammatory and anti-inflammatory cytokines (Peng et al, 2010). Another preferential p110α/γ PI3K inhibitor (compound 8C; PIK-75) that was found to be effective in inflammation-based assays, cell-based assays revealed that PIK-75 potently and dose dependently inhibits *in vitro* and *in vivo* production of TNF-α and IL-6, diminishes the induced expression of human endothelial cell adhesion molecules (E-selectin, ICAM-1, and VCAM-1), and blocks human monocyte-endothelial cell adhesion(Dagia et al, 2010). Most importantly, PIK-75, when given orally in a therapeutic regimen, significantly suppresses the macroscopic and histological abnormalities associated with dextran sulfate sodium-induced murine colitis. The efficacy of PIK-75 in attenuating experimental inflammation is mediated, at least in part, due to the downregulation of pertinent inflammatory mediators in the colon (Dagia et al, 2010).

In addition to the investigation performed to dissect the role of PI3K in inflammatory cells using human *in vitro* and mouse *in vivo* system in cancer progression, *in vitro* role of PI3K in mouse system was performed to confirm and crosscheck the observations. PI3K in gut-derived mouse MC was critical for degranulation and mouse colon carcinoma proliferation and invasion. LY294002 treatment of MC attenuated *in vitro* degranulation, MC associated mouse colon cancer proliferation and invasion.

Tissue infiltrating MC and macrophages are sensitive to inhibition of PI3K and are abundantly present in increasing numbers during progression to colitis and cancer. Therapies that target the PI3K pathway need to take into account that tumor cells may not be the primary target cells. These findings demonstrate the role of PI3K in tumor infiltrating cells and their communication with tumor cells, drawing attention to the role of PI3K signaling in the tissue and tumor environment in predisposition to cancer.

5.
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