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Toll-Like Receptors as Novel Therapeutic Targets for the Treatment of Pancreatic Cancer

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1. Introduction

Toll-Like Receptors (TLRs) are critical mediators of the innate immune response and subsequent activation of adaptive immune responses to pathogens that invade the body. TLRs on immune cells are the basis of our multigenic, innate immune, inflammatory response to pathogenic signature molecules that cause tissue damage. Functional TLRs are expressed not only on immune cells, but also on non-immune cells including cancer cells. TLRs are critical mediators of cellular transformation, tumor progression, and metastasis.

2. Toll-Like Receptors

TLRs are pattern recognition receptors that were originally identified in immune cells and described to recognize pathogen-associated molecular patterns (PAMPs) and initiate an innate immune response and subsequent adaptive immune responses against infection or tissue damage. They protect mammals from pathogenic organisms, such as viruses, by generating an "innate immune" response to products of the pathogenic organism (Takeda et al., 2003). This response results in increases in genes for several inflammatory cytokines and chemokines, major histocompatibility (MHC) genes I and II, as well as co-stimulatory molecules, and is critical for the development of antigen-specific adaptive immunity (Takeda et al., 2003). In addition to their presence on immune cells, TLRs are also found to be expressed normally on multiple non-immune cell types including, but not limited to epithelial cells that line the digestive system, lungs, and female reproductive tract, as well as on pancreatic beta cells, and keratinocytes in the skin where they function to regulate cellular proliferation and apoptosis in response to infection and/or other damaging environmental insults (radiation, chemicals, etc.)(Andonegui et al., 2003; Ortega-Cava et al., 2003; Giarratana et al., 2004; Schroder & Maurer, 2007; Nasu & Narahara, 2010; Yamasaki et al., 2010; Ayari et al., 2011). Regardless of cell type, TLRs recognize not only exogenous PAMPs, but also endogenous damage-associated molecular patterns (DAMPs) (Table 1). Eleven TLRs have been described in humans to date (TLR1 to TLR11) (Sato et al., 2009).

TLRs are located both on the cell surface and in the cytoplasm, are differentially expressed in different cell types under a variety of conditions and disease states, and recognize different PAMPs and DAMPs (Table 1).

Perhaps two of the most well studied TLRs are TLR3 and TLR4. In non-immune cells, as is the case for immune cells, dsRNA can activate two distinct anti-viral response pathways by activating TLR3 signaling. One, coupled via the adapter molecule, Toll-IL-1 receptor (TIR) domain-containing adapter inducing IFN-β (TRIF) (also called TIR domain-containing adaptor molecule-1 [TICAM-1]), i.e. (TRIF/TICAM)-1, activates IFN regulatory factor (IRF)-3 and the production of type 1 interferons (IFN-α or IFN-β) via TRAF-3 (Oganesyan et al., 2006; Schneider et al., 2006). The type 1 IFNs, acting as autocrine/paracrine ligands increase STAT-1 and IRF-1 activation, critical factors in expression of chemokines such as CXCL10 and genes such as VCAM-1. Specifically, TLR3 is expressed in the endosomal membrane and recognizes extracellular viral dsRNA and/or its synthetic analog poly I:C. Upon binding to dsRNA, TLR3 becomes dimerized, and two specific tyrosine residues (Tyr^{759} and Tyr^{858}) in the TIR domain of TLR3 become phosphorylated and are essential for dsRNA-induced recruitment of the adaptor protein TRIF/TICAM-1 (Sarkar et al., 2004). Phosphatidylinositol 3-kinase (PI3K) is then recruited to the two phosphorylated tyrosine residues and is required for phosphorylation and activation of IRF-3. Additionally, it has been shown that TLR3 associates with c-Src in response to dsRNA and that c-Src is necessary for PI3K-dependent activation of IRF-3, although the precise role of c-Src in this process is currently not well understood (Johnsen et al., 2006). TRIF/TICAM-1 dissociates from TLR3 and forms a complex with receptor interacting protein 1 (RIP1), TRAF-3 and NF-κB activating kinase (NAK)-associated protein 1 (NAP1). The TRIF/TICAM-1/TRAF-3/RIP-1/NAP-1 complex participates in the recruitment and activation of TBK-1 and IKKε which phosphorylate and activate IRF-3 (Sasai et al., 2005; Hacker et al., 2006; Oganesyan et al., 2006). Once phosphorylated IRF-3 translocates into the nucleus and together with nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and AP-1 induces IFN-β gene transcription (Sato et al., 2000). The second pathway is coupled to a different site on TRIF/TICAM-1 via TRAF-6 and activates NF-κB and MAP Kinase pathways important in the production of pro-inflammatory and inflammatory cytokines, e.g. TNF-α, IL-1β, and IL-6, as well as chemokines e.g. MCP-1.

TLR4 signaling has both a MyD88-dependent and MyD88–independent mechanism when activated [reviewed in (Lu et al., 2008)]. There is significant homology between TLR3 and TLR4 IRF-3/IFN signaling (the MyD88-independent TLR4 pathway); TRIF/TICAM-1 is a common signaling intermediate in both signaling pathways. As is the case with dsRNA-induced TLR3 signaling, activation of TLR4 signaling leads to TRIF/TICAM-1 recruiting TRAF-3 and RIP-1 which in turn leads to the recruitment and activation of TBK-1 and IKKε (Hacker et al., 2006; Oganesyan et al., 2006; Guo & Cheng, 2007). The TRIF/TICAM-1/TRAF-3/RIP-1/TBK-1/IKKε complex phosphorylates and activates IRF-3 (Fitzgerald et al., 2003; Hemmi et al., 2004). Once phosphorylated IRF-3 translocates into the nucleus and together with NF-κB and AP-1 induces IFN-β gene transcription (Sato et al., 2000). The MyD88-dependent pathway activates IRAK-4, IRAK-1, TRAF-6, and others to lead to the activation of transcription factors NF-κB, AP-1, and IRF-5, which induce the expression of pro-inflammatory cytokines.

Toll-Like Receptor	Predominant Cellular Localization	PAMPs/DAMPs/Other Ligands	Disease Associations
TLR1	Plasma Membrane	triacyl lipopeptides, modulin (phenol-solube)	cancer, psoriasis, sepsis, leprosy
TLR2	Plasma Membrane	glycolipids, triacyl lipopeptides, heat shock proteins, high mobility group box 1 protein, rare LPS species (*P. gingivalis*), lipopeptides, lipoteichoic acid, measles haemagglutinin, mannuronic acids, neisseria porins, peptidoglycan, zymosan (Beta-glucan), bacterial fimbriae, *Yersinia* virulence factors, CMV virions, saturated fatty acids	cancer, type 2 diabetes, psoriasis, pre-eclampsia, alzheimer's, herpes simplex encephalitis, rheumatoid arthritis, acne vulgaris, acute rheumatic fever, asthma, atherosclerosis, chronic obstructive pulmonary disease, diabetic nephropathy, subhorrheic dermatitis
TLR3	Endosomal Membrane	dsRNA (self and viral), poly I:C	cancer, type 1 diabetes, herpes simplex encephalitis, Hashimoto's thyroiditis, virus associated autoimmune disease
TLR4	Plasma Membrane	lipopolysaccherides, saturated free fatty acids, fibrinogen, fibronectin, heat shock proteins, flavolipins, *S. pneumoniae* pneumolysin, heparan sulfate, hyaluronic acid, high mobility group box 1 protein, MMTV envelope proteins, nickel, paclitaxel, RSV fusion protein, respiratory syncytial virus coat protein, mannuronic acid polymers, teichuronic acids, bacterial fimbriae, surfactant protein A, β-defensin 2	cancer, type 2 diabetes, toxic shock, ulcerative colitis, atherosclerosis, pre-eclampsia, alzheimer's, stroke, rheumatoid arthritis, chronic obstructive pulmonary disorder, Crohn's disease, periodontitis, peripheral arterial disease,

Toll-Like Receptor	Predominant Cellular Localization	PAMPs/DAMPs/Other Ligands	Disease Associations
TLR5	Plasma Membrane	flagellin	cancer, psoriasis, systemic lupus erthyematosus
TLR6	Plasma Membrane	diacyl lipopeptides, bacterial cell wall components, modulin (phenol-soluble)	cancer, leprosy (Hansen's disease), filarisis, asthma, hypersensitivity pneumonitis
TLR7	Endosomal Membrane	self ssRNA, ssRNA, bropirimine, loxoribine, imidazoquinoline	cancer, systemic lupus erythematosus, rheumatoid arthritis, arthritis
TLR8	Endosomal Membrane	self ssRNA, small synthetic compounds, imidazoquinoline	cancer, rheumatoid arthritis, viral keratitis, crimean-congohemorrhagic fever
TLR9	Endosomal Membrane	self DNA, unmethylated CpG DNA	allergies, asthma, cancer, multiple sclerosis, systemic lupus erythematosus, Graves' ophthalmopathy, crimean-congohemorrhagic fever, arthritis
TLR10	Plasma Membrane	Unknown	cancer, asthma, nephropathy, viral keratitis

Table 1. Toll-Like Receptors; Cellular Localization, Agonists, and Disease Associations

3. TLRs, chronic inflammation, and cancer

Overwhelming evidence suggests that chronic inflammation is crucial to the onset and progression of a multiplicity of human cancers, including pancreatic cancer (Kuper et al., 2000; Garcia et al., 2004; von Hafe et al., 2004; Berstein, 2005; Otake et al., 2005; Lu et al., 2006). The exact link between chronic inflammation and carcinogenesis is unclear, however many studies have shown that pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, etc.) are important for the proliferation, survival, metastasis, and escape from immune surveillance of many of these cancers (Garcea et al., 2005; Lu et al., 2006), and have identified nuclear factor-κB (NF-κB) as a key modulator of inflammation-induced carcinogenesis (Chen et al., 1995; Guttridge et al., 1999; Hinz et al., 1999; Perkins, 2000; Coussens & Werb, 2002; Esposito

et al., 2002; Ghosh & Karin, 2002; Karin et al., 2002; Karin & Lin, 2002; Li & Verma, 2002; O'Hanlon et al., 2002; Alexiou et al., 2003; Chen et al., 2003; Furbert-Harris et al., 2003; Lin & Karin, 2003; Greten et al., 2004; Huber et al., 2004; Luo et al., 2004; Niu et al., 2004; Pikarsky et al., 2004; Stoffel et al., 2004; Heyninck & Beyaert, 2005; Preciado et al., 2005; Yang et al., 2005a). Chronic inflammation as a result of disease, microbial infection, and/or obesity is an important risk factor for the development of a variety of cancers (Khatami; Kuper et al., 2000; Calle & Kaaks, 2004; Khatami, 2011) (Table 2). Chronic inflammation is thought to induce malignant transformation via activation of oncogenes, induction of immunosuppression, and inhibition of tumor suppressors.

It is now recognized that TLRs are important in development of carcinogenesis and tumor progression. The chronic inflammatory state observed in autoimmune disease, microbial infection, and obesity is mediated via activation of TLR signaling on both immune and non-immune cells. TLRs on immune cells recognize PAMPs and DAMPs and initiate an innate immune response and subsequent adaptive immune responses against infection or tissue damage. As part of this acute, innate immune response cytokines and chemokines are produced and released by the immune cells, which will subsequently upregulate TLR expression on non-immune cells. When this occurs, the TLRs on the non-immune cells can become stimulated by the same PAMPs and DAMPs that activated the TLRs on the immune cells, leading to very high levels of disease-causing inflammatory proteins. In a prolonged state of infection and/or tissue damage, sustained high levels of inflammatory proteins can lead to autoimmune, inflammatory diseases, and cancer in individuals with certain genetic and/or environmental susceptibilities (Table 1).

Multiple TLRs have been implicated in a variety of cancers including pancreatic cancer, melanoma, breast cancer, prostate cancer, colorectal cancer, lung cancer, cervical cancer, liver cancer, etc. (McCall et al., 2007; Sato et al., 2009; Schwartz et al., 2009) (Table 3). Activation of TLRs on cancer cells promotes chronic inflammation which stimulates cancer cell proliferation, migration, tumor angiogenesis, and creates a tumor microenvironment which impairs the anti-tumor function of the immune system allowing tumors to develop and survive.

4. Mechanisms of TLR regulation of carcinogenesis

4.1 Activation of TLR signaling leads to the production of cytokines that control growth

As previously described, chronic TLR activation and signaling in both immune and non-immune cells by environmental antigens are now linked to oncogenesis, tumor growth, and invasive spread (Schmausser et al., 2005; Kelly et al., 2006; Fukata et al., 2007; He et al., 2007; Ilvesaro et al., 2007; Goto et al., 2008; Kim et al., 2008; Yoneda et al., 2008; Curtin et al., 2009; Xie et al., 2009; Zhou et al., 2009). Activation of TLR signaling results in the activation of transcription factors NF-κB and AP-1, as well as Type I Interferon (IFN) signaling pathways with subsequent production of "oncogenic" cytokines, and the activation of MAPK and AKT signaling pathways (Figure 1). Multiple TLR-induced cytokines including TNF-α, IL-1, IL-6, IL-8, IL-10, IL-23, etc. have been linked to oncogenesis. TLR activation also upregulates many growth factors such as TGF-β, VEGF, CXCR4, and adhesion molecules such as ICAM-1 (Kelly et al., 2006; He et al., 2007; Ren et al., 2007; Zhou et al., 2009). These TLR-mediated processes have been linked to various cancers including colon, pancreas, melanoma, breast,

Type of Cancer	**Disease/Infection Association**
Thyroid Cancer	Hashimoto's Thyroiditis
	Obesity
Colorectal Cancer	Inflammatory Bowel Disease
	Colitis
	Crohn's Disease
	Obesity
Cervical Cancer	Human Papilloma Virus
Liver Cancer	Hepatitis Virus B and C
Pancreatic Cancer	*Helobacter pylori*
	Obesity
Prostate Cancer	Obesity
Hematologic Malignancies	Epstein-Barr Virus
	Cytomegalovirus
Esophageal Adenocarcinoma	Obesity
Renal Cancer	Obesity
Endometrial Cancer	Obesity
Gallbladder Cancer	Obesity
Breast Cancer	Obesity
Gastric Cancer	Epstein-Barr Virus
	Helobacter pylori
	Obesity
Non-Hodgkin's Lymphoma	Epstein-Barr Virus
	Human Herpes Virus 8
	Human Immunodeficiency Virus
Hodgkin's Disease	Epstein-Barr Virus
Nasopharyngeal Carcinoma	Epstein-Barr Virus
Burkitt's Lymphoma	Epstein-Barr Virus
Vulvar Cancer	Human Papilloma Virus
Anus Cancer	Human Papilloma Virus

Type of Cancer	Disease/Infection Association
Penis Cancer	Human Papilloma Virus
Head and Neck Cancer	Human Papilloma Virus
Kaposi's Sarcoma	Human Herpes Virus 8
	Human Immunodeficiency Virus
Casteleman's Disease	Human Herpes Virus 8
Adult T-cell Leukaemia	Human Thymus-Derived-Cell Leukaemia/Lymphoma Virus-1
Bladder Cancer	Schistosomes (*S. haematobium*)

Table 2. Associations Between Disease, Infection, Obesity, and Cancer.

prostate and many others (Sato et al., 2009). In addition, these cytokines also activate transcription factors that induce the expression of several tumor promoting and anti-apoptotic genes which will be discussed in Section 4.2 below.

Toll-Like Receptor	Type of Cancer
TLR1	Colon, Prostate
TLR2	Brain, Breast, Colorectal, Gastric, Hepatocellular Carcinoma, Laryngeal, Lung, Melanoma, Ovarian
TLR3	Breast, Colorectal, Hepatocellular Carcinoma, Laryngeal, Lung, Melanoma, Ovarian, Pancreatic
TLR4	Bladder, Brain, Breast, Cervical, Colorectal, Gastric, Hepatocellular Carcinoma, Laryngeal, Lung, Melanoma, Ovarian, Pancreatic, Prostate
TLR5	Cervical, Colorectal, Gastric, Ovarian
TLR6	Hepatocellular Carcinoma, Prostate
TLR7	Chronic Lymphocytic Leukemia, Lung
TLR8	Lung
TLR9	Breast, Cervical, Colorectal, Gastric, Glioma, Hepatocellular Carcinoma, Lung, Pancreatic, Prostate
TLR10	Nasopharyngeal, Prostate
TLR11	None

Table 3. TLRs are Associated with Human Cancers

These immune-response and tumor-associated cytokines have complex and often contradictory effects depending on the specific tumor, the specific TLRs activated, and the innate immune response to the malignancy. Indeed, the tumor microenvironment which includes tumor cells, tumor-derived fibroblasts, as, well as macrophages, T cells, and APCs each produce inflammatory cytokines forming a "milieu" which on one hand facilitates the differentiation and expansion of tumors and on the other tries to suppress this process.

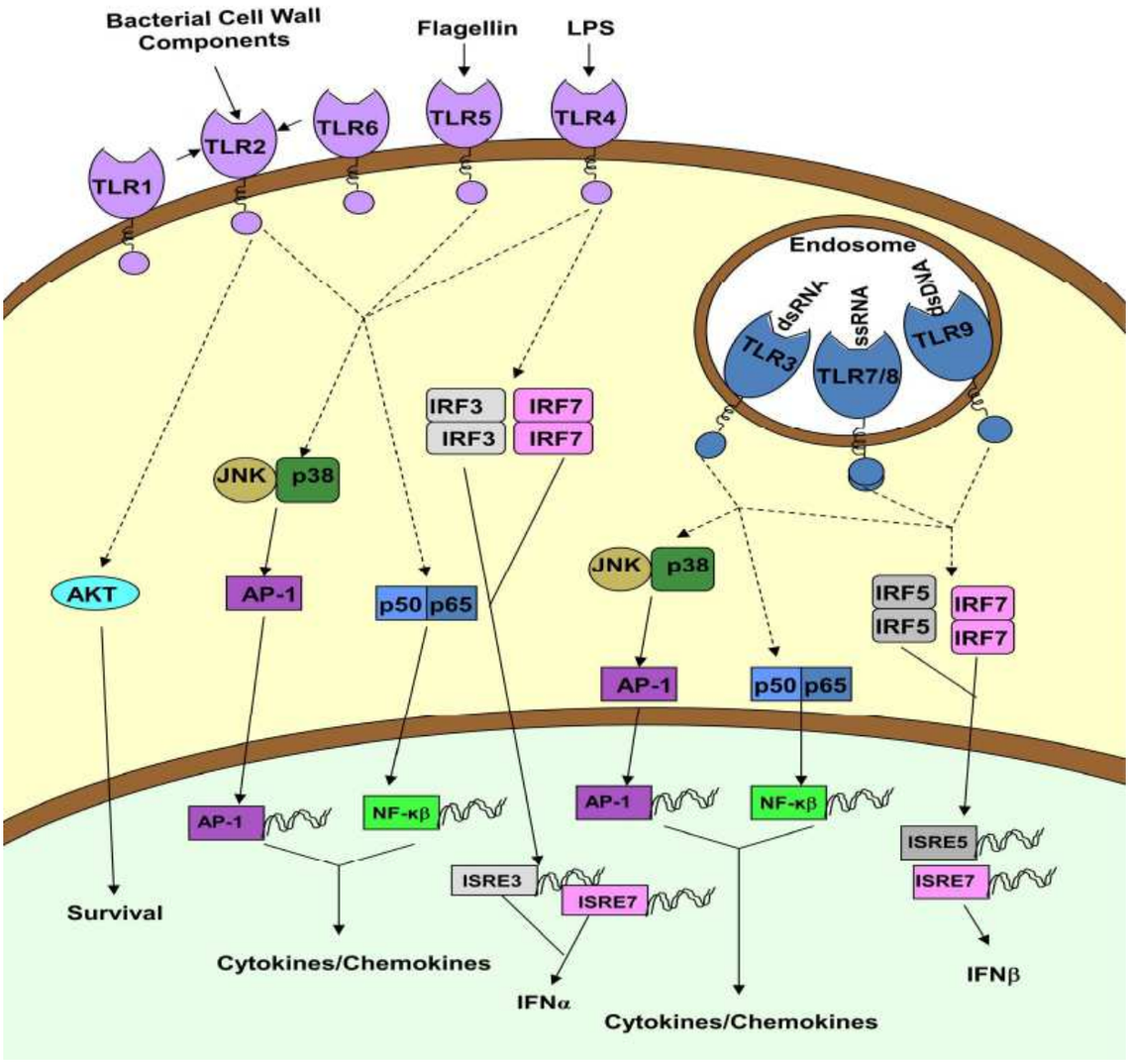

Fig. 1. TLR Signaling

The most important TLR-mediated cytokines involved in initiation and growth of malignant tumors appear to be tumor-derived IL-1, TNF-α and IL-6. TNF-α and IL-6 are potent activators of NF-κB pathways and JAK-STAT signaling pathways which also directly contribute to tumor induction and growth (See Section 4.2). TNF-α and IL-6 also induce anti-aptotic genes such as Mcl-1, Bcl-x, cell-cycle regulators (cyclins D1/D2, c-Myc), and inducers of angiogenesis (VEGF) (Calo et al., 2003). TNF-α in the tumor microenvironment also increases myeloid cell recruitment via IL-17. Importantly, IL-6 is one of the most potent activators of the oncogenic transcription factor STAT3 (Calo et al., 2003).

TLR mediated IL-1 expression within tumors directly stimulates tumor growth as well as stimulating angiogenesis which enhances a tumor's capacity to spread or metastasize (Gemma et al., 2001; Elaraj et al., 2011). Expression of IL-1 within tumors, the surrounding tissues, or endothelial cells is associated with aggressive growth characteristics (Elaraj et al., 2006; Sawai et al., 2006).

In contrast, IL-10 is normally an inhibitory cytokine which blocks NF-κB activity and the JAK-STAT signaling pathway but has been shown to help certain tumors escape normal immune surveillance (Linehan & Goedegebuure, 2005; Perrone et al., 2008; Strauss et al., 2009). IL-10 can induce CD4+CD25+Foxp3+ regulatory T cells (Tregs) in the tumor microenvironment which secrete additional IL-10 and TGFβ, which can then suppress the anti-tumor function of non-Treg T cells (Linehan & Goedegebuure, 2005; Perrone et al., 2008; Strauss et al., 2009).

4.2 TLR signaling activates transcription factors important for tumorigenesis

Two of the most notorious and well-studied oncogenic transcription factors are nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3). Both NF-κB and STAT3 are activated by a variety of stimuli (stressors, cytokines, etc.), and while they are regulated by entirely different signaling mechanisms, they both control the expression of proliferation-enhancing, anti-apoptotic, angiogenic, and immune-modulating genes. NF-kB and STAT3 also interact and mediate crosstalk between tumor cells and inflammatory cells within the tumor microenvironment to promote the development and progression of multiple types of human cancers including but not limited to pancreatic, colon, gastric, skin, head and neck, and liver cancers (Grivennikov & Karin; Lin et al.; Bromberg et al., 1999; Greten et al., 2004; Yu & Jove, 2004; Yu et al., 2009).

NF-κB, which is directly activated via the MyD88-dependent branch of TLR signaling, is one of the most studied transcription factors and arguably the most important for tumor promotion (Chaturvedi et al., 2011). NF-κB is constitutively activated in most cancers typically due to stimulation of TLRs, pro-inflammatory cytokine receptors (such as TNF-α and IL-1), and antigen receptors (Dinarello, 1994; Kruglov et al., 2008; Bezbradica & Medzhitov, 2009; Karin & Gallagher, 2009). This constitutive activation of NF-κB has been linked to inflammation, transformation, proliferation, angiogenesis, invasion, metastasis, chemoresistance and radioresistance (Beg & Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996; Huang & Miyamoto, 2001; Joyce et al., 2001; Luo et al., 2004; Mantovani et al., 2008; Chaturvedi et al., 2011). Activation of NF-κB results in the upregulation of several genes and cytokines which are associated with cell growth and proliferation. These include critical cellular progression genes and anti-apoptotic genes including specific inhibitor of caspase 8, c-FLIP29, the caspase inhibitors cIAP1 & cIAP2, the anti-apoptotic member of the B-cell leukaemia/lymphoma 2 (Bcl2) family, Bcl-XL BCL2 and BCL-XL, c-myc, c-myb. Cyclin D1/2 stimulation by NF-kB activation has also been shown to stimulate cell-cycle progression. Many of these together upregulate JNK activity and subsequent activation of the AP-1 transcription factor which also enhances cell survival (Karin, 2006). Inhibitors of NF-κB have been shown to decrease tumor cell proliferation and aid in the potency of chemotherapeutics (Luo et al., 2004; Chaturvedi et al., 2011).

In non-stimulated cells, STAT3 is kept in an inactive form in the cytoplasm (Darnell et al., 1994; Yu & Jove, 2004). Upon stimulation STAT3 is phosphorylated at two critical residues; tyrosine 705 (Tyr^{705}) and serine 727 (Ser^{727}) (Wen et al., 1995; Barboza et al., 2004; Gartsbein et al., 2006; Yeh et al., 2006), dimerizes (Yoshimura et al., 2007), and translocates to the nucleus where it activates a wide array of genes critical for tumor development and

progression. STAT3 is activated by many cytokines, including those cytokines that are products of the TLR signaling pathway, including cytokines of the IL-6 family that signal through gp130 (IL-6, IL-11, IL-27, etc.), the IL-10 family (IL-10, IL-22, IL-19, IL-20), and the epidermal growth factor (EGF) family (VEGF, IL-21, IL-23, HGF). Like NF-κB, STAT3 also activates anti-apoptotic genes such as Bcl-xL, Bcl-2, and c-IAP2 (Yu & Jove, 2004; Chang et al., 2006; Rebouissou et al., 2009), cell cycle and proliferation genes such as Cyclin D1 and c-Myc (Levy & Darnell, 2002; Naugler & Karin, 2008; Bollrath et al., 2009), and members of the AP-1 family such as c-Jun and c-Fos (Hirano et al., 2000; Yang et al., 2005b; Yang et al., 2007), whereas others such as Mcl-1 and Survivin are STAT-3-dependent (Yu & Jove, 2004). Thus, STAT3 is an attractive target for anti-cancer therapy and numerous strategies have been employed to inhibit constitutive STAT3 signaling in cancer cells (Meydan et al., 1996; Turkson et al., 2001; Coleman et al., 2005; Song et al., 2005; Duan et al., 2006; Schust et al., 2006; Iwamaru et al., 2007; Siddiquee et al., 2007; Goel et al., 2008; Hatcher et al., 2008; Lin et al., 2010a; Lin et al., 2010b).

Activator Protein 1 (AP-1) transcriptional complexes also play a pivitol role in malignant cellular transformation (Lopez-Bergami et al., 2009). AP-1 can be activated through the TLR Myd88-dependent pathway by a variety of growth factors and cytokines (Akira, 2006). Increased exposure to environmental carcinogens such as tobacco, nicotine and asbestos have been shown to increase AP-1 activity and correlate with tumorgenesis, tumor invasion and metastasis (Lopez-Bergami et al., 2009). AP-1 can induce the activation of well-known oncogenes including FOS and Jun which are observed in many cancers (Lopez-Bergami et al., 2009). The expression of Jun can further activate other oncogenes including Ras, BRAF and EGFR. Increased FOS expression has been associated with poor prognosis and progression (Lopez-Bergami et al., 2009). Inhibition of AP-1 complexes as well as Jun and FOS have been shown to inhibit tumor formation suggesting that AP-1 may be a viable target for therapeutic intervention (Lopez-Bergami et al., 2009).

4.3 Activation of TLR signaling leads to the production of cytokines and upregulation of other proteins that control metastasis

An important effect of inflammation on cancer is the ability to stimulate tumor invasiveness and metastasis. This ability depends in part, on activation of TLRs on immune cells and cancer cells. The pro-inflammatory microenvironment contributes to shaping the gene expression profile that is required for metastatic behavior of cancer cells.

4.3.1 NF-κB

TLR activation of MyD88, TRAF6, and NF-κB in inflammatory cells and tumor cells within the tumor microenvironment play a key role not only in cancer development but also in tumor progression (Gohda et al., 2004; Greten et al., 2004; Pikarsky et al., 2004; Kaisho & Akira, 2006; Inoue et al., 2007). A correlation was found between the amount of tumor associated macrophages (TAMs) present in the tumor and prognosis; the higher the density of TAMs, the poorer the prognosis (Duncan et al., 1998). TLR-mediated pro-inflammatory cytokine production from tumor associated macrophages (TAMs) play a key role in tumor progression and metastasis (Gohda et al., 2004; Inoue et al., 2007). For example, activation of the TLR-TRAF6- NF-κB pathway in tumor associated inflammatory cells, and the

subsequent release of pro-inflammatory cytokines from those cells has been shown to result in the activation of NF-κB in precancerous cells which drives the growth and malignant transformation of those cells (Gohda et al., 2004; Inoue et al., 2007). In addition, TLR-mediated activation of NF-κB is necessary for the differentiation and maturation of osteoclasts, which drive the resorption of bone and generate a microenvironment ideal for tumor cells to proliferate and colonize, thus enhancing bone metastasis of certain types of cancer cells, particularly breast cancer cells (Coleman & Rubens, 1987; Sasaki et al., 1995; Lomaga et al., 1999; Naito et al., 1999; Kobayashi et al., 2001). Several genes regulated by NF-κB encode adhesion molecules, MMPs, serine proteases, heparanase, and chemokines that have been shown to be important for tumor invasion and metastasis (Karin & Greten, 2005). Blockade of NF-κB activation reduces proliferation, metastatic ability, and enhances apoptosis of cancer cells (Nakanishi & Toi, 2005; Inoue et al., 2007).

4.3.2 TLR4

TLR4 signaling has been directly implicated in the regulation of cancer cell metastasis. Activation of TLR4 signaling in cancer cells by lipopolysaccharide (LPS), an exogenous ligand for TLR4, induces synthesis of IL-6, inducible nitric oxide synthase (iNOS), and IL12 p40. This effect on cancer cells is similar to what is observed during the activation of macrophages. Blockade of the TLR4 pathway has been found to delay tumor growth and prolong survival in mice (Huang et al., 2005a). Recently it was demonstrated that LPS increases the invasiveness of pancreatic cancer cells and that increases in invasiveness by LPS was hampered by blocking the NF-κB signaling pathway. More specifically, the LPS-dependent invasiveness was decreased by blocking the TLR4/MyD88/NF-κB signaling pathway, once again connecting TLR-mediated inflammation with cancer invasion and progression (Ikebe et al., 2009). In fact, a clinical study of 30 cases of pancreatic ductal adenocarcinoma found that TLR4, NF-κB and hypoxia-inducible factor 1-alpha (HIF1α) were over-expressed compared to surrounding tissues. Survival of patients with an absence of TLR4 expression in tumor tissues was significantly longer than those with TLR4 expression (Zhang et al.).

4.3.3 Wnt5a

Wnt proteins are a family of secreted glyocoproteins involved in critical cellular processes during embryogenesis. Wnt5a is a member of the Wnt family that has been implicated in carcinogenesis and inflammation. Non-canonical Wnt5a activates B-catenin-independent pathways important for cell migration and polarity.

Wnt5a has been implicated in pancreatic cancer for many years. A decade ago the gene expression of Wnt5a signaling members were found in tissue samples of pancreatic adenocarcinomas (Crnogorac-Jurcevic et al., 2001). Wnt5a expression was found to be gradually increased in pancreatic intraepithelial lesions and highly expressed in advanced pancreatic cancer (Ripka et al., 2007). Despite these associations, the role of Wnt5a in cancer remains controversial as some studies show that Wnt5a may work as a tumor suppressor while others show an oncogenic effect (McDonald & Silver, 2009). Although the exact role of Wnt5a in cancer remains unclear, the expression of Wnt5a in tumor samples has been correlated with advanced stages and poor prognosis in gastric, colon, prostate, lung, and malignant melanoma (Iozzo et al., 1995; Lejeune et al., 1995; Saitoh et al., 2002; Weeraratna et al., 2002; Huang et al., 2005b).

In vitro studies have shown that Wnt5a induces cell migration, proliferation and invasion in a variety of cancer cell lines (Weeraratna et al., 2002; Kurayoshi et al., 2006; McDonald & Silver, 2009), lending support to the hypothesis that Wnt5a may be involved in invasion or metastasis of several different cancers. At present the precise mechanisms linking Wnt5a with cancer invasion and metastatis are still largely unknown, however recent studies have hinted at possible molecular mechanisms. The Ca2+ signaling and subsequent protein kinase C (PKC) activation was suggested as the mechanism for enhanced motility and invasiveness for malignant melanoma (Weeraratna et al., 2002). In addition, a recent study associated polarized cell migration with the Wnt5a-ROR2 signaling pathway (Nishita et al.). Wnt5a involvement during EMT in pancreatic cancer was also suggested (Ripka et al., 2007). Wnt5a was described as a target of the homeobox transcription factor CULT1, enhancing migration, proliferation and invasiveness during pancreatic tumorigenensis (Ripka et al., 2007).

An IL-6 / STAT3 / Wnt5a signaling loop has been described by different groups (Katoh, 2007; McCall et al., 2007). Our group demonstrated that IL-6, a TLR signaling product, can activate STAT3 with resulting overexpression of Wnt5a in papillary thyroid carcinoma cells and that phenylmethimazole (C10), a derivative of the anti-thyroidal medication methimazole, has the ability to block TLR3 signaling, IL-6 production, as well as decrease growth and migration of papillary thyroid carcinoma cells (McCall et al., 2007). We hypothesized that the C10 effect on growth and migration of the papillary thyroid cancer cells was related to its suppressive effect on TLR3 signaling which led to the downregulation of TLR-mediated STAT3 and Wnt5a signaling (McCall et al., 2007). This was the first study that linked TLR signaling with Wnt5a and cancer cell growth and migration.

More recently our group has shown that TLR3 and Wnt5a RNA are constitutively expressed in human pancreatic cancer and malignant melanoma cell lines in culture. In similar findings to what we reported in human papillary thyroid cells, C10 inhibits TLR3 expression and signaling in addition to growth and migration of these human pancreatic cancer and malignant melanoma cells. Moreover, in this report we established *in vivo* efficacy by showing that C10 delays tumor growth in mouse models of human pancreatic cancer and malignant melanoma. These studies showed that this phenomenon was also associated with inhibition of STAT3 activation (Schwartz et al., 2009). Since STAT3 activation is a strong regulator of Wnt5a expression, and since C10 can block migration of these pancreatic and malignant melanoma cells *in vitro* as well as Wnt5a expression and signaling, we suspect that C10 may also act to prevent metastasis in *vivo*.

4.3.4 Other TLR-related molecules

Tumor necrosis factor alpha (TNF-α), a product of TLR signaling, is a critical cytokine that induces expression of other inflammatory mediators and proteases important for tumor invasiveness and metastasis. Although at high doses extrinsic TNF-α cause hemorrhagic necrosis, at low concentrations it acts as an endogenous tumor promoter. It can be produced by malignant epithelial cells or stromal cells (Balkwill, 2002). The tumor promotion capacity depends on activation of NF-κB (Luo et al., 2004; Pikarsky et al., 2004). TNF-α expression/production is associated with poor prognosis, loss of hormone responsiveness

and cachexia (Luo et al., 2004). TNF-α increases vascular permeability, can stimulate the migration and extravasation or intravasation of cancer cells or can acts as a growth factor (Luo et al., 2004).

TLR signaling products IL-1α and IL-1β in the tumor microenvironment both contribute to increased invasiveness and metastasis (Gemma et al., 2001; Elaraj et al., 2011). IL-1 can promote metastasis by different mechanisms; first by increasing the adhesiveness of the endothelium via VCAM-1 or mannose receptor expression in endothelial cells. A second mechanism may involve induction of MMPs, cytokines and chemokines in tumor or stromal cells (Anasagasti et al., 1997; Song et al., 2003). A third mechanism is via the induction of angiogenic factors such as VEGF and IL-8 (Lewis et al., 2006). In 2003 two groups independently established that IL-1α and IL-1β were critical for the invasiveness and metastasis of pancreatic cancer and melanoma tumor cells (Sawai et al., 2003; Voronov et al., 2003). IL-1β is mainly produced by myeloid cells, with intricate transcriptional and post-transcriptional control. IL-1β increases tumor invasiveness and metastasis by promoting the production of angiogenic factors by stromal mononuclear cells (Saijo et al., 2002). IL-1α is secreted mainly by epithelial cells undergoing necrosis (Sakurai et al., 2008). In liver it was found that IL-1α released by necrotic hepatocytes induces IL-6 synthesis by Kupffer cells which activates pro-oncogenic transcription factor STAT3 (Naugler et al., 2007). IL-1 receptor activation by either form of IL-1 can lead to induction of IL-6. In multiple myeloma IL-6 promotes survival and proliferation of cancer cells via activation of STAT3 and extracellular signal – regulated kinase ERK signaling (Honemann et al., 2001). IL-6 - STAT3 signaling was also found in chemically induced liver carcinogenesis as well as many other types of cancers (Calo et al., 2003; McCall et al., 2007; Naugler et al., 2007; Schwartz et al., 2009).

COX-2, a TLR4 signaling product (Fukata et al., 2006), is highly expressed in a variety of cancers such as colorectal, gastric, esophageal, breast and prostate carcinomas. COX-2-produced prostaglandin E2 (PGE2) increases tumor invasiveness and metastasis and enhances production of IL-6, IL-8, VEGF, iNOS, MMP2 and MMP9 among others (Gasparini et al., 2003). COX-2 inhibition shows chemopreventive and antimetastatic activity in a variety of human cancers through disruption of the inflammatory microenvironment (Baek & Eling, 2006).

TLR-induced TGF-β is produce by myeloid cells, mesenchymal cells and cancer cells in hypoxic and inflammatory conditions (He et al., 2007; Ren et al., 2007; Zhou et al., 2009). It is one of the most highly expressed cytokines in the tumor microenvironment and has a large influence on tumor cell invasiveness and metastasis (Yang et al., 2010). The induction of angiopoietin-like 4 (ANGPTL4) in cancer cells by TGF-β disrupts vascular endothelial cell cell to cell junctions, increases the permeability of lung capillaries, and facilitates the trans-endothelial passage of tumor cells (Padua et al., 2008).

Versican is an aggregating chondroitin sulfate proteoglycan highly expressed in several cancers (Pirinen et al., 2005). Versican enhances tumor cell migration, growth and angiogenesis (Zheng et al., 2004). Versican has pro-inflammatory activity, it induces macrophages activation and stimulates the secretion of TNF-α and other cytokines (Wight, 2002; Kim & Karin, 2011). In addition, versican interacts with several adhesion molecules

expressed by inflammatory cells (Wight, 2002) and activates endothelial cells, fibroblasts and macrophages in the tumor microenviroment (Wang et al., 2009). Importantly, versican activates TLR2 on macrophages to induce NF-κB and MAP kinase (MAPK) signaling with the subsequent production of pro-inflammatory cytokines such as IL-6 and TNF-α (Wang et al., 2009). The inhibition of versican expression in LCC cells eliminates their metastatic behavior (Kim et al., 2009). In addition, a related proteoglycan, biglycan was found to activate macrophages through TLR2 and TLR4 (Schaefer et al., 2005).

Helix-loop-helix protein Twist, is a key transcription factor that regulates cell movement and tissue reorganization during early embryogenesis with a role in the epithelial-mesenchymal transition (EMT) process during normal development. Suppression of Twist in metastatic 4T1 mammary carcinoma cells specifically inhibits their ability to metastasize to lung but not their ability to form primary tumors (Yang et al., 2004) and loss of Twist expression prevents the entry of metastatic cells into the circulation (Yang et al., 2004). Together, these findings suggest that Twist can contribute to invasion and metastasis by promoting the EMT developmental program in cancer. Interestingly, Twist expression can be induced in response to NF-κB activation and is therefore upregulated in response to inflammation (Pham et al., 2007). This could be a mechanism through which tumor-associated inflammation may stimulate metastatic progression through induction of Twist-dependent EMT (Yang et al., 2004; Pham et al., 2007).

Another mechanism of cancer invasiveness and metastasis was identified in a metastatic prostate cancer model that involves the repression of Maspin. Maspin is a member of the serpin family with well-established anti-metastatic activity in breast and prostate cancers. It was found that the metastatic behavior of isolated cells was dependent on the activation and nuclear accumulation of IKKα (Preciado et al., 2005). Repression of Maspin transcription required nuclear translocation of catalytically active IKKα, which occur only in advanced prostate tumors that contain inflammatory infiltrates and cells that express receptor activator of nuclear factor kappa-B ligand (RANKL) and LTα:β (Preciado et al., 2005). RANKL can lead to repression of maspin-expression in an IKKα-dependent manner (Zou et al., 1994; Luo et al., 2007). Thus, IKKα infers its pro-mestastatic effect by repressing transcription of the maspin gene.

Although the molecular mechanisms involved in these metastatic processes seem to be complex and not well understood, TLR signaling and related proteins appear to play an important role.

4.4 TLRs have an important role in tumor angiogenesis

In 1995, Judah Folkman wrote (Folkman, 1995) that "recent discoveries of endogenous negative regulators of angiogenesis, thrombospondin, angiostatin, and glioma-derived angiogenesis inhibitory factor, all associated with neovascularized tumors, suggest a new paradigm of tumorigenesis. It is now helpful to think of the switch to the angiogenic phenotype as a net balance of positive and negative regulators of blood vessel growth. The extent to which the negative regulators are decreased during this switch may dictate whether a primary tumor grows rapidly or slowly and whether metastasis grows at all." Folkman recognized that cancer has the ability to spread to adjacent or distant organs; he recognized that tumor cells could penetrate blood or lymphatic vessel walls, spread through

the blood or lymphatic vessels to another site, where they could proliferate; but he further recognized that the key to the metastatic or tumor growth process was angiogenesis. At that time he did not envision the importance of the Toll-like receptor and signal system in regulating this switch from normal to pathologically driven angiogenesis.

Cell growth, cell development, and cell migration, independent of whether immune or non-immune cell in origin, require blood vessel formation to feed the inflamed areas with nutrients and oxygen, particularly chronically inflamed and growing tissues. This is reviewed in Grote, et al, whose work can be construed not only as an important component of the following discussion but the "bible" on which it is based (Grote et al., 2011). Vascular growth is termed angiogenesis or vasculogenesis. Angiogenesis is the formation of new blood vessels by sprouting or by intussusception of preexisting vessels; vasculogenesis defines a process whereby progenitor cells differentiate into endothelial cells (Ribatti, 2010). Both processes often occur together and are often termed neovascularization, as for example in atherosclerotic lesions.

Tumor angiogenesis is a multistep process (Nishida et al., 2006). Simplistically, first the basement membrane in tissues is injured locally with resultant tissue destruction and hypoxia. Second, endothelial cells activated by angiogenic factors migrate into the damaged area. Third, endothelial cells stimulated by angiogenic factors proliferate and stabilize. Then, angiogenic factors continue to influence tissue nutrient supply and waste removal. It is intuitive that this is not a simple process and must be highly controlled (Carmeliet, 2000).

One example of this is the role of hypoxia in tumor angiogenesis. The irregular pattern and organization of the tumor vasculature result in some cells being more than 100 μm from a blood vessel, the accepted diffusion limit for oxygen. Progressive hypoxia with distance from the oxygen source results in induction in hypoxia-inducer factor 1 alpha (HIF-1α) and 1 beta (HIF-1β) and, upregulated gene expression (VEGF, Ang2, iNOS, PDGF-B), increased glycolysis, and stimulation of angiogenesis.

Secondly, blood vessels have endothelial cell walls, a media composed of fibroblasts, fat cells, collagen molecules, and mesenchymal tissue, and a surrounding smooth muscle and epithelial cell layer in arteries or just an epithelial cell layer in non arterial vasculature. Blood vessels form in a regular pattern as part of a normal vascular network but in a disordered array in chronic inflammatory states or cancer. In examining angiogenesis, one must thus consider multiple cell types, multiple coordinated interactions, and complex regulatory networks.

The complexity is evidenced molecularly (Carmeliet, 2000; Olsson et al., 2006). VEGF (vascular endothelial growth factor) is now recognized to be 5 VEGF ligands (A-D) in different spliced or processed variants, yet each is a dimeric glycoprotein of about 40K. Placenta growth factor (PLGF) is also a family member. They bind to 3 receptor tyrosine kinases (VEGFR 1-3) which can have an overlapping functional pattern, and can have multiple co-receptors, including neuropilins, proteoglycans, and heparin-sulfate. Each VGFR has a different function: VEGFR1 is important in hematopoetic progenitor cell recruitment; it also regulates monocyte migration; VEGFR2 and 3 control endothelial cell function during angiogenesis. Lest this complexity overwhelm us, Tie receptors and their angiopoitin (Ang) ligands are a second endothelial cell-specific receptor tyrosine kinase

system which interacts with the VEGF-VEGFR kinase system during angiogenesis (Partanen et al., 1992; Augustin et al., 2009). Ang-Tie interactions normally control signals leading to vessel quiescence and the last steps of vessel maturation.

Capillaries develop and grow with a VEGF gradient. Endothelial cells at the leading edge of the capillary tube, tip cells, have filopodia and express multiple VEGFR family members. "Behind them" in the advancing gradient of growth and development are highly proliferative, differentiating "stalk" cells and resting cells expressing components of the Ang-Tie system. A key component regulating the "sprouting" tip cells vs stalk cells is the Delta/Notch Signaling system (Gridley, 2010). Again complexity exists. There are 4 different Notch Receptors, Notch 1-4. Notch receptors have a single transmembrane domain binding to membrane ligands Delta-like (Dll) 1-4 and Jagged. Notch signaling in stalk cells induces a quiescent endothelial cell phenotype whereas TIP cells enriched in DLL4 promote sprouting activity and capillary growth (Gridley, 2010). Other factors in associated cells, such as basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) control "endothelial cell coverage" by pericytes and smooth muscle cells to establish vasculature stability and maturation (Distler et al., 2003).

In sum, the regulated action of a multiplicity of angiogenic factors and receptor kinases control capillary sprouting, growth, differentiation (e.g., tip vs stalk) and endothelial cell "coverage" and "stabilization." Moreover this list does not even consider a multiplicity of cytokines, chemokines, and growth factors with pro-angiogenic importance (Bussolino et al., 1991). Together, these, plus controlling inhibitory factors such as angiostatin, define a balanced pro- or anti-angiogenic system in normal tissues. It is now accepted that the "angiogenic switch" is "off" when the effect of pro-angiogenic molecules is balanced by that of anti-angiogenic molecules and is "on" when the net balance is tipped in favor of angiogenesis. A list of angiogenic stimulators and angiogenic inhibitors was summarized in "Angiogenesis in cancer and other disease" an Insight Review Article by Peter Carmeliet and Rakesh Jain (Carmeliet & Jain, 2000). This multiplicity is much expanded now, and offers an array of targets to stimulate angiogenesis, for example after a myocardial infarction (Vandervelde et al., 2005), or to inhibit angiogenesis to control dysregulated cell growth precipitated by chronic inflammation, for example in cancer, atherosclerosis, or obesity-induced diabetes (Cao, 2009). A VEGF-neutralizing monoclonal, anti-TNF-α, is one of many therapies targeting single gene products in this complex cascade. Of interest, numerous individual specific antibodies and inhibitors of specific tyrosine kinases have been evaluated in the past 10-15 years; however, not surprisingly, this approach has had limited success in a complex, interrelated, redundant signal system/pathway.

4.4.1 Inflammation and angiogenesis

Accumulating evidence supports a link between inflammation and angiogenesis. The two processes are intimately intertwined in inflammation-associated wound healing and tissue regeneration. Thus, the acute gene response set off by the innate immune process results in cytokines, chemokines, and growth factors produced at the site of the injury, which not only induce inflammation but also successive tissue repair. Angiogenesis is an important component of this tissue repair as evidenced by increased expression of VEGF. The inflammation-induced wound healing includes, in addition, pro-angiogenic factors such as basic fibroblast growth factor (bFGF), TGF-β, TNF-α, insulin-like growth factor-1 (IGF-1),

monocyte chemotactic protein-1 (MCP-1), IL-6 and IL-8, PDGF, to name but a few, which attract endothelial cells, smooth muscle cells, and epithelial cells needed for vessel growth and maturation.

Chronic inflammation leading to different pathological states, such as obesity-induced diabetes, ulcerative colitis, rheumatoid arthritis, systemic lupus erythematosus, and cancer, nevertheless have a common gene activation process and angionesis component. That chronic inflammation leads to diverse pathologic states involving the same molecular events should not be a surprise if one recognizes the commonality of inflammation and wound repair in all tissues, despite their varied differentiated states. Organ specific diseases do not negate common mechanisms of wound repair and angiogenesis in each.

4.4.2 Toll-like receptor signaling in inflammation and angiogenesis

To date, most studies linking TLR signaling to angiogenesis are *in vitro* and are largely descriptive. Thus, there is literature describing the use of several TLR agonists to induce expression and secretion of multiple angiogenic factors in an array of different cell types *in vitro*. The majority, however use LPS to stimulate TLR4 signaling with increased VEGF as a marker of angiogenesis.

1. LPS activation of TLR4 signaling *in vitro* increases adenosine promotion of angiogenesis through the A_{2A} Receptor system to increase VEGF (Hara et al., 2009). This cardio-protective nucleoside stimulates angiogenesis by increasing VEGF in macrophages, thereby likely offering tissue protection after ischemic injury. Moreover, there is a synergistic down-regulation of TNF-α (Leibovich et al., 2002). This angiogenic "switch" was noted also with TLR2, TLR7, and TLR9 agonists (Pinhal-Enfield et al., 2003). There is an *in vivo* correlate, since MyD88-deficient mice had decreased generation of new capillaries in response to an A_{2A}R agonist (Macedo et al., 2007).
2. LPS-increased TLR4 signaling was shown to increase endothelial sprouting *in vitro* via a TRAF6, NF-κB, JNK stimulatory process (Pollet et al., 2003). The requirement for TRAF6 *in vitro* and *in vivo* was established using a retrovirally expressed dominant negative TRAF6 in endothelial cells (Pollet et al., 2003). Moreover, inhibition of c-Jun N-terminal kinase (JNK) activity or NF-κB activity downstream of TRAF6 inhibited LPS-induced endothelial sprouting. Inhibition of only NF-κB but not JNK activity blocked bFGF, not TRAF6 induced angiogenesis. In sum, a direct endothelial role of TLR4 activation via TRAF6 is important in inducing angiogenesis in endothelial cells (Pollet et al., 2003). This has a pathologic counterpart in pathological corneal neovascularization that can cause impaired vision when induced by infections wherein TLR4 and VEGF are increased (Rodriguez-Martinez et al., 2006).
3. *Bactonella henselae* infections increase MCP-1 in endothelial cells, which chemotactically attracts monocytes to produce VEGF and increase angiogenesis (McCord et al., 2005). This is an NF-κB dependent process independent of TLR4 or LPS.
4. The TLR4/MyD88 signal system is important in VEGF production and angiogenesis in liver endothelial cells stimulated with LPS (Jagavelu et al., 2010).
5. *Mycoplasma* infections of the pulmonary tract causing a chronic inflammatory process increase angiogenesis and vascular remodeling (McDonald, 2001). This appears to be associated with a TLR2/6-dependent induction of NF-κB and a MAPK cascade by a 2 kDa macrophage activating diacylated lipopeptide (MALP-2) present in the

mycoplasma and secretion of GM-CSF from endothelial cells and monocytes. MALP-2 induced angiogenesis *in vitro* and *in vivo* is suppressed by GM-CSF, explaining both angiogenesis and remodeling in the same tissues.

6. Increased VEGF in chondrocytes and VEGF and IL-8 in fibroblasts are associated with inflammatory cell induced angiogenesis in chronically inflamed joints associated with arthritis. This progressive self-destructive angiogenic process involves a peptidoglycan (PGN) TLR2 ligand from Gram-positive bacteria (Cho et al., 2007).

4.4.3 Toll-like receptor signaling in tumor angiogenesis

Cancer is associated with, or induced, by chronic inflammation. Nevertheless, a clear definition of TLR induced angiogenesis varies because of the diversity of cancers and incomplete studies of all.

In gastric cancer linked to chronic *H. pylori* infections, cyclooxygenase 2 (COX-2) plays a critical role. Thus, *H. Pylori* activation of TLR2 and TLR9 signals activate the MAPK cascade leading to increased COX-2 and COX-2-dependent prostaglandin E_2 (PGE_2) release. This contributes to cancer cell invasion and angiogenesis (Chang et al., 2005). The COX-2 increase can be attenuated by the specific COX-2 inhibitor, NS398 or celecoxib. The cAMP response element (CRE) and AP-1 sites, but not NF-κB on the COX-2 promoter, are involved in MAPK-regulated COX-2 expression. Differential binding of CREB-1, ATF-2, and c-jun to the CRE site and c-fos, c-jun, and ATF-2 to the AP1 site were demonstrated and attenuated by different MAPK inhibitors as well as mutants of TLR2 and TLR9. In sum, these results showed that *H. pylori* activated TLR2 and TLR9 to activate MAKs, particularly p38, and downstream transcription factors (CREB-1, ATF-2, c-jun, and c-fos) resulting in activations of CRE and AP-1 on the COX-2 promoter (Chang et al., 2005).

Sustained pro-inflammatory processes in cancer, as well as diabetes, atherosclerosis, and rheumatoid disease are associated with increased angiogenesis and disease progression. Necrotic cells release high mobility group B1 (HMGB1), a pro-inflammatory cytokine, which signals TLR2 and TLR4 and the receptor for advanced glycation end products (RAGE) to increase angiogenesis by up-regulating NF-κB and VEGF in hematopoietic cells and endothelial cells (van Beijnum et al., 2008). HMGB1 seems to be involved in a positive feedback mechanism that sustains inflammation and angiogenesis contributing to disease progression. Endothelial cells express HMGB1 as well as RAGE receptors, TLR2 and TLR4. The HMGB1 can increase NF-κB activity, which can, in turn, increase HMGB1 receptors.

Inflammation-induced oxidative stress and angiogenesis are a common theme in tissue regeneration and remodeling in cancers. End products of lipid oxidation, such as ω-(2-carboxyethyl)pyrrole (CEP), are generated, accumulate, and are recognized by TLR2 in endothelial cells, leading to a MyD88-dependent angiogenic responses independent of VEGF (West et al., 2010). These endogenous ligands accumulating during cancer-induced tissue disruption promote angiogenesis via a TLR-dependent pathway. Grote, et. al., summarize this visually in Figure 4 of their review (Grote et al., 2011).

Nevertheless, a counter-regulatory TLR path can be activated as well, which inhibits angiogenesis and cancer progression. For example, the immune-modulatory TLR9 agonist, IMO, inhibits micro-vessel formation and cancer growth (Damiano et al., 2006). Similarly the

TLR7 agonist imidazoquinoline and the TLR9 agonist un-methylated cytosine- phosphate-guanosine (CpG) oligonucleotide exhibit strong local activity against leukemia in Phase I/II trials at different centers (Spaner & Masellis, 2007). Their importance appears, however, to be to sensitize CLL cells to other cytotoxic agents so any future lies with combined chemotherapy, radiotherapy, or other more "toxic" agents.

Resistance to anti-HER2 monoclonal antibody, trastuzumab is an issue in breast cancer patients. The novel Toll-like receptor 9 (TLR9) agonist termed IMO, immune modulatory oligonucleotide, potentiates the anti-EGFR/HER2 signaling of monoclonal trastuzumab. It modulates a functional interaction between TLR9 and HER receptors at a membrane level, producing a cooperative antiangiogenic effect (Damiano et al., 2009).

In skin cancer, strategies to inhibit neovascularization and angiogenesis include blockade of COX-2, m-TOR, sonic hedgehog, growth factor receptor activation, and activation of TLR by imiquimod (Li & Li, 2008). Separately, Myricetin, a phytochemical from onions, berries and red wine, suppresses ultraviolet (UV) B-induced angiogenesis by inhibiting PI-3 kinase activity *in vivo* in mouse skin. The chronic UVB exposure induced neovascularization that is associated with increased VEGF, matrix metalloproteinase (MMP)-9 and MMP-13 expression. The myricetin inhibited UVB-induced hypoxia inducible factor-1α expression. The myricetin effect was associated with attenuation of UVB-induced PI-3kinase activity and phosphorylation of Akt/p-70(S6K) (Jung et al., 2010).

The complexity of TLR3 action in individual tumors with both increased and decreased angiogenic activity was suggested *in vitro* by Paone, et al. (Paone et al., 2010). These authors had shown TLR3 activation in LNCaP and PC3 lines, with more efficiency in the former cells from a less aggressive tumor. They subsequently describe novel pro-tumor machinery. Triggered by TLR3 activation by polyI:C in PC3 cells, they show increased expression of the specific 1.3 isoform of HIF-1 alpha and nuclear accumulation of this complex in PC3 cells with decreased apoptosis and in secretion of functional VEGF. This is not the case in less aggressive LNCaP cells. However, in both cell lines, transfection of the 1.3 isoform of HIF-1 alpha causes decreased apoptosis and increased secretion of functional VEGF. They suggest basal levels of the 1.3 isoform of HIF-1 alpha distinguish differential responses to TLR activation.

In sum, the role of TLR in inducing signaling to increase tumor growth and angiogenesis is clear but largely poorly defined. Nevertheless, Grote et al. and others (Chang et al., 2005; Spaner & Masellis, 2007; Damiano et al., 2009) suggest that future modulation of TLR signaling could be the basis for a therapeutic approach to cancer and inhibition or control of abnormal angiogenesis to limit tumor growth. However, this is potentially difficult because not only are there TLR-induced pro-angiogenic signals but also anti-angiogenic signals. Moreover, the different cells interacting in the process present a complex network to control. Trials of anti-VEGF monotherapy provide a caution as well (Freedman et al., 2002; Henry et al., 2003) since they have not yielded consistent beneficial results. It is now recognized that not one (VEGF) but a multiplicity of potent angiogenic factors act in concert with VEGF for proper vessel formation and maturation (Augustin et al., 2009; Gridley, 2010). Rather than a unique anti-angiogenic "bullet," what may be needed is a broadly acting agent acting on a multiplicity of cells and a multiplicity of steps in the TLR stimulated cascade as in the case of diabetes.

5. TLR Involvement in pancreatic cancer

Toll-like receptors were first implicated in the pathogenesis of pancreatic cancer in 2009 in two separate reports. First, TLR3 was first described by our laboratory to play a role in the regulation of pancreatic cancer growth and migration. In this report we demonstrated that TLR3 and Wnt5a were coordinately constitutively expressed in a human pancreatic cell line (PANC-1) derived from a human pancreatic ductal adenocarcinoma, and that phenylmethimazole (a TLR signaling inhibitor) inhibits growth and migration of these pancreatic cancer cells in cell culture and inhibits pancreatic cancer tumor growth *in vivo* in a *nude* (*nu/nu* mice, which lack T cells) mouse model of human pancreatic cancer (Schwartz et al., 2009). In a separate 2009 report, Ikebe et. al., showed that LPS activation of the TLR4/MyD88 signaling pathway increases the invasive ability of PANC-1 and Aspc-1 (another pancreatic cancer cell line derived from a human pancreatic ductal adenocarcinoma) cells, while blockade of TLR4, MyD88, or NF-kB signaling decreases the LPS-dependent increased invasive ability (Ikebe et al., 2009). Together, these studies were the first to implicate TLR expression and signaling in pancreatic cancer cells as playing a role(s) in pancreatic tumor growth and migration. These studies helped establish that TLR expression and signaling in the pancreatic cancer cells (i.e. non-immune cells) themselves may be an important contributor to disease development, an idea that is now widely accepted for a multitude of autoimmune/inflammatory diseases including cancer. As briefly mentioned earlier, clinical relevance of these findings has recently been noted in a study that investigated the expression and clinical relevance of TLR4, NF-κB, and hypoxia-inducible transcription factor-1α (HIF-1α) in pancreatic adenocarcinoma (Zhang et al., 2010). In this study, TLR4 and HIF-1α expression was measured via real time polymerase chain reaction (PCR) in 30 cases of pancreatic ductal adenocarcinoma and its adjacent tissues, and TLR4, NF–κB, p65, and HIF-1α protein expression was measured by immunohistochemistry in 65 cases of pancreatic ductal adenocarcinoma and 38 cases of corresponding adjacent tissues. In addition, the relationship between TLR4 or HIF-1α and pathologic features, and the association between TLR4 and HIF-1α, were also analyzed. The Kaplan-Meier method was used to assess the impact of expression of TLR4 and HIF-1α on survival of the patients with pancreatic cancer. Results of these analysis revealed that TLR4, NF-κB, and HIF-1α are all overexpressed in pancreatic adenocarcinoma, that TLR4 may regulate HIF-1α expression, and that TLR4 and HIF-1α act synergistically to promote the development of pancreatic adenocarcinoma.

6. TLRs as potential therapeutic targets for pancreatic and other cancers

The therapeutic use of TLR agonists has been investigated in several cancer models. The rationale for inducing TLR signaling in a tumor setting is that: (*a*) TLR signaling will target tumor cells, inducing apoptosis or inhibiting the generation of factors that augment tumor growth or, (*b*) TLR signaling will enhance a resident or therapy-driven antitumor immune response that will eventually lead to tumor cell destruction. At present, mixed results have been obtained using TLR agonists against different TLRs. For example, with respect to the effect of TLR agonists on tumor cells, it has been shown in mouse breast xenograft cancer models that the antitumor effect of TLR3 agonists was dependent on the expression of TLR3 receptors in tumor cells, and that dsRNA treatment improved outcomes in patients harboring TLR3-positive breast tumors (Salaun et al., 2011). Similarly, CpG treatment was

able to trigger tumor cell death in human neuroblastoma cells, and tumor-targeted delivery of this TLR9 agonist increased survival in a xenograft model of mouse neuroblastoma (Brignole et al., 2010). In human patients harboring low-grade B-cell lymphoma, when CpG was delivered intratumorally in combination with radiotherapy almost 50% of the patients showed complete regression (Brody et al., 2010). CpG molecules were able to interact with the transformed B cells that express TLR9 receptors.

Treatment with TLR agonists has also shown to induce an antitumor response by either enhancing dendritic cell (DC) vaccination or T cell adoptive therapies. For example, tumor-localized delivery TLR agonists such as poly(I:C) or CpG combined with adoptive transfer immunotherapy was effective to control tumor growth in an established model of aggressive murine B16F10 melanoma(Amos et al., 2011). In particular, it is proposed that TLR agonists enhance T cell adoptive therapy by inducing a better interaction of these cells with activated resident DCs and by augmenting the activity of these T cells through IFNγ induction (Amos et al., 2011). TLR agonists have also been proposed as adjuvants for DC antitumor vaccination. In particular, TLR agonists have been shown to enhance the efficacy of DC vaccines in mouse models of melanoma and brain tumors (TLR7/8 agonist) (Prins et al., 2006; Ma et al., 2010), sarcoma (TLR3/9 agonists) (Zheng et al., 2008); or lung tumors (TLR9)(Cho et al., 2009) among others.

On the other hand, it has also been shown that TLR agonists can promote cancer cell survival and migration, and tumor progression. For example, TLR agonists have been shown to increase tumor viability and metastasis of human lung cancer cells (TLR7/8) (Cherfils-Vicini et al., 2010); proliferation of human myeloma cells (TLR3) (Chiron et al., 2009); adhesion and metastasis of human colorectal cancer cells (TLR4) (Hsu et al., 2011); and migration of human glioblastoma (TLR4) or human breast cancer cells (TLR2) (Thuringer et al., 2010).

We considered that these contradictory results are due to the complex nature of the tumor microenvironment. Tumors are more than cancer cells; they are also composed of non-tumor cells and the extracellular matrix. In particular, in pancreatic cancer the tumor microenvironment is composed of endothelial cells, leukocytes (lymphocytes, macrophages, dendritic cells, mast cells and neutrophils), mesenchymal cells (stellate cells and fibroblasts), neural cells and an extracellular matrix rich in fibronectin, collagen and periostin (Farrow et al., 2008; Erkan et al., 2011). These components of the tumor microenvironment often support tumor cell growth, or suppress immune responses against tumor cells. For example, stellate cells mediate fibrosis by generating high amounts of extracellular matrix components (Masamune et al., 2009), while fibroblasts release hepatocyte growth factor (Xu et al., 2010) facilitating tumor cell proliferation. Similarly, macrophages generate cytokines and growth factors (Pinhal-Enfield et al., 2003) that stimulate both tumor cell proliferation and angiogenesis. However the tumor microenvironment can also hamper therapeutic efforts. In addition, the nature of the tumor endothelium can prevent the delivery of antitumor factors within the tumor as has been previously shown for ovarian cancer (Buckanovich et al., 2008). In the same way, the presence of tumor-associated regulatory T cells or myeloid derived suppressor cells can render attempts to generate a powerful antitumor immune response by therapeutic vaccination ineffective (Whiteside, 2008).

Interestingly, although both tumor cells and tumor-associated leukocytes can express TLR, their signaling can induce the generation of different molecules (Palha De Sousa et al., 2010).

Thus, TLRs are present both in tumor cells and leukocytes, but may have different activities. Further, the signaling pathway may be not the same among different leukocyte populations such as T cell or antigen presenting cells. Indeed, TLR signaling on mast cells (Oldford et al., 2010) or Tregs cells (Zhang et al., 2011) may contribute to tumor inhibition, while TLR signaling on macrophages can contribute to tumor progression (Pinhal-Enfield et al., 2003). Thus there are diverse effects that TLR signaling can induce on different cells within the tumor microenvironment. Together, this data argues for specific targeting of tumor microenvironment components when applying TLR agonist therapies for cancer. For example, TLR agonists can be prepared for their delivery to particular cells within the tumor microenvironment (Bourquin et al., 2010). This type of strategy was successfully used to activate tumor-associated DCs in ovarian cancer, promoting antitumor immune response *in vivo* (Cubillos-Ruiz et al., 2009; Scarlett et al., 2009).

On the other hand, the use of TLR antagonists may prove beneficial for those tumors in which the tumor microenvironment promotes tumor cell survival and metastasis upon TLR signaling. TLR antagonists might also decrease the levels of activation of stromal cells, such as tumor-associated macrophages. Macrophages express an array of TLRs and are able to produce several growth factors via TLR signaling (Pinhal-Enfield et al., 2003). Moreover, abrogation of TLR-4 signaling in tumor-associated macrophages was able to decrease tumor growth (Lee et al., 2009). In particular for pancreatic cancer, it has been shown that TLR3 and TLR4 signaling promotes the invasiveness of pancreatic tumor cells (Ikebe et al., 2009). In this context, our recently published manuscript showing that C10 inhibited tumor growth in an *in vivo* model of pancreatic cancer highlights the relevance of using TLR antagonists for tumor therapies (Schwartz et al., 2009). Since C10 abrogates both TLR3 and TLR4 signaling (Schwartz et al., 2009; McCall et al., 2010), this molecule will be extremely relevant as a novel therapeutic agent for the treatment of those cancers whose microenvironment induces tumor progression via TLR signaling.

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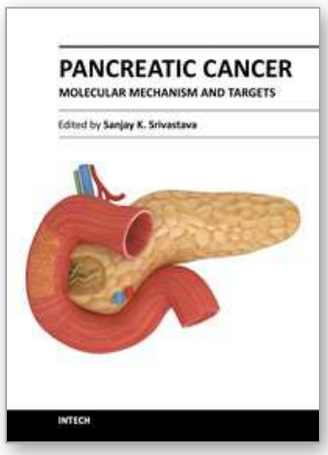

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This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

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