Chapter Two:
Review of Literature

2.1 Cancer epidemiology: The global trend and burden

Cancer is one of the leading cause of morbidity and mortality in low-income and middle-income countries (Harlan and Warren, 2015). The incidence of cancer continues to rise in developed and developing countries alike. The global cancer surveillance program reported an individual record of 37.5 million patients during the 15-year period from 2000 to 2014 for 18 cancer type that collectively represents 75 percent of all cancers diagnosed worldwide annually (Allemani et al., 2018). The incidence of cancer is projected to increase to 21.6 million patients annually by 2030. The age-standardised incidence rates of cancer are low in demographically young country such as India (Sullivan et al., 2014). More than 1 million new cases are diagnosed annually in a population of over 1.3 billion, representing a combined incidence of approximately a third of that recorded in developed countries of western Europe in age-adjusted terms (Sharma, 2016). However, a significant difference exists between the ratios of cancer incidence to morality. In India, the cancer mortality rate is about 68 percent of the total annual incidence (Mallath et al., 2014). This ratio suggest that fewer than 30 percent of Indian patients with cancer survive 5 years or longer after diagnosis.Therefore, to reduce cancer mortality rate, reduction of cancer incidence and improvement of cancer survival are both necessary.

2.2 Breast cancer epidemiology in India

Breast cancer is the most common malignancy diagnosed in women after lung cancer and one of the major cause of cancer-related deaths in women worldwide. Reportedly, about 1.7 million women were diagnosed with breast cancer worldwide and almost half a million died from this disease during 2012 (Ferlay et al., 2015). One in eight to ten women would be diagnosed with breast cancer during their lifetime. The breast cancer mortality rate in the European Union and North America has decreased significantly by 10 percent in 2016, mostly due to to early diagnosis and efficient systemic therapies (Harbeck and Gnant, 2017). In contrast, the global burden of breast cancer exceeds all other cancers and the incidence rates are increasing in less developed countries such as India (Torre et al., 2015). A consolidated reports of 27 population-based and 17 hospital-

based registries for 2012–14 show that breast cancer is the most frequent form of neoplastic disease among women in India and continues to increase in incidence (Sharma, 2016). In 2012, breast cancer became the most common cause of cancer-related mortality in the country followed by cervical cancer. Delayed diagnosis, inadequate treatment and altered lifestyle are the chief factors responsible for poor cancer survival rate in rural populaions as compared to urban populaions (Sullivan et al., 2014). For most cancers, the 5-year net survival is highest among Australia, New Zealand, Nordic countries , North America and Canada (Allemani et al., 2018). For women diagnosed with breast cancer during 2010-2015, the 5-year net survival in India is as low as 66 percent as compareed to more than 90 percent in USA and Australia (Mallath et al., 2014). Hence, an early diagnosis and optimal treatment is critical to significantly increase their survival in Indian contexts.

2.3 Tumor heterogeniety of breast cancer

The heterogeniety of breast cancer makes them a challenging solid tumor to diagnose and treat. Breast cancer is broadly categorized into atleast four clinically different molecular subtypes: luminal A, luminal B, HER2-positive and basal-like (Visvader, 2009). Ten different molecular subtypes have been characterized based on the analysis of gene copy number and expression profile (Perou et al., 2000; Sorlie et al., 2001). The original subtypes have been categorized on the basis of absence or presence of steroid hormone receptors (estrogen receptor [ER], progesterone receptor [PgR]) and HER2 status. Luminal A-like subtype (ER or PgR positive, or both, HER2 negative), luminal B-like subtype (ER or PgR positive, or both, HER2 negative) are characteristic feature of primary breast cancer whereas HER2 subtype, non-luminal (HER2 positive and ER and PgR negative)or luminal (HER2 positive and ER or PgR positive, or both) and basal-like subtype (HER2 negative and ER and PgR negative, triplenegative breast cancer) potentially leads to metastatic breast cancer (Harbeck and Gnant, 2017).

A primary breast tumor without any distant metastases is potentially curable. The surgical removal of the tumor combined with hormonal and systemic therapy is considered the best treatment option (McLaughlin, 2013). However, the high rate of relapse, estimated to

be approximately 30 percent, strongly suggests the existence of clonal heterogeniety within a specific tumor subtype and continues to pose a major clinical challenge in the elimination of primary breast cancer (Benson and Wishart, 2013). In contrast, the metastatic breast cancer is currently considered as incurable with a long-term survival rate of less than 5 percent (Greenberg et al., 1996). These tumor subtypes are associated with poor prognosis and do not respond to hormonal or HER2-targeted therapies. Therefore, current therapeutic goals in metastatic breast cancer are controlling disease symptoms and maintaining quality of life post-diagnosis. The therapy concepts are usually more individualized in metastatic breast cancer because of its highly heterogeneous structure and composition.

The intratumoral heterogeneity contradicts the model of linear succession by clonal expansions which is triggered by the acquisition of strong driver mutations that provides strong clone-specific selective advantages (Marusyk et al., 2014). The spatial restriction within the solid tumors leads to the formation of individual niche in different parts of the tumors which favors the outgrowth of cancer cells with different characteristics. Recent data from tumor genome sequencing and single-cell based analyses of human breast cancer has revealed the existence of at least two genetically diverse subpopulations present within individual tumors (Burrell et al., 2013; Gerlinger et al., 2012). The analysis of intratumor clonal heterogeneity based on iFISH data (combined immunostaining and FISH) and molecular typing of tumor suppressor (TP53) or oncogenes (RAS, PIK3CA) from early ductal carcinoma *in situ* (DCIS) and invasive breast tumors of different subtypes revealed the presence of stem cell-like $CD44^+$ and more-differentiated $CD24^+$ breast cancer cells (Park et al., 2010). Similarly, a cell-lineage tracing approach identified two distinct breast cancer subclones, namely basal and luminal subclones, displaying aberrant expression of signaling molecule Wnt 1 and its downstream effector molecule β-catenin in a mice model of mammary tumor (Cleary et al., 2014). Interestingly, paracrine interaction by Wnt 1 from luminal cells was required for the metastatic behavior of basal cells by β-catenin signaling. It is widely hypothesized that intratumor genetic heterogeneity underlies the phenomenon of therapeutic resistance and metastatic progression of breast cancer, however the mechanisms are not well understood. A recent study suggested a mechanism where in vivo fusion between neoplastic cells and leukocytes (for example,

macrophages) lead to the generation of a heterogenous cancer cell population with increased metastatic behavior and acquired macrophage phenotypes (Gast et al., 2018). Despite the importance of tumor heterogeneity in therapeutic resistance and metastatic progression whether it's a cause or consequence of tumor progression remains unclear.

2.4 Tumor microenvironment: a premetastatic niche

Tumor development resembles, to some extent, the development of a multicellular organisms, except that the coordination of events such as cell division and differentiation and tissue organization is dysregulated. Normal cells progressively develop to a neoplastic state by acquiring a succession of six hallmark characteristics namely sustained chronic proliferation, evading tumor suppressor signaling, resistance to cell death, unlimited replication capacity, neovasculature and metastasis (Hanahan and Weinberg, 2000). Underlying these hallmarks is tumor-promoting inflammation which fosters multiple hallmark functions by orchestrating a supportive tumor microenvironment (TME) comprised of neoplastic cells, non-malignant stromal cells, neovasculature and a gamut of immune cells, often embedded within a robust extracellular matrix (Hanahan and Weinberg, 2011). In fact, tumor progression depends on alterations of the TME mediated by the factors acting non-cell-autonomously such as cytokines. *In vivo* studies using mice xenograft model of breast cancer suggested that these cytokines could potentially contribute to the intratumoral heterogeneity and emergence of metastatic phenotypes (Esquivel-Velazquez et al., 2015). Therefore, understanding the molecular mechanism of cytokine actions and the cancer cell intrinsic pathways which modulates the inflammatory TME to support proliferation and immune evasion, would help in improving existing anti-cancer therapies as well as would further allow the designing of novel therapeutic strategies aimed at controlling human breast carcinoma.

2.5 Chronic inflammation and cancer

Clinical and epidemiologic studies suggest that chronic inflammation is intricately associated with tumor initiation and progression. About 20% of the global cancer burden is

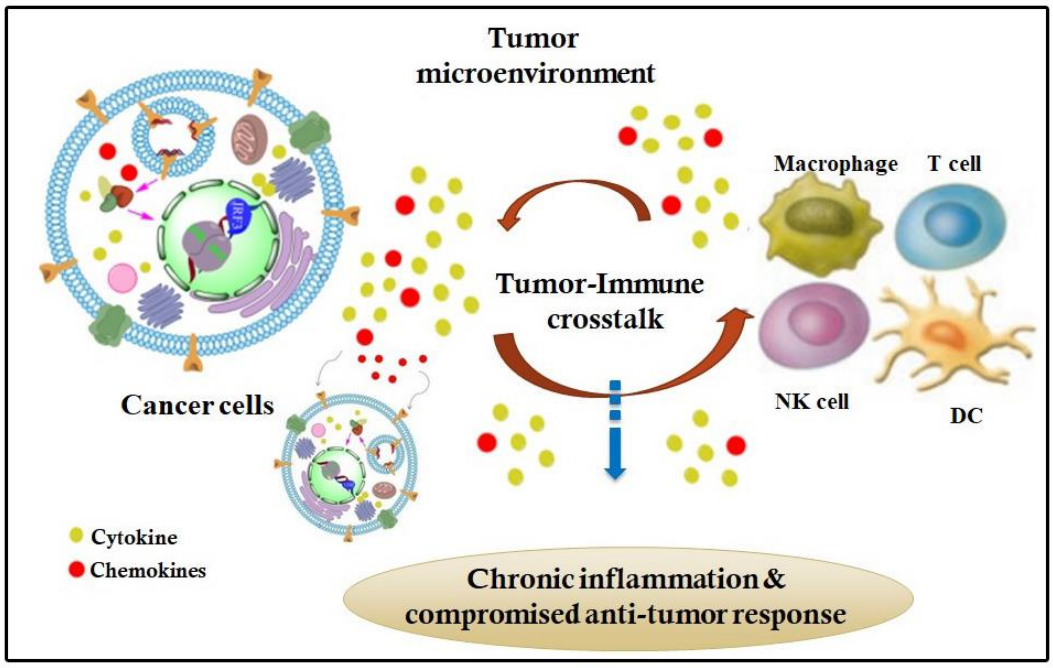

Figure 2.1: Tumor-Immune crosstalk: *The crosstalk between cancer cells and cells of the innate and adaptive immune system, mediated through the production of proinflammatory cytokines and chemokines leads to chronic inflammation and immunological tolerance in the TME.*

linked to chronic infections (Grivennikov et al., 2010). In gastric, hepatic and colorectal cancer, an induction of chronic inflammatory response by bacterial or viral infection precedes the development of malignancy. For example, a persistent *Helicobacter pylori* infection is strongly associated with development of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (Wu et al., 2009). Similarly, viral infections with hepatitis B (HBV) or C (HCV) viruses potentially increases the risk of hepatocellular carcinoma (HCC) (Karin, 2006). Nearly 30% of worldwide cancer incidence is attributed to alcohol abuse, tobacco smoking and pollutants exposure (such as asbestos and silica), each associated with inflammation of hepatocellular and lung carcinoma (Aggarwal et al., 2009).

In addition to the extrinsic inflammatory conditions which initiates and promote cancer, a similar chronic inflammatory response is the one that follows tumor development. Most

solid malignancies trigger an intrinsic inflammatory response that progressively builds up a pro-tumorigenic microenvironment *(Figure 2.1)*. All solid tumors at some point outpace their blood supply and become oxygen and nutrient limited. This results in necrotic cell death at the tumors core and the release of proinflammatory mediators such as cytokines and chemokines (Hanahan and Weinberg, 2000). These mediators induce a transcriptional program through inflammatory signaling cascade in positive feedback loop manner that leads to remodeling of TME through the infiltration of immune cells and induction of neoangiogenesis (Lin and Karin, 2007).

Thus, inflammation, irrespective of its origin, is strongly associated with accelerated tumor growth, invasion of surrounding tissues, angiogenesis and metastasis. The inflammatory milieu of solid tumors is primarily occupied by myeloid cells (macrophages and neutrophils) and adaptive immune cells (T cells and NK cells) (Restifo, 2013). Tumor-associated macrophages (TAMs) exert tumor-promoting functions at primary and metastatic sites and play supportive roles in cell proliferation, angiogenesis, invasion and suppression of antitumor cytotoxic T lymphocytes (CTLs) responses (Galdiero et al., 2018). A high TAM content generally correlates with poor prognosis. Cancer-associated inflammation closely mimics a wound-healing response that induces an exhausted/anergic-like $CD8^{+}$T cell phenotype and suppression of antitumor functions (Ruffell et al., 2012). Both TAMs and a subset of T cells promotes tumor progression and metastasis through secretion of pro-inflammatory cytokines which also plays a critical role in maintenance of immunological tolerance (Noy and Pollard, 2014). During persistent infections and chronic inflammation, $CD8^{+}$T cells fails to form effector or memory cells and undergo exhaustion as observed during viral infections. For instance, during chronic viral infection of HCV, viral replication is held in check by immune check-points that prevent an over-zealous antiviral response while also avoiding immunopathological damage to host liver (Sharma and Allison, 2015). These evolutionary conserved controls have also been implicated in T-cell tolerization during cirrhosis and hepatocellular carcinoma. However, the underlying mechanisms of the tumor-promoting immunosuppressive actions of the innate immune system remain obscure.

2.6 Immunosuppressive TME in breast cancer

Chronic inflammation could contribute to the development of breast carcinoma via multiple mechanisms. A common pathological feature of chronic inflammation is the generation of an immunosuppressive microenvironment that promotes tumor formation and progression (Wang and DuBois, 2015). The accumulation of proinflammatory mediators and infiltration of immune suppressor cells is observed in tumor microenvironment. Leukocytic infiltration such as TAMs increases in the neoplastic breast stroma and parallels with primary breast carcinoma and secondary metastasis. During breast tumorigenesis, chronic activation of leukocytes including TAMs and B cells is found in tumor-associated stroma of premalignant breast tissue, including hyperplasia and early ductal carcinoma *in situ* (DCIS) as well as breast tumors of high histological grade (lymph node metastasis) (DeNardo and Coussens, 2007).

In primary breast cancer, the presence of infiltrating T cells with high $CD4^+/CD8^+$and Th2/Th1 ratio is indicative of poor prognosis (Chin et al., 1992; Kohrt et al., 2005). Paradoxically, Th2 $CD4^+$T cells stimulate mammary cancer progression and metastasis by enhancing protumorigenic properties of TAMs to produce angiogenic and metastatic factors (Mantovani et al., 2002). The gene expression profiling of myoepithelial (basal) cells of DCIS and invasive breast tumor suggested an increased expression and secretion proinflammatory cytokines and chemokines such as TNF-α, IL-6, IL-1α, IL-1β, TGF-β, MMP9 and CXCR4, which corelated with tumor growth, invasion and metastasis (Esquivel-Velazquez et al., 2015). The proinflammatory activity of myoepithelial cell was associated with enhanced proliferation and polarity of luminal epithelial cells. These evidences indicate that increased accumulation of proinflammatory mediators released by TAMs, malignant cells and exhausted T cells, act in autocrine and paracrine manner to support chronic inflammation.

Consequently, chronic inflammation could induce T cell tolerization characterized by accumulation of proinflammatory mediators and immune suppressor cells. Therefore, understanding the mechanisms by which chronic inflammatory condition leads to accumulation of proinflammatory mediators and how these mediators could induce the immuno-

suppressive microenvironment may provide a rationale for developing more effective therapeutic strategies to control breast cancer progression.

2.7 Molecular mediators of chronic inflammation:

2.7.1 Cancer cell-intrinsic control of inflammatory pathways

Inflammation is a classical feature of innate immune cells and non-immune cells of host tissues (epithelium and mesenchyme) and play a primary role in cell-autonomous response to injury, infection and intracellular oxidative stress (Kotas and Medzhitov, 2015). An innate immune response is triggered by detection of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). PAMPs are conserved molecular motifs found in microbial pathogens which include viral nucleic acids and bacterial molecules such as flagellin, lipopolysaccharide (LPS), lipoproteins, peptidoglycan, CpG DNA and cyclic dinucleotides (CDNs). In contrast, DAMPs are emerging class of host self-molecules that normally reside in specific cellular compartments but are actively secreted into the cytosol or passively released into extracellular milieu during oxidative stress, cellular injury or cell death (Medzhitov, 2008). Both PAMPs and DAMPs are detected by families of host sensor molecules collectively known as pattern recognition receptors (PRRs) (Jin et al., 2013). PRRs are germ-line encoded immune receptors, broadly classified into three most-studied protein families : Toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) *(Figure 2.2)*.

TLRs are transmembrane receptors that detect extracellular or luminal PAMPs or DAMPs. The oxidative stress generated by increased ROS production in hypoxia-limited tumor leads to dsDNA breaks that is released into cytosol. The dsDNA is recognized as intracellular DAMPs by TLR3 or TLR9 which triggers the activation of NF-κB responsive genes and type-I IFN (Lim and Staudt, 2013). Indeed, a significantly increased expression of TLR3 and TLR9 in epithelial breast cancer cells was associated with poor prognosis (Bhatelia et al., 2014b). Similarly, clinical studies showed a significant associa-

tion of high TLR4 expression with primary tumor progression and lymph node metastasis. Increased proliferation and tumor invasion was mediated through TLR4-induced canonical NF-κB pathway via p65-mediated activation and transcription (Liao et al., 2012). *In vitro* studies using MDA-MB-231 epithelial breast cancer cell line (HER2 negative and ER and PgR negative, basal-like subtype) showed enhanced invasion upon activation of TLR3, TLR4 and TLR9. Similarly, TLR3 was found to be highly expressed in MCF-7 (ER and PgR positive and HER2 positive, luminal-like subtype) breast cancer cell line (Bhatelia et al., 2014b).

RIG-I-like receptors are potent activators of interferon production triggered by virus infection or sterile inflammation caused by accumulation of host RNA species in the cytosol. The RNAs are detected by a family of three PRR proteins, RIG-I, MDA5 and LGP2 (Sun et al., 2010). While the role of LGP2 extends beyond from type-I IFN signaling to TRAF-mediated NF-κB activation, RIG-1 and MDA5 specifically activates type-1 interferon production through MAVS adaptor protein in response to recognition of cytosolic dsRNA released during mitochondrial stress or viral infection. For example, LGP2 suppresses TRAF- ubiquitin ligase activity, a common downstream mediator during MAVS-dependent and MAVS-independent NF-κB activation pathways including TNF-α, IL-1β and cGAMP/STING (Parisien et al., 2018). An increased expression of LGP2 was associated with high-grade breast carcinoma and conferred prosurvival functions following genotoxic stress caused by DNA-damaging agents (Widau et al., 2014). RIG-1 was found to be highly expressed in ER and PgR negative breast cancer tissues than ER and PgR positive tissues. A similar expression was observed in breast cancer cell lines (Shyu et al., 2005).

The NLR proteins function as cytosolic sensors to detect intracellular PAMPs and DAMPs. A fundamental role of the NLR receptors is to regulate production of proinflammatory cytokine and chemokine that drive host immune response (Meunier and Broz, 2017). A major mechanism of regulation is a two-step process, known as priming and activation leading to the assembly of a multi-protein complex known as inflammasomes which activates caspase-1 to further process and secrete IL-1β and IL-18 (Prochnicki and Latz, 2017). A recent study demonstrated the critical role of inflammo-

some formation and IL-1β accumulation in promoting tumor growth and metastasis in breast cancer. Inflammasome activation in mice model of human breast cancer revealed an increased infiltration of TAMs and myeloid-derived suppressor cells suggesting that inflammasome/IL-1 pathway provides an inflammatory microenvironment to promote breast cancer progression (Guo et al., 2016a).

Additionally, cGAS/STING-mediated activation of activation of NF-κB responsive genes is an alternate innate immune signaling response that is triggered upon detection of cytosolic DNA. cGAS (Cyclic GMP-AMP synthase) is a cytosolic DNA sensor which induces the synthesis of cyclic GMP-AMP (cGAMP) upon binding with intracellular DNA (self or foreign) *(Figure 2.2)*. cGAMP acts as a secondary messenger and bind to STING resulting in STING-dependent activation of innate immune signaling cascade (Barber, 2015). In contrast to other PRRs-based inflammatory responses, cGAMP diffuses via gap junctions to communicate the signal directly to the STING of neighboring cells (Ablasser et al., 2013). Recent reports suggested an increased activation of cGAS/STING cytosolic DNA-sensing pathway in metastatic breast cancer cells was associated with high genomic instability as compared to primary breast cancer cells. The activation of cGAS/STING pathway was consistent with increased NF-κB activity in these cells which showed enhanced ability to colonize to distant metastatic sites (Bakhoum and Cantley, 2018; Bhatelia et al., 2014a).

Taken together, these evidences suggest that all innate immune signaling pathways triggered by the detection of DAMPs by various PRRs in breast cancer cells converges on the activation of atleast three tumor promoting transcription factors- NF-κB, STAT1/3 and IRF3/7 *(Figure 2.2)*. These transcription factors act as a key molecular link between the generation of inflammatory microenvironment and tumor promotion and progression. NF-κB is a master transcription factor that regulates the expression of a plethora of genes whose products suppresses tumor cell death, stimulates cell cycle progression and enhance tumor invasion. Importantly, NF-κB signaling pathway induces the production of several proinflammatory cytokines which supports a persistent inflammatory microenvironment in a positive feed-forward loop manner (Karin, 2009).

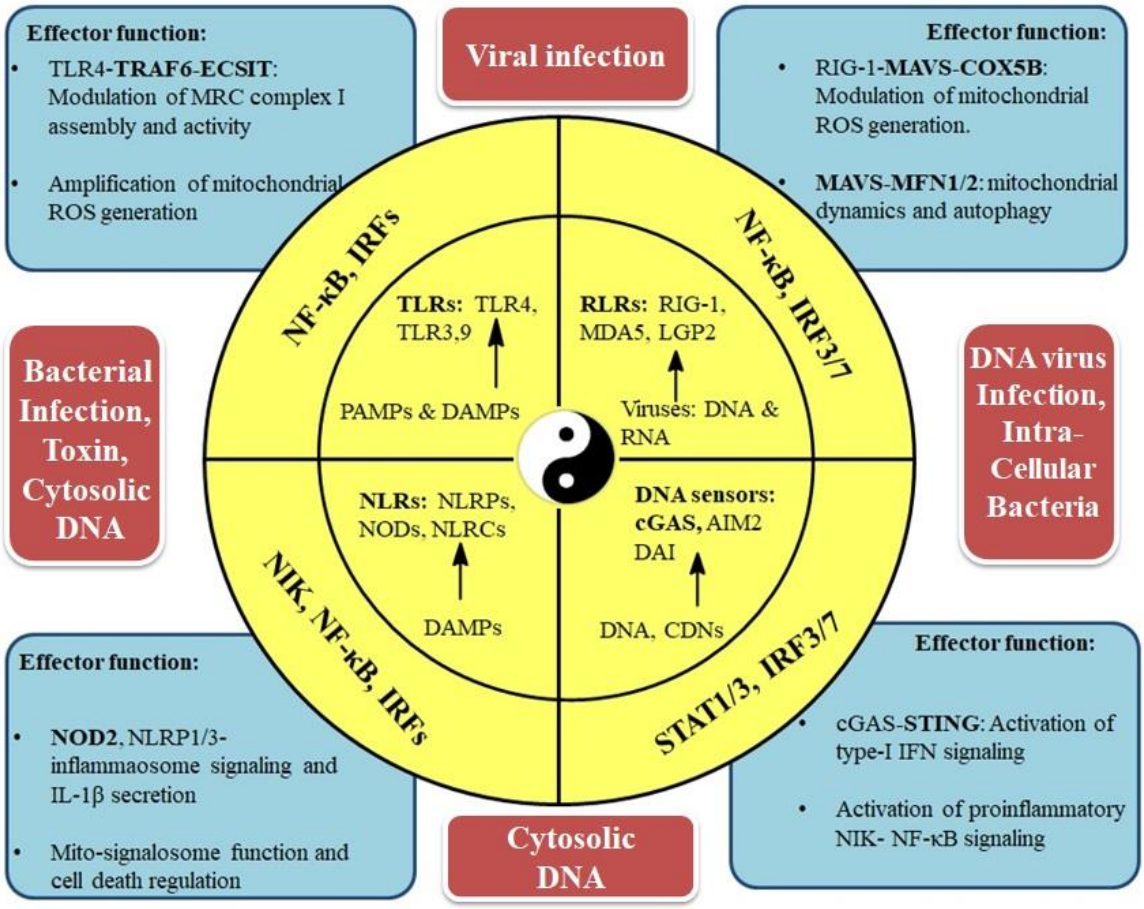

Figure 2.2: Cell-intrinsic sensors of danger signals: *The three classes of PRRs: TLRs, RLRs and NLRs and the DNA sensors recognizes intracellular PAMPs and DAMPs which leads to the activation of NF-κB, STAT1/3 and IRF3/7 transcription factors and secretion of type-I IFN and proinflammatory cytokines. Importantly, these immune sensors communicate with mitochondria through adaptor proteins (shown in bold) to alter mitochondrial function and innate immune response during intracellular bacterial or viral infection as well as self-DNA/RNA.*

2.7.2 Inflammation-induced cytokines in intratumoral crosstalk

Cytokines secreted by tumor cells or immune cells are key modulators of inflammatory response resulting in local tumor immune escape or tumor-induced immune suppression (Lin and Karin, 2007). The clinical studies of patients with cancer displayed an enhanced

expression and a complex interaction network of the proinflammatory cytokines. The increased level of proinflammatory cytokines results in active but dysfunctional immune response in patients with several tumor type (Galdiero et al., 2018). These studies suggested that malignant tumors such as metastatic breast cancer utilize local network of inflammatory cytokines within its microenvironment to prevent activation of immunological effector function and develops an effective immune escape mechanisms (Mendez-Garcia et al., 2018). Indeed, an increased level of several intratumoral cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, IL-10, IL-18, transforming growth factor (TGF)-β, and macrophage migration inhibitory factor (MIF) have been reported with various experimental and clinical tumor types such as breast, gastric, colorectal, lung, pancreatic, renal and hepatocellular carcinomas (Lippitz, 2013) *(Table 2.1)*. This specific cytokine pattern is associated with negative prognosis in patients diagnosed with advanced breast carcinoma suggesting the role of these immunostimulatory cytokines in promoting local cancer-associated inflammation and immune evasion (Esquivel-Velazquez et al., 2015). Delineating the complex intracellular signaling pathways of cytokines which regulates chronic inflammatory response will provide better understanding of immunostimulative and immunosuppressive pathways and will help in development of improved therapeutic interventions.

Cytokines	Lung	Breast	CRC	HCC	Pancreatic
TNF-α	+	+	+	+	+
MIF	+	+		+	+
IL- 6	+	+	+	+	
IL-8	+	+	+		+
IL-18	+	+		+	
IL-17	+	+	+	+	
Pluses indicates published evidence exists					

Table 2.1: Presence of specific cytokines within the TME of various cancer types from published reports. *TNF-α levels in all cancer types is shown in bold. CRC-Colorectal cancer, HCC- Hepatocellular carcinoma.*

2.7.2.1 TNF-α: role in chronic inflammation and cell death

Tumor necrosis factor-alpha (TNF-α) is a potent inflammatory mediator which play a central role in innate immune responses of normal and malignant cells. First identified in the 1970s, as an endotoxin-induced serum factor with oncolytic property against certain solid tumor, TNF-α is one of the most intensely studied cytokine (Balkwill, 2009). TNF-α acts as a master regulator of a complex signaling network which plays a critical role in balancing cell survival and programmed cell death (Wang et al., 2008). Thus, TNF-α has a double-edged function in regulating innate immune response and maintaining cellular homeostasis. A localized administration of high concentration of TNF-α show antitumoral response in a murine model of cancer whereas systemically administered TNF-α was associated with severe organ failure. Similarly, a local administration of TNF-α-expressing vehicle combined with chemotherapy proved to be safer and effective in randomized trial for advanced pancreatic and esophageal cancer (Landskron et al., 2014).

In contrast, chronic production of TNF-α have a paradoxical effect on limiting tumor growth. A low, sustained production of TNF-α by host and tumor cells is an important factor involved in initiation, proliferation, angiogenesis and metastasis of primary breast tumors. TNF-α has been strongly implicated in cancer cachexia, a late-stage cancer-associated symptom with inferior survival outcome (Fearon et al., 2012). As a major pro-inflammatory cytokine in the TME, TNF-α promotes the activation and recruitment of neutrophils and monocytes as well as impairs antitumor effector functions of T cells and cytotoxic activity of macrophages (Mantovani and Dejana, 1989). It also induces the expression of adhesion molecules on the vascular endothelial cells and contributes to the maintenance of a proinflammatory microenvironment (Newton and Dixit, 2012). A computational text-mining approach, encompassing entire PubMed entries (approx. 16 million articles), identified a large-scale cell-cytokine interaction network with TNF-α forming one of the top hubs (Kveler et al., 2018). Similarly, clinical data mining revealed an

elevated, chronic levels of intratumoral TNF-α were consistently associated with more aggressive tumor phenotype in patients with breast cancer (Lippitz, 2013). The genetic polymorphisms that enhances TNF-α expression and production are associated with increased risk of breast cancer (Lin and Karin, 2007). Anti-TNF-α monotherapy have shown potential therapeutic benefits for the treatment of few advanced solid tumors. In patients with progressive metastatic breast cancer, Etanercept (Human TNFR2–Fc fusion protein) treatment led to a significant decline in the levels and activity of proinflammatory cytokines but a limited antitumor activity was noted in this small phase II settings (Madhusudan et al., 2004). On the other hand, a CD4+ T cell-based adoptive immunotherapy led to a TNF-α-dependent intensification of oxidative stress and tumor cell death in a preclinical mice model of colorectal cancer. An increased level of TNF-α in TME synergized with chemotherapeutic agents and induces extensive metabolic changes in tumor and intensifies cellular ROS levels leading to cell death and tumor regression (Habtetsion et al., 2018). Conversely, neutralization of oxidative stress by preventing TNF-α signaling in tumor cells antagonizes the therapeutic effects. Therefore, successful therapeutic use of modulating intratumoral TNF-α signaling for the treatment of various solid tumors such as breast cancer needs to be further explored.

TNF-α is a pleiotropic cytokine produced by tumor cell or inflammatory cells and promotes cell survival through the induction of genes encoding NF-κB-dependent anti-apoptotic molecules. TNF-α, in its soluble (sTNF-α) or membrane-bound (mTNF-α) form is recognized by two receptors: TNFR1 and TNFR2 (Blaser et al., 2016). sTNF-α, a non-covalent homotrimer of 17 kDa monomer is a potent ligand which binds and activates the TNF receptors. TNFR1 is ubiquitously expressed on all cell types and can be activated by both form of TNF-α, whereas TNFR2 is predominantly expressed by immune and endothelial cells which is dependent on mTNF-α for its activation (Grell et al., 1995). Further, TNFR1 is a more potent activator of NF-κB transcription factor than TNFR2, therefore, TNFR1 is an important receptor for the activation of proinflammatory signaling pathways. The binding of TNF-α to TNFR1 results in the stimulation of prosurvival pathway through the formation of proximal plasma membrane bound complex I *(Figure 2.3)*, consisting of TNF receptor-associated protein with death domain (TRADD), TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein kinase 1 (RIP1) (Wang et

al., 2008). The formation of complex I leads to rapid transcriptional activation of RIP1-mediated NF-κB responsive genes and antiapoptotic response by cIAP-1 and TRAF2 through intermediate kinase cascade. Alternatively, in a process that is not well characterized, complex I undergoes ubiquitin-dependent modifications, proteolysis and internalization to form a sub-cytosolic prodeath multiprotein complex called complex II *(Figure 2.2)*. The association of complex II with Fas-associated death domain (FADD) and pro-caspase-8 initiates a non-reversible proteolytic cascade by activating caspase-8 (Micheau and Tschopp, 2003).

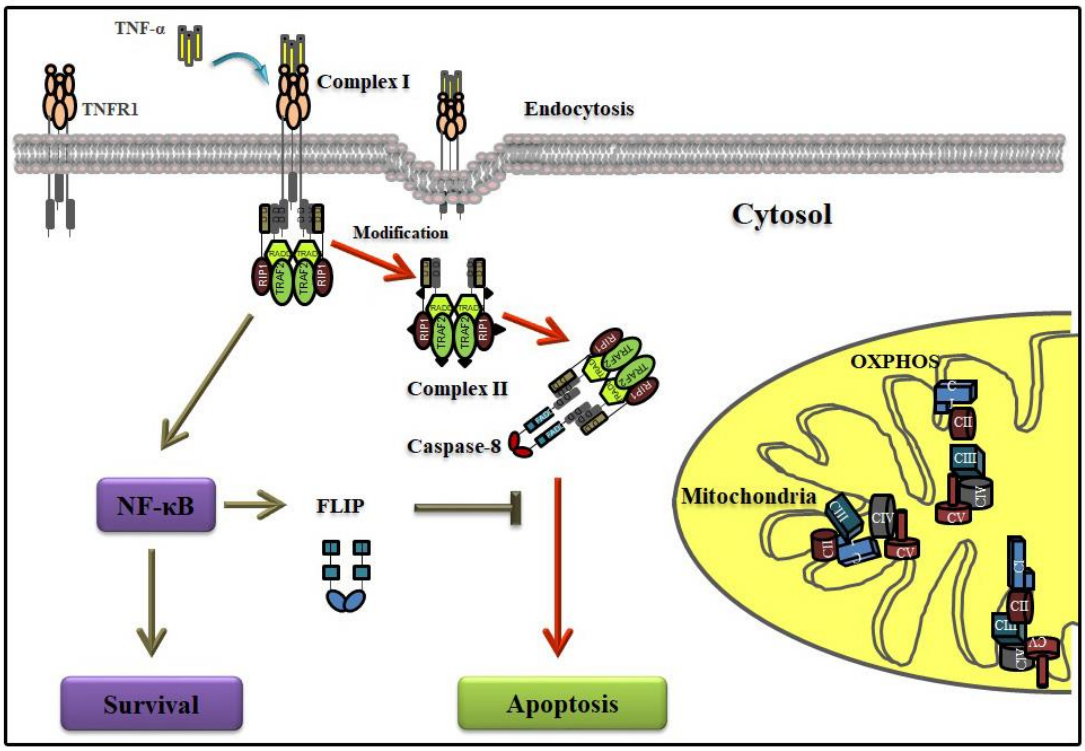

Figure 2.3: TNF-α signaling pathway: *TNF-α dually regulates cell survival through transcriptional activation of NF-κB responsive genes or cell death through activation of caspase-8.*

TNF-α is emerging as an important regulator of the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). TNF-α contributes to tumor initiation and progression by stimulating the production of genotoxic molecules such as nitric oxide ($NO^{\bullet}$) and superoxide (O_2^{-}) radicals that leads to DNA damage and genomic instability

(Blaser et al., 2016). The chronic levels of ROS have important function in regulating NF-κB responsive genes during TNFR1 signaling such as enhanced transcription of cFLIP and Bcl-XL antiapoptotic genes (Brenner et al., 2015). Interestingly, TNF-α-dependent programmed cell death pathway involve ROS derived from either mitochondrial or non-mitochondrial sources. Accordingly, RIP1-deficient cells failed to undergo programmed cell death upon abrogation TNF-α-induced mitochondrial ROS generation demonstrating that both TNF-α signaling and ROS generation influence each other in a positive feedback loop (Blaser et al., 2016). ***Therefore, the molecular mechanisms mediating the crosstalk between TNF-α signaling and mitochondrial ROS generation is important for the regulation of the tumor cell survival and promotion of the inflammatory microenvironment. A detailed understanding of molecular connections between the inflammatory pathways and cancer cell metabolism could reveal novel immune or metabolic therapeutic targets for solid tumors***.

2.8 Metabolic alterations in the tumor microenvironment

2.8.1 Tumor cell-intrinsic metabolic reprogramming

An early solid tumor faces two major metabolic challenges as it advances - a bioenergetic and biosynthetic crisis due to increased cell proliferation and external nutrient and oxygen limitations as (Lunt and Vander Heiden, 2011). In order to meet the increased demands of proliferation, cancer cells reprograms the energy metabolism and nutrient uptake by oncogenic gene amplifications, translocations and loss of tumor suppressor function (Jones and Thompson, 2009). A key metabolic change associated with proliferating tumor cells is the increased dependence on glycolysis for ATP generation rather than the energetically more efficient oxidative phosphorylation (OxPhos) in the presence of oxygen, a metabolic phenotype also known as Warburg effect (Pavlova and Thompson, 2016). The shift in the bioenergetic metabolism provides multiple advantages for proliferating cells. Firstly, the percentage of total cellular ATP produced from glycolysis through substrate-level phosphorylation exceeds that produced from OxPhos. Although the ATP yield per molecule of glucose is much lower for aerobic glycolysis, the glyco-

lytic flux to lactate exceeds by more than an order of magnitude in comparison to low-flux pathway from pyruvate dehydrogenase complex (PDH) to mitochondrial respiratory chain (MRC) (Lunt and Vander Heiden, 2011). In proliferating cancer cells, more than 90 percent of pyruvate generated from glucose is converted to lactate by lactate dehydrogenase (LDH-A) and secreted into the extracellular microenvironment (DeBerardinis et al., 2008). During the conversion of pyruvate to lactate, LDH-A regenerates the NAD+ required to maintain glycolytic flux. This metabolic phenotype of tumors is exploited in clinical settings through the application of ^{18}F-deoxyglucose positron emission tomography (FDG-PET) to image solid tumors (Ward and Thompson, 2012).

Secondly, a high glycolytic rate generates metabolic intermediates for the control of nucleotide biosynthetic pathway through pentose phosphate pathway (PPP). The oxidative arm of PPP generates NADPH, which function as a cofactor and provides reducing equivalents for both nucleotide and fatty acid biosynthesis (Pavlova and Thompson, 2016). Importantly, a preferential expression of pyruvate kinase-M2 (PK-M2), an isoform of the rate-limiting enzyme for pyruvate generation, has been observed in all solid tumors including breast cancer. PK-M2 isoform directly regulates the pyruvate generation to direct the glucose carbon flux into ribose-5-phosphate (Rib-5-P) and NADPH generation (Vander Heiden et al., 2010). Similarly, glycolytic intermediates including 3-phosphoglycerate (3-PG) supply carbon precursor for amino acid and lipid biosynthesis (Jones and Thompson, 2009). Thus, high rate of glycolysis allows proliferating cancer cells to generate abundant ATP as well as maintain biosynthetic demand through low-flux oxidative pathways.

In contrast to the permanent defective OxPhos metabolism resulting in a compensatory increase in glycolytic flux as proposed by Warburg and his contemporaries, it is now well established and widely accepted that mitochondrial metabolism of cancer cells is rewired to meet the demands of macromolecular synthesis (Birsoy et al., 2014; Cairns et al., 2011). In fact, mitochondrial ATP production becomes dispensable with access to sufficient glucose for supporting amino acid and fatty acid biosynthesis. Approximately 10 percent of the remaining pyruvate enters the mitochondria and shunted to Krebs cycle (tricarboxylic acid cycle, TCA) to generate citrate for the lipid biosynthesis in the cytosol

(Lunt and Vander Heiden, 2011). The cytosolic citrate is converted back to acetyl-CoA by ATP citrate lyase (ACL). Genetic or pharmacological inhibition of ACL diminishes tumor cell proliferation *in vivo* (Hatzivassiliou et al., 2005). The high rate of glycolytic flux provides sufficient amount of glycolytic carbon despite the low overall percentage of pyruvate available for biosynthesis. The shunting of TCA intermediates for amino acid and lipid biosynthesis may result in collapse of the cycle or a "truncated" cycle due to the relative decrease in the fraction of mitochondrial citrate (Vander Heiden et al., 2009). Therefore, to maintain the integrity of TCA cycle, glutamine, a second principle growth-supporting substrate, undergoes stepwise oxidation to generate a pool of α-ketoglutarate, which is further metabolized through TCA cycle to regenerate oxaloacetate or citrate consumed by biosynthesis (Mullen et al., 2014). A high anaplerotic flux through oxidative glutamine metabolism allows proliferating cancer cells to channel a significant fraction of TCA cycle intermediates as precursor for amino acid and nucleotide biosynthesis. This explains glutamine's central role as an essential carbon source for TCA cycle in aggressive glioblastoma and triple negative breast carcinoma (Wise and Thompson, 2010).

Overall, cancer cells display altered metabolic circuitry by performing aerobic glycolysis for ATP generation and utilizing the mitochondrial metabolic pathways for different anaplerotic reactions. This fundamental metabolic transformation of cancer cells confers enhanced proliferation advantage and resistance to cell death in the TME (Jones and Thompson, 2009). However, increased proliferation poses an inherent challenge for malignant cells. A growing tumor continues to increase in size, the increased metabolic demands of the cells outstrips nutrient supply. Therefore, cancer cells must engage evolve strategies for metabolic adaptation to maintain cellular bioenergetics and survive the periods of metabolic stress.

2.8.2 Metabolic adaptation by autophagy

Macroautophagy (hereafter referred to as autophagy) is a catabolic self-degradation process whereby intracellular components such as proteins, lipids and organelles are engulfed by the double-membrane vesicles, known as autophagosomes, that are targeted to lysosomes for degradation and recycled into cytosol (Kimmelman and White, 2017). A

critical stage in autophagy pathway is the autophagosome maturation and its degradation, which involves fusion with endosome-lysosome to form autolysosome. Lysosome is a key subcellular organelle involved in the execution of the autophagic process. Therefore, lysosomal biogenesis and its function is tightly controlled in the process of autophagy as evident by the pathological consequences resulting from the loss of lysosomal function during neurodegeneration and lysosomal storage disorders (Gyparaki and Papavassiliou, 2014). Transcription factor EB (TFEB) is the master regulator of lysosomal biogenesis during cellular response to lysosomal stress. TFEB is localized on lysosome in complex with mTORC1 and translocates to nucleus during lysosomal biogenesis. TFEB transcriptionally regulates the CLEAR (Coordinated Lysosomal Expression and Regulation) gene network closely related to lysosomal structure and function, including lipases, hydrolases, lysosomal membrane proteins and the V-ATPase complex (Saftig and Haas, 2016). Whereas, the biogenesis of lysosomes during the course of autophagy is relatively well defined, the mechanisms regulating its function remain poorly understood.

In contrast to its early role in limiting tumor initiation by inducing cell death, it is now evident that autophagy promotes the growth and survival of multiple cancer types including breast cancer . In established tumor, autophagy is elevated in hypoxic tumor regions, which provides a mechanism to generate building blocks for macromolecular synthesis and maintain energy homeostasis through intracellular recycling in periods of nutrient limitations (White et al., 2015). Autophagy also promotes survival during chemotherapy and radiotherapy and thus confer therapeutic resistance. A high frequency of tumor relapse in patient with invasive breast carcinoma strongly correlates with high tumor grade and an elevated basal autophagy (Guo et al., 2013).

Given the diverse substrates that undergo autophagic degradation, autophagy feed into multiple intermediary pathways of central carbon metabolism to provide cancer cells with tremendous metabolic plasticity. Autophagy also mitigates intrinsic cellular damage by degrading toxic protein aggregates and damaged organelles specifically mitochondria (Guo et al., 2011). Thus tumor-promoting function of autophagy is twofold: firstly, providing substrate for biosynthesis during nutrient stress in TME and secondly, preserving organelle function to maintain energy homeostasis. This was evident in mice model

of aggressive breast cancer where an upregulated basal autophagy enabled the survival and progression of early breast tumors by maintaining mitochondrial metabolic function in a stressed TME (Karantza-Wadsworth et al., 2007). Aggressive tumors, particularly those associated with poor prognosis, display an increased dependency on autophagy for normal proliferation. Apparently, the pharmacological inhibition of autophagy in aggressive tumors have shown improved efficacy in cancer treatment but with varied result suggesting that targeting mitochondrial metabolism of cancer cell may provide a valuable approach to therapy for aggressive cancer (White and DiPaola, 2009).

2.8.3 Metabolic interactions with tumor microenvironment

Heterocellular metabolic interaction in the TME leads to the clonal heterogeneity within a tumor that exhibit varying metabolic requirements and properties. Metabolic remodeling of cancer cells regulates the nutrient states of the non-malignant and immune cells in TME (Lyssiotis and Kimmelman, 2017). The abnormal metabolic activities of cancer cells drive the metabolic changes in innate and adaptive immune cells and dictates their overall metabolic fitness. In TME, nutrient availability is proportional to tumor vasculature and efficient waste removal is inversely proportional *(Figure 2.4)*. For instance, enhanced glycolytic activity in hypoxic region of tumor leads to build up of high lactate levels which acidifies TME (Brand et al., 2016). While normoxic region of tumor readily consume lactate discarded by hypoxic cancer cells to fuel mitochondrial metabolism, an acidic microenvironment influences how immune cells recognizes and respond to tumors. This symbiotic intratumoral metabolism was observed in a breast cancer model following antiangiogenic therapy (Pisarsky et al., 2016).

Cancer and immune cells share similarities in nutrient utilization and metabolic regulation to sustain proliferation. Similar to cancer cells, innate immune cells (macrophages and dendritic cells) and adaptive immune cells (B and T lymphocytes), engage metabolic reprogramming upon cytokine or receptor stimulation, to drive their activation, differentiation and effector function (Kouidhi et al., 2017). However, abnormal vasculature, nutrient limitations and inefficient removal of metabolic waste product in the TME poses a physical challenge in which cancer and immune cells must compete for nutrients to per-

form biosynthesis and effector function (Lyssiotis and Kimmelman, 2017). Immune cells tend to be less adapted for this metabolic competition which is a principle mechanism regulating antitumor immunity (Gajewski et al., 2013).

Similar to glucose-lactate metabolism, an increased utilization of extracellular amino acids through alanine, glutamine, arginine and tryptophan metabolism in cancer cells and its

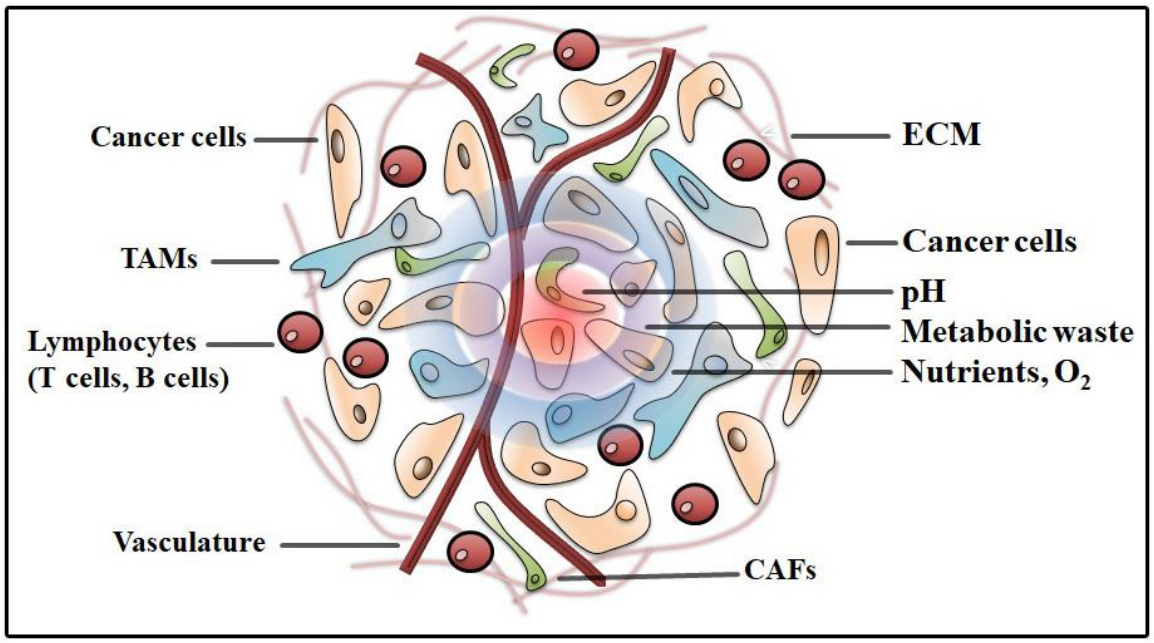

Figure 2.4: Intratumoral Metabolic crosstalk in TME: *Heterocellular interaction within TME affects the metabolic properties of cancer and immune cells. Tumor extrinsic features such as heterogenous cell population, nutrient availability, pH gradient, vasculature, lactate deposition and hypoxia regulate the metabolic crosstalk in TME. Gradients are indicated by shadowed circles, ECM-extracellular matrix, CAFs- Cancer-associated fibroblasts, TMAs-Tumor-associated macrophages.*

availability in the TME regulates the antitumor response of T cell (Sousa et al., 2016). Cytotoxic T cells are sensitive to fluctuations in local amino acid availability and thus display significantly impaired antitumor response when amino acids are depleted locally. For instance, metabolism and availability of tryptophan and arginine amino acids regulates immune activation across many cancer types (Murray, 2016). The depletion of local tryptophan and its breakdown to immunosuppressive catabolites such as kynurenine by

tumor cells impairs dendritic and CD4+ T cell activation and effector functions. Similarly, an active catabolism of extracellular arginine by tumor cells creates a state of arginine deficiency for the effector T cells (Lyssiotis and Kimmelman, 2017; Mezrich et al., 2010). These evidences strongly suggest that intratumoral mechanism of metabolic communication act symbiotically to support biosynthesis and tumor growth and competitively to impair antitumor response. Heterocellular metabolic interaction in the TME is repurposed to counteract nutrient limitation by cancer cells and represent critical nodes for designing and targeting anti-cancer therapies (Anastasiou, 2017).

2.8.4 Role of TNF-α in metabolic remodeling in cancer cells

Intrinsic and extrinsic features of the TME including abnormal vasculature, tumor heterogeneity, chronic inflammation, growth factors, cytokines and DAMPs imposes multiple layers of regulations that influences the overall metabolic properties of a tumor (Gouirand et al., 2018). In normal cells, extracellular signals control the uptake and metabolism of the nutrients. The recognition of PAMPs/DAMPs or activation of immune cells by cytokines results in profound metabolic alteration which is required for its effector function. Thus, the metabolic reprogramming in normal cell is tightly regulated by the availability of the extracellular signal (Palm and Thompson, 2017). In contrast, metabolic reprogramming in cancer cells is intrinsically controlled where extracellular factors in the TME fine tune cellular metabolic pathways to promote tumor development and progression (Pavlova and Thompson, 2016). For instance, elevated levels of TGF-β in metastatic breast tumor induce acetyl-CoA carboxylase 1 (ACC-1)-dependent metabolic reprogramming was frequently associated with metastases and tumor recurrence (Rios Garcia et al., 2017).

Similarly, aberrant TNF-α signaling represents one of the key features of the cytoprotective mechanisms in promoting growth and migration of breast cancer. As discussed above, intratumoral TNF-α levels were significantly elevated in breast cancer patients and proportionally increased with the degree of the tumor grade. A chronic activation of TNF-α signaling has been linked to the breast cancer aggressiveness and prognosis in epidemiological studies (Esquivel-Velazquez et al., 2015). The molecular mechanism of

TNF-α signaling as a potent activator of canonical NF-κB prosurvival pathway in cancer cells has been well elucidated (Wang et al., 2008). Unexpectedly, pharmacological inhibition and genetic ablation of NF-κB activity led to an enhanced inflammation and organ failure in mice models and human subjects. Termination of the clinical development of the programs focusing on directly targeting NF-κB activity has led to the identification of alternative strategies to activate TNF-α-induced programmed cell death in cancer cells (Zhong et al., 2016).

More than a decade of intensive investigation has led to the identification of ROS generation critically involved in caspase-dependent (apoptosis) or caspase-independent (necroptosis) programmed cell death. Earlier studies suggested that mitochondria-derived ROS could be a major source regulating TNF-α-induced cell death (Blaser et al., 2016). Subsequent studies identified a critical role of mitochondrial ROS (mROS) in inducing mitochondrial outer-membrane permeabilization (MOMP) and positive regulation of TNF-α-induced apoptosis or necroptosis (Huai et al., 2013; Kim et al., 2010). These experimental findings have been successfully validated in tumor-bearing mice model, where tumor cell intrinsic TNF-α signaling combined with chemotherapy drives increased ROS generation and tumor regression (Habtetsion et al., 2018). These findings further indicate that tilting tumor redox balance towards oxidative stress may augment the efficacy of immunotherapy that would otherwise be less effective. Conversely, chronic levels of intracellular ROS may promote TNF-α-mediated NF-κB activation and cell survival (Gloire et al., 2006; Kastl et al., 2014). Therefore, understanding the mechanisms of intracellular communication between tumor cell intrinsic TNF-α signaling and mitochondrial metabolism regulating TNF-α-induced oxidative stress is important.

The emerging evidences suggest that caspase-8 may translocate to different subcellular sites including mitochondria in response to different stimuli. TRADD, TRAF2 and RIP1 subunits of complex-II together with procaspase-8 have been reported to translocate to mitochondria during the TNF-α induced apoptosis (Kim et al., 2010; Scorrano, 2008). The functional relevance of Caspase-8 and other subunits of complex-II translocation to mitochondria is not clear. Similarly, additional studies provided evidence that TNF-α induces aerobic respiration and increases pool size of anaplerotic intermediates of TCA cy-

cle during necroptosis in cancer cells. Importantly, the authors showed that RIP3 activates pyruvate dehydrogenase complex (PDC) through phosphorylation and enhances aerobic respiration and mROS generation during TNF-α-induced necroptosis (Yang et al., 2018; Zhang et al., 2009). These evidences indicate a positive regulation of aerobic respiration and its by-product ROS in cancer cells, however the regulatory mechanism of this metabolic reprograming during TNF-α signaling is unknown and demands further investigation.

2.9 Diverse role of mitochondria in metabolism and immune responses

2.9.1 Mitochondria : an essential organelle for bioenergetic adaptation in cancer cells

Cancer cells display a dramatic alteration in metabolic circuitry from performing aerobic glycolysis for ATP generation to use mitochondria as a biosynthetic hub as discussed above (Lunt and Vander Heiden, 2011). Metabolic reprogramming of mitochondrial function in cancer cells represents a fundamental bioenergetic switch which confers enhanced proliferation and resistance to cells death (Pavlova and Thompson, 2016). Recent evidences indicate that dynamic remodeling of mitochondrial electron transport chain (mETC) complexes play a critical role in bioenergetic adaptation during activation/proliferation of lymphocytes and starvation (Buck et al., 2016; Couvillion et al., 2016). Tailoring of mETC to stimulus-specific requirements is central to metabolic reprogramming observed in proliferative immune cells upon PRR stimulation and nutrient stress conditions (Sander and Garaude, 2018). Thus, investigating the mechanism of biogenesis and remodeling of mETC complexes through nuclear-encoded regulatory factors is critical for understanding bioenergetic adaptation of cancer cells in response to inflammation and nutrient limitations.

The OxPhos system embedded in inner mitochondrial membrane is composed of five multiprotein complexes forming the mitochondrial respiratory chain (MRC). The MRC complexes are assembled from nearly 100 protein subunits, of which approximately 90

subunits are encoded by nuclear genes and imported into mitochondria. The remaining 13 subunits are encoded by mitochondrial DNA (mtDNA) (Meisinger et al., 2008). In addition, mitochondrial genome encodes 22 tRNAs and 12S and 16S mt-rRNAs that are essential for the synthesis of mitochondria-encoded proteins. All protein factors involved in mtDNA replication, transcription, post-transcriptional modification and translation are nuclear encoded and imported to mitochondria to exert their function. Nuclear transcription factors and activators coordinate mitochondrial and nuclear gene expression to orchestrate the timely synthesis of MRC complexes in proper stoichiometry (Couvillion et al., 2016).

mtDNA-encoded polypeptides of respiratory chain complex are transcribed on both strands to form three continuous polycistronic transcripts (Montoya et al., 1983). These precursor transcripts are usually punctuated by tRNAs that are further excised by RNase P at the 5'and by RNase Z at the 3' ends of the tRNAs (Rackham et al., 2012). The post-transcriptional processing of mt-mRNAs and mitoribosome assembly is spatially organized within distinct foci termed as mitochondrial RNA granules (MRGs) *(Figure 2.5)* (Jourdain et al., 2016). These are dynamic mitochondrial sub-domains to which mt-RNAs processing enzymes such as RNase P, RNase Z, GRSF1, RNA methyl transferases and other unidentified proteins are recruited (Pearce et al., 2017). MRGs interactome capture studies have identified novel class of nuclear encoded RNA binding proteins that translocates to mitochondria and lack the canonical RNA binding domain, however, interacts with mt-mRNAs in human cells (Antonicka and Shoubridge, 2015; Baltz et al., 2012). Mutations in genes encoding these proteins have been associated with a range of heritable disorders with overlapping phenotypes, as observed in mitochondrial monogenic diseases (Van Haute et al., 2015).

Human Fas-activated serine/threonine kinase (FASTK) family, an emerging class of RNA-binding proteins, acts as a central regulator of mitochondrial post-transcriptional RNA processing. The family comprises of six structurally related proteins named FASTK and its homologs FASTKD1-5. All members share an N-terminal mitochondrial targeting signal and a putative RNA-binding module, RAP domain (RNA-binding domain abun dant in Apicomplexans) (Jourdain et al., 2017). FASTKD1, FASTKD2 and FASTKD5

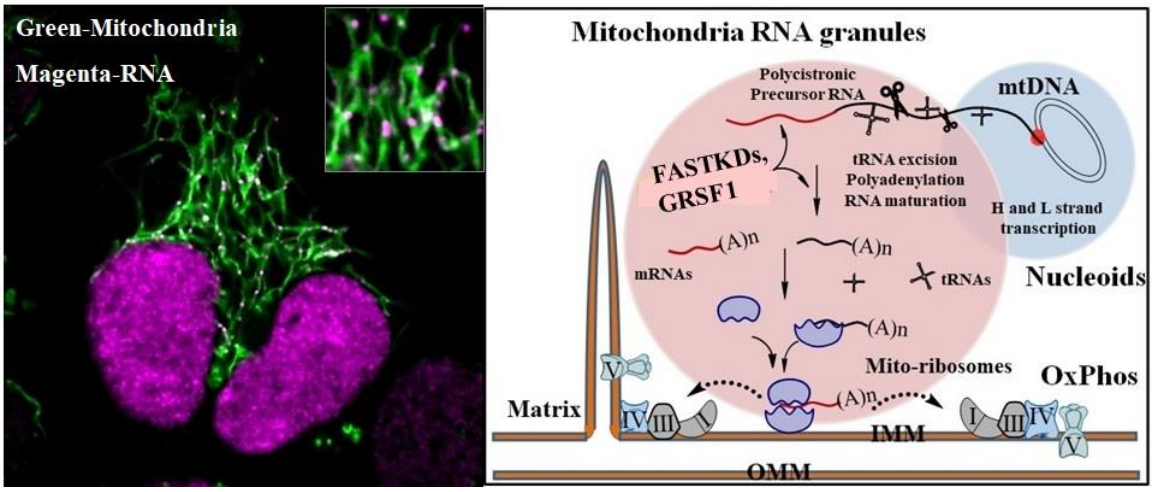

Figure 2.5: Mitochondria RNA granules (MRGs): *MRGs are the submitochondrial compartment inside matrix where the post-transcriptional processing of polycistronic precursor RNA transcribed from mtDNA in nucleoid is performed by nuclear-encoded proteins including GRSF1 and FASTKD1-5. The confocal image on left shows HEK293 cells stable expressing mtGFP (labeling mitochondrial network) immunostained with anti-BrU (for labeling nascent RNA both inside nucleus and mitochondria).*

have been shown to localize within MRGs, which differentially regulate the processing of mitochondrial RNAs (Pearce et al., 2017). Distinct role of FASTK family proteins and recruitment of other RNA-binding proteins to regulate processing and maturation of mitochondrial RNA during patho-physiological condition is still lacking.

2.9.2 Mitochondria: a fundamental hub for innate immune signaling

While the role of mitochondria is well established as a biosynthetic and bioenergetic organelle, mitochondria have emerged as a signaling organelles during other important cellular process such as ion homeostasis, autophagy, programmed cell death and innate immune signaling (Chandel, 2015). Mitochondrial outer membrane as well as MAMs provides a molecular platform for the assembly of immune adaptor protein complexes and execution of innate immune signaling pathways upon PRR engagement as discussed above (West et al., 2011b). Mitochondria act as a coordinating site where innate immune

signals generated from the activation of extracellular PRRs or cytokine receptors are conveyed to mitochondrial inner membrane and matrix *(Figure 2.6)* (Arnoult et al., 2011). These findings came from the initial observation that activation of TLR4 by LPS upregulated the expression and secretion of proinflammatory cytokines such as TNF-α which in turn depended on generation of mROS (West et al., 2011a). Thereafter, a variety of innate immune regulatory proteins have been identified which modulates mitochondrial bioenergetic states during innate immune signaling. Interestingly, damage mitochondria are itself sensed by the cytosolic PRRs due to their bacterial ancestry and trigger inflammatory response comparable to those induced by microbial infection. Stressed mitochondria release cytosolic DAMPs such as mtDNA, mtRNA, ROS, ATP, *N*-formyl peptides, which are sensed as a cellular damage signals and causes abnormal activation of innate immune responses (Newman and Shadel, 2018). Therefore, to maintain the close interaction between mitochondrial metabolism and immune signaling and to prevent aberrant mitochondria-targeted responses, its form and functions are tightly regulated.

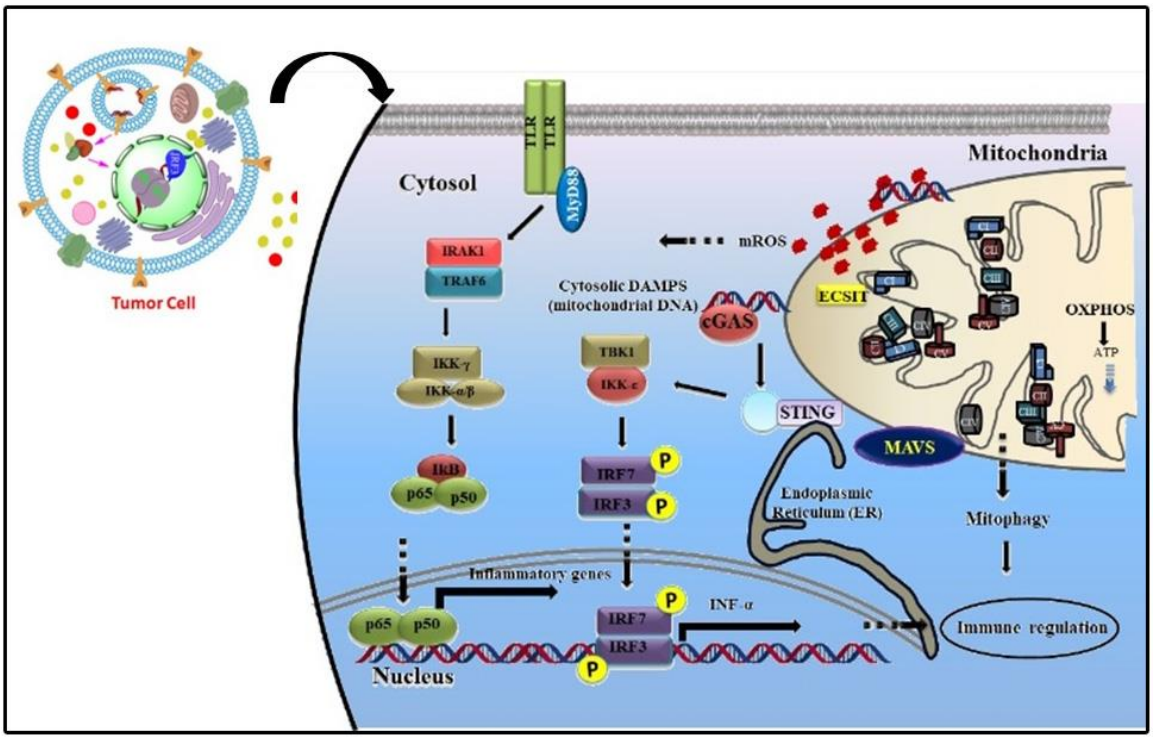

Figure 2.6: Mitochondria in innate immunity: *In cancer cells, mitochondria acts as a molecular platform for the assembly and activation of innate immune adaptor proteins including STING and MAVS in response to cytosolic DAMPs. The engagement of TLR4*

signaling in response to extracellular PAMPs, leads to association of TRAF6 with EC-SIT, an assembly factor of mitochondrial complex I. ECSIT modulates the assembly and activity of CI to regulate ROS generation during NF-κB activation. Similarly, MAVS interacts with COX5B to modulate ROS generation during type-I IFN signaling.

2.9.2.1 MAVS and STING: Mitochondrial Adaptor proteins regulating cytosolic DNA and RNA induced innate immune pathways

2.9.2.1.1 MAVS -Mitochondria Anti-Viral Signaling protein

Previous studies focusing on understanding the adaptation of mitochondrial bioenergetics during innate immune signaling has led to identification of various mitochondria membrane-localized adaptors proteins which may provide singling cue to mitochondrial inner membrane and matrix to reprogram mitochondrial functions (Weinberg et al., 2015). MAVS (Mitochondria Anti-Viral Signaling protein) is the first identified immune adaptor protein of RLR signaling pathway which localizes to mitochondrial outer membrane (OMM) following interaction with cytosolic dsRNA sensor RIG-1 and MDA5 (McWhirter et al., 2005) *(Figure 2.6)*. MAVS oligomerization at the OMM recruits TBK1 and IKK complex leading to activation of IRF3/7 and NF-κB and synthesis of antiviral proteins (IFN-1, IFN-III and IFN-stimulated genes) and proinflammatory cytokines. Subsequent studies demonstrated that MAVS contain a C-terminal transmembrane domain, which targets it to OMM and is indispensable for its function (Scott, 2010).

MAVS activation and function is tightly regulated by mitochondrial dynamics and its bioenergetic state. Healthy mitochondria, frequently present in an elongated, fused network maintain a robust mitochondrial membrane potential ($\Delta\Psi_m$) whereas a fragmented/fissioned network is indicative of damaged depolarized mitochondria. Mfn 1 (Mitofusin 1) and Mfn 2 (Mitofusin 2), which regulates mitochondria fission/fusion interacts with MAVS and reorganizes its distribution at OMM for the robust execution of RIG-1-induced antiviral response (West et al., 2011b). Similarly, several studies have demonstrated that physiologically normal $\Delta\Psi_m$ and generation of mROS is essential for the

proper induction of MAVS-mediated antiviral signaling further emphasizing the role of mitochondria as a key component of innate immune defense pathways (Pourcelot and Arnoult, 2014).

In addition to the regulatory role of mitochondrial dynamics and membrane potential in controlling MAVS-mediated innate immune response, three additional mitochondria-localized proteins namely COX5B, TUFM and NLRX1 negatively regulates MAVS activation. Cytochrome c Oxidase (CcO) complex subunit, COX5B of mETC complex downregulates MAVS signaling through direct interaction and by repressing ROS generation from mitochondria. Further, COX5B positively regulates autophagy to control MAVS aggregation and thereby balances the antiviral signaling activity (Zhao et al., 2012). COX5B is predominantly localized to the matrix face of the mitochondrial inner membrane and is an integral component of MRC complex IV (Calvo et al., 2016). Thus, it is difficult to envision as to how COX5B interacts with MAVS, a OMM protein and regulates its function under patho-physiological condition, unless one has to include the possibility of varied localization of COX5B which may be relevant to its multiple functions. Interestingly, these evidences provide novel insight in regulating innate antiviral immunity through maintenance of normal bioenergetic state and function of mitochondria.

2.9.2.1.2 STING -STimulator of INterferon Genes

MAMs provides a mitochondria/ER inter-organelle communication that allow the exchange of ions and metabolites which regulates mitochondrial bioenergetic and mitochondrial dynamics. MAMs also function as a platform for the assembly of innate immune signaling modulators which adapt mitochondrial metabolism to immune changes (Rieusset, 2018). STING (stimulator of interferon genes) is an ER-associated immune adaptor protein which is essential for controlling the activation of type I IFN and proinflammatory cytokines following detection of cytosolic DNA species or CDNs *(Figure 2.6)* (Abe et al., 2013). STING in coordination with cGAS has been recently identified as the central mechanism of cytosolic DNA sensing upon microbial infection and intracellular escape of mtDNA. cGAS (Cyclic GMP-AMP synthase) is a cytosolic protein which

enzymatically converts cytosolic DNA species to CDN, cyclic GMP–AMP (cGAMP). cGAMP acts as a secondary messenger that binds to STING adaptor protein which in turn induces secretion of type-I IFNs and proinflammatory cytokines such as TNF-α (Barber, 2015). cGAS-STING pathway is activated by cytosolic double-stranded DNA released from genomic damage or mitochondrial stress irrespective of the sequence (Li and Chen, 2018). However, the degree of inflammatory gene induction by cGAS-STING pathway upon detection of cytosolic DNA is usually lower than that induced by viral infection or chemotherapy and radiation treatment of advanced tumors. *In vivo* studies in tumor-bearing mice models revealed that chemotherapy and radiation treatment induce type-I IFN-dependent cell death in tumors and promote antitumor immunity (Deng et al., 2014; Sistigu et al., 2014).

Tumor cells which accrue genomic instability due to loss of tumor suppressor genes such as p53 or in response to genotoxic stress induced by DNA-damaging agent or irradiation override cell cycle arrest and apoptosis (Hanahan and Weinberg, 2000). Damaged DNA is driven out of the nucleus in the form of micronuclei during the formation of daughter cells. The membrane integrity of micronuclei is comprised as they progress through cell cycle and sensed by cGAS-STING pathway resulting in the production of type-I IFN and cytokines promote inflammation and immunosuppression in TME (Bakhoum and Cantley, 2018). Recently, a genome-wide study reported that chromosomal aberrations in primary tumor cells is associated with missegregation of anaphase chromosome and generate chromosomal instability (CIN), a hallmark of cancer cells (Bakhoum et al., 2018). Tumor cells with high CIN displayed more micronuclei and an increased incidence of lymph node metastasis (Seton-Rogers, 2018). Thus, primary cancer cells subvert the lethal response to genomic instability by co-opting the chronic activation of innate immune signaling pathways for distant metastasis. These evidences suggest the role of cell-intrinsic innate immune response in promoting growth and metastasis of solid tumors such as breast tumors, however the mechanisms are not well understood.

In non-malignant cells, activation of executioner caspases such as caspase-3/7 or caspase-9 suppresses the sterile inflammation induced by cGAS-STING pathway upon MOMP and enable the apoptotic event to be immunologically silent (Minton, 2015). In contrast,

defect in intrinsic apoptotic program of cancer cells or induction of caspase-independent cell death program leads to chronic production of type-I IFN and proinflammatory cytokines such as TNF-α, IL-1β and IL-18 (Kretschmer and Lee-Kirsch, 2017). These evidences further strengthen the concept that mitochondria act as a central participant in the innate immune signaling by identifying mitochondrial stress as a cell-intrinsic of antiviral signaling. Intracellular monitoring and sensing of mitochondrial DAMPs such as mtDNA, mtRNA, ROS, ATP, *N*-formyl peptides and TFAM engages canonical pathogen-sensing mechanisms such as NF-κB, type-I IFN signaling and autophagy pathway to regulate mitochondrial function and homeostasis (Rongvaux, 2018).

2.9.2.2 NOD-like receptors - Emerging family of intracellular PRRs

The nucleotide-binding domain and leucine-rich repeat containing (NLR) proteins (also known as CATERPILLERs, NODs, NALPs or NACHT-LRRs) in mammals share a highly significant sequence homology with plant NBS-LRR proteins which are important in acquisition of disease resistance. NLRs are an emerging class of intracellular PRRs which senses cytosolic PAMPs and DAMPs. At least 23 distinct NLR and NLR-like protein have been identified in humans and 34 family members identified in mice (Meunier and Broz, 2017). These proteins share a highly conserved tripartite domain structure containing a variable N-terminal effector domain which function to recruit adaptor or effector molecules that drives downstream signaling. The central domain of the protein is comprised of a conserved NACHT nucleotide-binding domain, which facilitates oligomerization. The C-terminal domain of the protein contain multiple LRR element (up to 33 LRR element, each comprising of 28-29 amino acid residues in length) which are essential for ligand sensing *(Table 2.2, Figure 2.7)* (Kufer and Sansonetti, 2011).

2.9.2.2.1 Inflammosome forming NLRs

A fundamental role of NLR family proteins is to regulate pro-inflammatory cytokines and chemokines that drive host innate immune response to pathogens and cellular insults. A controlled release of proinflammatory cytokine such as IL-1β and IL-18 which are in-

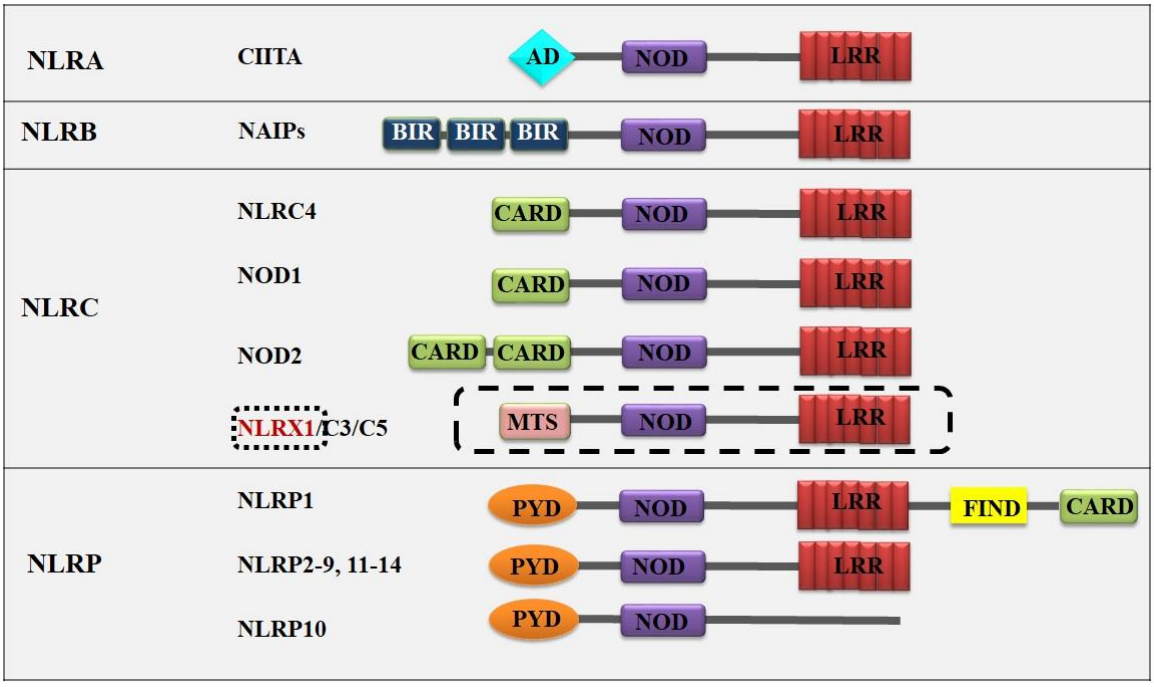

Table 2.2: The human NLR gene family. *The human NLR gene family consists of 22 members that share a tripartite structure, consisting of an N-terminal signaling domain, a central nucleotide-binding and oligomerization domain, and a C-terminal agonist sensing/ligand-binding domain. The NLR family is sub-divided into four sub-groups NLRA, NLRB, NLRC, and NLRP based on the nature of the N-terminal domain consisting respectively of an acidic transactivation domain, a baculovirus IAP repeat (BIR), a caspase-recruitment and activation domain (CARD), and a Pyrin domain (PYD). NLRX1, highlighted here, has an N-terminal mitochondrial targeting sequence.*

volved in tissue repair following inflammation is key to this response (Allen, 2014). A functional sub-group of NLRs have been identified to drive this process through the formation of a multi-protein complex termed the inflammasome *(Figure 2.8)*. Inflammosome forming NLRs such as NLRP3, recognizes oxidized form of mitochondrial DNA (increased mROS converts mtDNA into an oxidized form) in cytosol in an initial step called priming leading to rapid transcriptional activation of NF-κB responsive genes such as pro-IL-1β and pro-caspase-1 (Murphy, 2018). In a second activation step, initiated by common intracellular intermediates such as ATP, ROS or mtDNA fragments leads to the

NLRP3 inflammosome assembly and caspase-1 activation and uncontrolled secretion of pro-IL-1β and pro-IL-18 in its active form (Newman and Shadel, 2018).

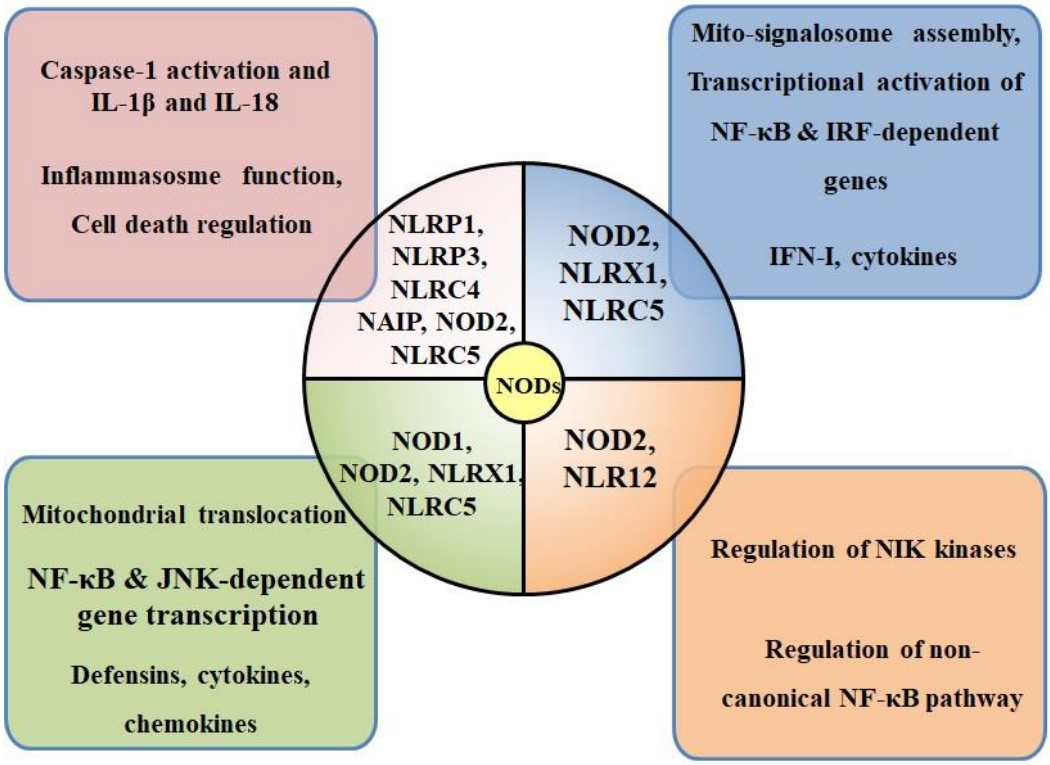

Figure 2.7: NLR family proteins in innate immune signaling : *The NLR family proteins function through different multimeric protein complexes regulating caspase-1-activating inflammasomes, the IFN/cytokine-inducing RLR signaling complex, the NF-κB/MAPK-activating NOD1/2 complex and the non-canonical modulation of NF-κB via NIK. The figure also depicts that one NLR can serve multiple functions, whereas multiple NLRs can also serve similar functions.*

2.9.2.2.2 NLRX1: Modulator of innate immune signaling

Recent studies have also identified a functional group of non-inflammosome forming NLRs that negatively regulates inflammation during host anti-viral immune response *(Figure 2.8)* (Allen, 2014). NLRX1 (Nucleotide-binding domain (NBD) and Leucine-rich repeat (LRR)-containing family member X1), originally characterized as a negative regulator of canonical NF-κB signaling, is unique among the NLRs due to its mitochondrial

localization. Human NLRX1 is a ubiquitously expressed, highly conserved multidomain protein of 975 amino acids having N-terminal mitochondria-targeting sequence (MTS), a central nucleotide-binding and oligomerization domain (NOD) and a C-terminal putative ligand-binding and regulatory leucine-rich repeat (LRR) domain (Tattoli et al., 2008).

NLRX1 negatively regulates host innate immune response to bacterial and viral infections. NLRX1 attenuated the nuclear translocation of p65 subunit and NF-κB activation by binding to the upstream IKK complex through LRR domain during LPS-triggered TLR4 signaling (Xia et al., 2011). Similarly, NLRX1 negatively regulates type-I IFN signaling by preventing the formation of RIG-1-MAVS signalosome at the OMM during viral infection and chronic pulmonary inflammation (Kang et al., 2015; Moore et al., 2008). NLRX1 also function as a positive regulator of virus-induced autophagy through its interaction with another mitochondrial protein TUFM (Tu translation elongation factor, mitochondrial also known as Ef-Tu). NLRX1-TUFM complex at the mitochondria, similar to Mfn2, dually controls the downregulation of IFN-1 and induction of autophagy through the recruitment of ATG12-ATG5 and ATG16L1 (Lei et al., 2012). Further, increased autophagy flux negatively regulates IRF3 signaling and type-I IFN production by inhibiting MAVS aggregation and ROS generation to facilitate its degradation via mitophagy (Arnoult et al., 2011).

In addition to negative regulation of STING activation by apoptotic caspases, a recent study reported that NLRX1 attenuates cGAS-STING-dependent antiviral response upon cytosolic infection with DNA viruses by localizing to mitochondria. NLRX1 sequesters DNA-sensing adaptor STING to block its interaction with TBK1 and inhibit antiviral immune response to HIV-1 and DNA viruses (Guo et al., 2016b). However, the mechanism through which NLRX1 may regulate STING-dependent activation of antiviral signaling in response to cytosolic DNA released from mitochondria or micronuclei in cancer cells has not been investigated. Studying the regulatory functions of NLRX1 could reveal the mechanisms by which cancer cells regulate the activation of NF-κB and type-I IFN signaling in response to intracellular DAMPs such as mitochondrial RNA or DNA.

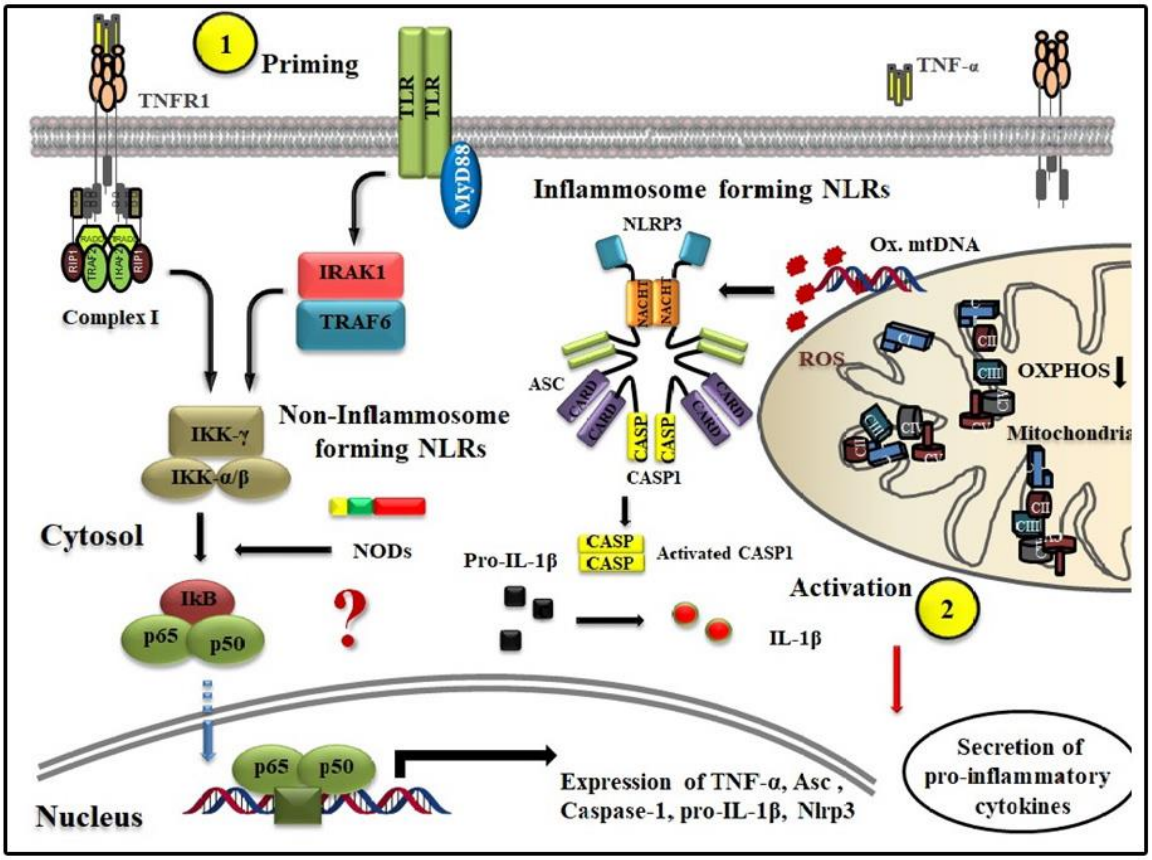

Figure 2.8: Inflammasome-forming and Non-inflammasosme-forming NLRs in innate immune signaling: *The inflammasome-forming NLRs regulates the secretion of proinflammatory cytokines including IL-1β and IL-18 through the formation of a multi-protein complex termed inflammasomes in a two-step NF-κB-dependent signaling. The non-inflammasome forming NLRs may regulates the NF-κB signaling and production of pro-inflammatory cytokines in a direct or indirect manner.*

Although earlier line of evidences supported the concept that NLRX1 functions as a checkpoint inhibitor of early innate immune responses to both DNA viruses and RNA viruses, subsequent studies in *Nlrx1*$^{-/-}$ mice and *Nlrx1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) reported an unchanged level of MAVS-dependent phosphorylation of IRF3 and production of IFN-β and chemokine CXCL10 during infection with RNA viruses (Rebsamen et al., 2011; Soares et al., 2013). A few studies also suggested a positive regulatory role for NLRX1 in inducing antiviral responses (Feng et al., 2017). These evidences suggest that NLRX1 may modulate the assembly of dynamic signaling complexes re-

cruiting positive or negative regulators of innate immune signaling depending upon the stimuli, however this demands further investigation.

2.9.2.2.3 NLRX1 : Unique NLR protein, regulator of mitochondrial function

Emerging evidences suggested the role of NLRX1 beyond the regulation of host innate immune response. Overexpression of NLRX1 potentiated ROS generation induced by TNF-α which further amplified the canonical NF-κB signaling and IFN-β production in response to *Shigella flexneri, Chlamydia trachomatis* and double-stranded RNA virus infection of epithelial cells (Abdul-Sater et al., 2010; Tattoli et al., 2008). The authors also reported that NLRX1 was targeted to the mitochondrial matrix through a functional N-terminal addressing sequence. In the matrix, NLRX1 interacted with UQCRC2 (Ubiquinol-Cytochrome C Reductase Core Protein 2), a matrix-facing protein subunit of MRC complex-III (bc1 complex) (Arnoult et al., 2009). Based on this interaction, it was subsequently proposed that NLRX1 may potentiate the generation of ROS by directly associating with UQCRC2 at the complex-III site during stimulus-specific condition such as TNF-α signaling or microbial infection. Although no functional effect of this interaction was elucidated further, these results suggested a possible link between NLRX1 function and control of mitochondrial metabolism.

The high-throughput proteome analysis of human innate immunity network identified NLRX1 interactome during unstimulated and MAVS-dependent activation of NF-κB signaling. A subcellular compartment localization analysis of the NLRX1 interactome revealed a high percentage of mitochondrial proteins as interacting partners (Lei et al., 2012; Li et al., 2011). A partial structure of C-terminal fragment (amino acid 629-975) of NLRX1, determined by X-ray crystallography, revealed that extensive intramolecular and intermolecular interactions between NLRX1-LRR and its associated α-helical domains forms a higher order oligomer which may promote cooperative interactions between NLRX1 and its target proteins (Xiao and Ting, 2012). Interestingly, the authors also demonstrated that the affinity of LRR domain of NLRX1 for single or double stranded RNA using an *ex situ* binding assay, however the effector function and potential ligands of NLRX1 within mitochondria is still unknown (Hong et al., 2012). These findings are

puzzling given that the preponderance of evidences favors a functional role of NLRX1 in controlling mitochondrial metabolism over its negative regulatory role in host innate immune response.

It is also possible that NLRX1 could be a potential regulator of immunometabolic functions which may integrate intratumoral-stress induced innate immune responses and mitochondrial function to provide survival advantage and immune escape to cancer cells. For example, NLRX1-TUFM complex as been reported to inhibit the formation of RIG-1-MAVS signallosomes through direct sequestration of MAVS at the OMM and thereby prevents the activation of MAVS-dependent NF-κB and type-I IFN signaling (Lei et al., 2012). Separate studies have confirmed the subcellular localization of these proteins to mitochondrial matrix (Arnoult et al., 2009; Calvo et al., 2016), it is more difficult to envision how NLRX1-TUFM complex can access the RIG-1-MAVS signallosome at the OMM. The role of NLRX1 in regulation of mitochondrial metabolism could be the adaptive mechanism during inflammation-induced stress condition. This in turn could potentially regulate tumor initiation and progression, however these possibilities need to be further investigated using different model systems.

In the present study, we systematically investigated the role of NLRX1 in modulating mitochondrial function and cell death and its contribution in regulating the tumorigenic potential of human breast cancer cells in presence of TNF-α. We further demonstrated the role of NLRX1 in preserving the mitochondrial homeostasis of breast cancer cells during TNF-α induced inflammatory stress condition. NLRX1 resides in mitochondrial RNA granules and regulates mitochondrial RNA processing and hence reprogramming of mitochondrial metabolism in breast cancer cells. The study also elucidates a functional role of NLRX1-regulated mitochondrial function in controlling TNF-α-induced autophagy flux which have important implication in tumor cell metabolism.