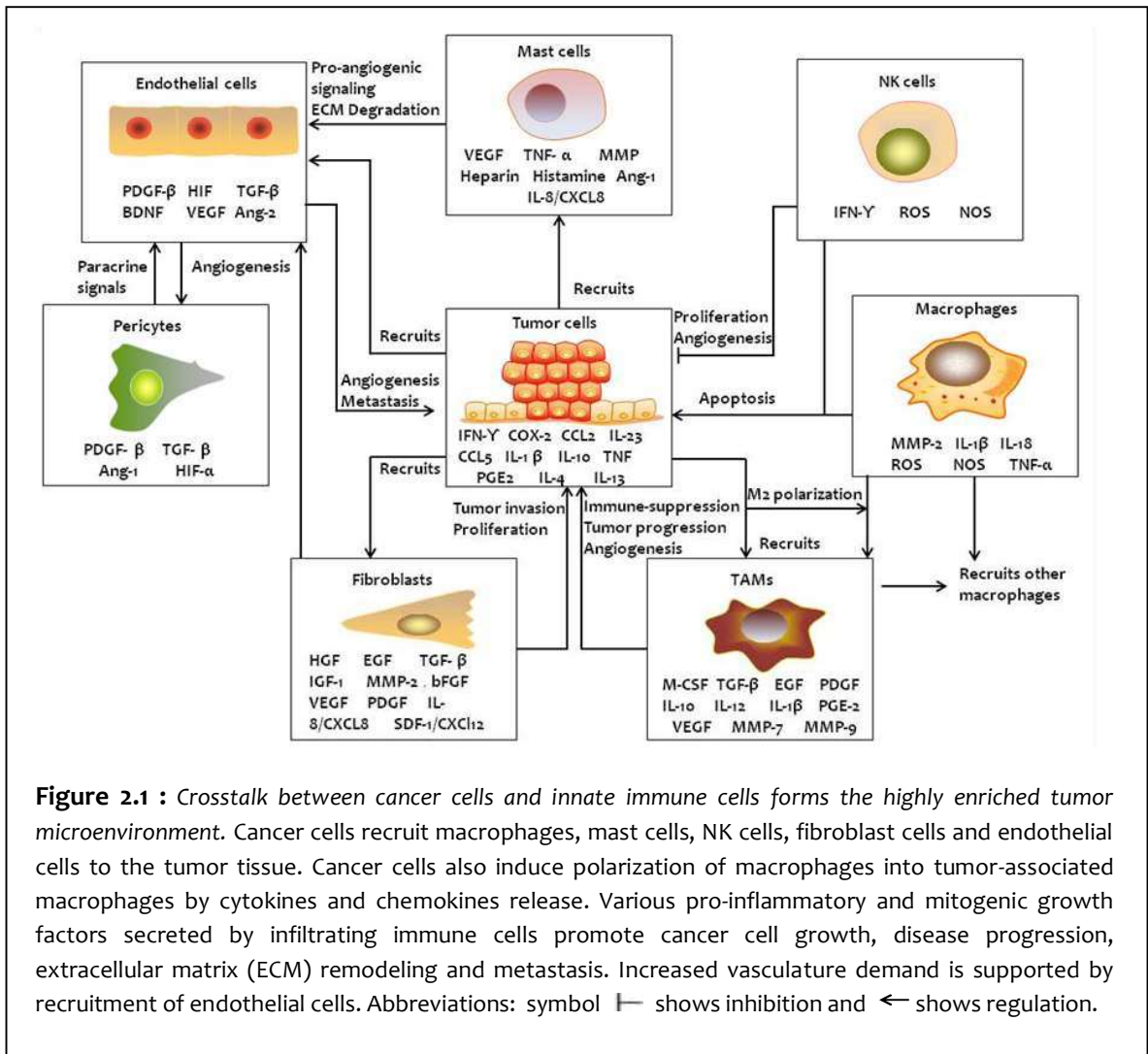# 2
Review of Literature

2.1 ABSTRACT

NLRs (nucleotide-binding domain, leucine-rich repeat containing receptors) are pattern recognition receptors associated with immunity and inflammation in response to PAMPs and DAMPs stimuli. Dysregulated NLR function is associated with several diseases including cancers, metabolic diseases, autoimmune disorders and autoinflammatory syndromes. In the last decade, distinct cell and organ specific roles for NLRs have been identified however; their roles in cancer initiation, development and progression remain controversial. In this chapter, I summarize the emerging role of NLRs in inflammation, cancer and their promising future as targets for cancer therapeutics.

Innate immune cells trigger inflammation to eliminate pathogen and combat tissue injury. Inflammatory responses resolve infection as a protective response. However, prolonged aberrant inflammation causes disruption in immune surveillance and cellular homeostasis. The unprecedented ability of inflammation to regulate core cellular and molecular mechanisms related to tumorigenesis is one of the major hallmarks of cancer. The association of chronic inflammation with cancer is well established [Balkwill and Mantovani, 2001; Philip, Rowley *et al.*, 2004; Mantovani, Allavena *et al.*, 2008; Grivennikov, Greten *et al.*, 2010]. Epidemiological data suggests that up to 15% of human cancer incidence is associated with inflammation [Balkwill and Mantovani, 2001][Colotta, Allavena *et al.*, 2009; Hanahan and Weinberg, 2011]. Sustained chronic inflammation gives rise to a tumor promoting microenvironment. The tumor microenvironment comprises of stromal cells, extracellular matrix and dense vasculature as depicted in Figure 2.1. Stromal cells consist of innate immune cells including macrophages, mast cells, natural killer cells, as well as other cell types such as fibroblasts, endothelial cells and pericytes. Both cancer and stromal cell population exhibit active communication through exchange of various soluble factors including mitogenic growth factors, pro-angiogenic molecules, pro-inflammatory mediators and tumor promoting inflammation-induced factors [Balkwill, Charles *et al.*, 2005; Lin and Karin, 2007; Mantovani, Allavena *et al.*, 2008; Porta, Larghi *et al.*, 2009; Kasinski and Slack, 2011; Charles, Holland *et al.*, 2012; Hanahan and Coussens, 2012; Elinav, Nowarski *et al.*, 2013]. Cytokines produced by innate immune and stromal cells have been shown to promote cancer initiation, development and progression (Figure 2.1).

Figure 2.1 : *Crosstalk between cancer cells and innate immune cells forms the highly enriched tumor microenvironment.* Cancer cells recruit macrophages, mast cells, NK cells, fibroblast cells and endothelial cells to the tumor tissue. Cancer cells also induce polarization of macrophages into tumor-associated macrophages by cytokines and chemokines release. Various pro-inflammatory and mitogenic growth factors secreted by infiltrating immune cells promote cancer cell growth, disease progression, extracellular matrix (ECM) remodeling and metastasis. Increased vasculature demand is supported by recruitment of endothelial cells. Abbreviations: symbol ⊢ shows inhibition and ← shows regulation.

2.2 THE NLR GENE FAMILY

NLRs are a newly discovered family of pattern recognition receptors [Ting, Lovering *et al.*, 2008]. NLRs are evolutionarily conserved from plants to animals showing high structural similarity with the R proteins [DeYoung and Innes, 2006]. NLRs recognize molecular patterns associated withPAMPs and DAMPs as well as irritants (Figure 2.2). These pattern recognition receptors are regulatory proteins of innate immunity as well as adaptive immunity [Takeuchi and Akira, 2010].

In humans, the NLR family consists of 22 individual genes with a characteristic tripartite domain structure comprising of an N-terminal domain, a central domain and a C-terminal domain [Ting and Davis, 2005] (Table 2.1). The N-terminal domain may be; a PYRIN domain (PYD), a caspase activation and recruitment domain (CARD), an acidic transactivation or a baculovirus inhibitor of apoptosis protein repeat (BIR) domain. These domains are responsible for homotypic/heterotypic interactions with downstream signaling molecules. The central domains of NLRs, consisting of a nucleotide binding and oligomerization domain (NBD) play crucial role in the conformational switch during receptor activation and subsequent oligomerization [Cai, Chen *et al.*, 2014; Lu, Magupalli *et al.*, 2014; Dick, Sborgi *et al.*, 2016]. The C-terminal domain represents a series of leucine rich repeats (LRRs) which are responsible for ligand binding [Ye and Ting, 2008].
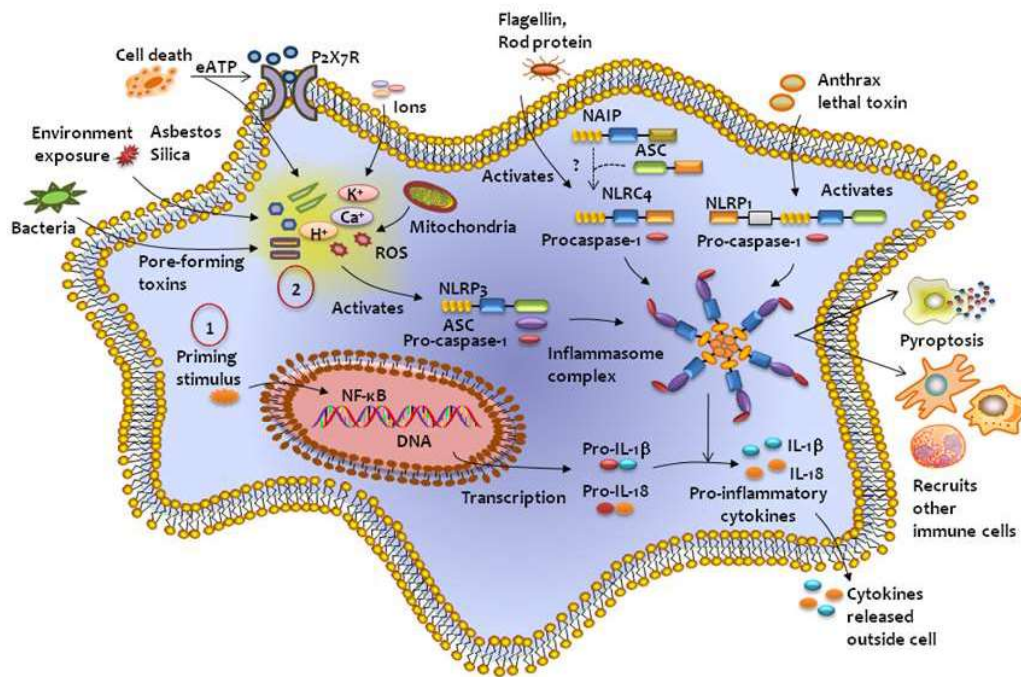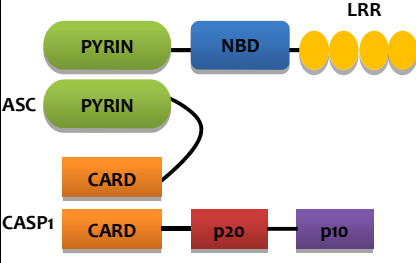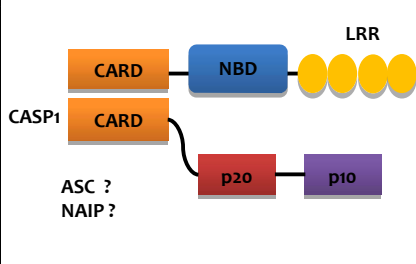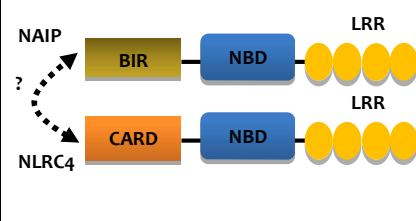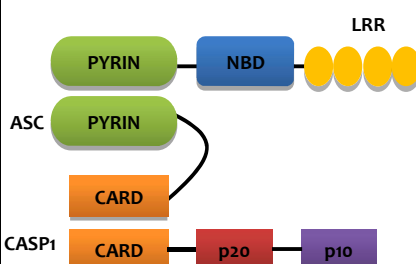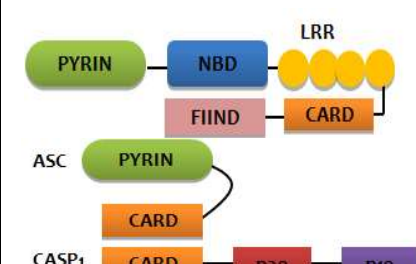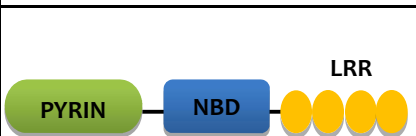
Figure 2.2 : *Inflammasome activation pathways.* NLRs are activated in response to specific PAMPs and DAMPs. NLRP3 inflammasome activation is a two-step process. The first step includes a priming stimulus for cell priming and second stimuli leads to NLRP3 activation. NLRP3 then along with ASC and pro-Caspase-1 forms inflammasome complex resulting in the maturation and release of pro-inflammatory cytokines, IL-1β and IL-18 into the extracellular environment, recruitment of other immune cells and pyroptosis (if required). Similarly, other NLRs like NLRP1, NLRC4 also form inflammasome complex upon activation. Though the activation mechanism doesnot require any priming stimulus, it is largely similar to that of NLRP3 inflammasome.

Table 2.1 : Ligand sensing mechanisms of inflammasome and non-inflammasome forming NLRs

Inflammasome and non-inflammasome forming NLRs	Structure	Function	Ligands	References
NLRP3		- Interacts with ASC and Caspase-1 - Activation of NF-κB pathway and release of IL-1β and IL-18	MDP, LPS, Bacterial and viral RNA/ DNA, silica, amyloid-β fibrils, extracellular ATP	[Guo, Callaway *et al.*, 2015]
NLRC4		- Interacts with Caspase-1 - Interaction with ASC and NAIP unclear - Elevates NF-κB signaling and IL-1β and IL-18 release	Bacterial flagellin, PrgJ, component of T3SS	[Zhao, Yang *et al.*; Latz, Xiao *et al.*, 2013]
NAIP		- NAIP/NLRC4 inflammasome complex	Cprl T3SS needle protein, Protects during *L. pneumophila* infection	[Vinzing, Eitel *et al.*, 2008]
NLRP6		- Inflammasome complex with ASC and Caspase-1 - NF-κB activation and IL-1β and IL-18 release	Alterated gut flora	[Anand, Malireddi *et al.*, 2012]
NLRP1		- Inflammasome complex with ASC and Caspase-1	Muramyl dipeptide, *Toxoplasma gondii* and *Bacillus anthracis* lethal toxin	[Chavarría-Smith and Vance, 2015]
NLRP12		- Inhibits IRAK1, TRAF3 and NIK - Attenuates canonical/ non-canonical NF-κB	Protects from *Yersinia pestis* and *S.typhimurium* infection	[Vladimer, Weng *et al.*; Zaki, Man *et al.*, 2013]

NLRX1		- Inhibits TRAF6 - Attenuates canonical NF-κB signaling	Viral RNA	[Allen, Jania *et al.*; Lei, Wen *et al.*, 2012]
NLRC3		- Inhibits TRAF6 - Attenuates canonical NF-κB signaling - Inhibits STING induced signaling	Affects LPS, intracellular DNA and c-di-GMP induced immune responses	[Zhang, Mo *et al.*; Schneider, Zimmermann *et al.*, 2012; Karki, Man *et al.*, 2016]
NOD1		- Interacts with RIP2, recruits RICK and CARD9	Bacterial iE-DAP containing peptidoglycan	[Chamaillard, Hashimoto *et al.*, 2003; da Silva Correia, Miranda *et al.*, 2006]
NOD2		- Recruits RIP2 and activates NF-κB and MAPK pathways. - Negative regulation by CARD8	Intracellular sensor for muramyl dipeptide	[Yang, Yin *et al.*, 2007; Hsu, Ali *et al.*, 2008]

2.3 NLRs IN CANCER

NLR family members are involved in multiple functions of innate immune signaling, from acting as activators of the inflammasome, nuclear factor-kB (NF-κB) and mitogen activated protein kinase (MAPK), to acting as inhibitors of inflammatory signaling, and trans-activators of MHC expression [Kufer and Sansonetti, 2011]. Dysregulation in NLR activation leads to various inflammatory diseases, metabolic disorders and auto-inflammatory immune disorders [Zhong, Kinio *et al.*, 2013]. In the past decade, there has been an exponential increase in research linking inflammasome signaling to human diseases. NLRs play dual pro- and anti-tumor roles in the initiation, progression and regression of cancer [Kent and Blander, 2014]. In this section, we discuss emerging research showing the association of NLRs with cancer and the prospective future implications in developing targeted cancer therapeutics (Table 2.2).

Table 2.2 : Beneficial and detrimental roles of NLR family members in cancer.

NLR	Synonyms	Cancer Types	Interactions	References
NAIP	BIRC1; NLRB1; psiNAIP	Oral squamous cell carcinoma (SCC), Breast cancer	- NAIP overexpression in SCC and breast cancer Increased NF-κB activation	[Choi, Hwang *et al.*, 2007; Mazrouei, Ziaei *et al.*, 2012]
		Colon cancer (CC)	- NAIP expression more in normal tissues in case of colon cancer	[Nemoto, Kitagawa *et al.*, 2004]
NLRP3	AII; AVP; FCU; MWS; FCAS; CIAS1; NALP3; C1orf7; CLR 1.1; Cryopyrin; CIAS1; PYPAF1; AGTAVPRL; NALP3	Hepatocellular carcinoma (HCC) Colorectal cancer	- Protective role in HCC and colitis associated CRC	[Allen, TeKippe *et al.*, 2010; Hu, Elinav *et al.*, 2010; Zaki, Boyd *et al.*, 2010; Zaki, Vogel *et al.*, 2010; Wei, Mu *et al.*, 2013; Oficjalska, Raverdeau *et al.*, 2015]
		Melanoma	- NLRP3 constitutively expressed Enhanced NF-κB activation and positive IL-1 feedback loop is formed	[Verma, Bivik *et al.*, 2012]
		Gastric cancer	- Promotes migration and tumor invasion	[Xu, Li *et al.*, 2013]
		Glioblastoma (GBM)	- Aberrant IL-1 expression in malignant glioma cells	[Fathima Hurmath, Ramaswamy *et al.*, 2014; Tarassishin, Lim *et al.*, 2014]
NLRP6	AVR; CLR 11.4; NALP6; NAVR; PYPAF5; NAVR/AVR; PAN3	Colitis-associated Colorectal cancer	- Negative regulator of colorectal tumorigenesis. Role in self-renewal of intestinal epithelium. Upregulation of SMARRC1 and Mycl1 (primarily expressed in epithelial cells) in *NLRP6* $^{-/-}$ mice.	[Martinon, Burns *et al.*, 2002; Chen, Liu *et al.*, 2011; Normand, Delanoye-Crespin *et al.*, 2011; Hu, Elinav *et al.*, 2013]
NLRP12	CLR 19.3; FCAS2; NALP12; RNO2; PYPAF7; PAN6; RNO; Monarch 1	Colitis-associated colorectal cancer	- Decreased inflammatory response by inhibition of NF-κB, ERK and STAT3 pathways. *Nlrp12* $^{-/-}$ mice are hypersusceptible to inflammation induced tumorigenesis	[Xiao and Ting; Zaki, Vogel *et al.*, 2011] [Allen, Wilson *et al.*, 2012]
NLRC4	CLR 2.1; CARD12; CLAN; CLAN1; CLANA; CLANB; CLANC; CLAND; Ipaf	Colitis-associated colorectal cancer	- Protective role of NLRC4 via Caspase-1 mediated inflammasome formation. Controls/ inhibits epithelial cell and tumor cell proliferation	[Hu, Elinav *et al.*, 2010]

NLRP1	NALP1; CARD7	Inflammatory bowel disease and colitis-associated cancer	- Protects against gastrointestinal inflammation and progression of colitis-induced tumorigenesis. Regulates inflammation through non-hematopoietic cell compartment.	[Girardelli, Maestri *et al.*, 2012; Williams, Leeth *et al.*, 2015]
NLRX1	CLR 11.3; NOD5; NOD9; NOD26; DLNB26	Colorectal cancer Urethane based model of tumorigenesis Colon cancer	- Regulates apoptosis in cancer cells - Attenuates tumorigenesis by negative regulation of AKT and NF-κB pathways. - Tumor suppression by sensitizing cells to TNF-α induced cell death via Caspase-8 regulation	[Xia, Cui *et al.*, 2011; Singh, Poteryakhina *et al.*, 2015] [Soares, Tattoli *et al.*, 2014] [Koblansky, Truax *et al.*, 2016]
NOD1	CARD4; CLR7.1; NLRC1;	Colon-associated tumorigenesis Gastric, breast, prostate, lung and other cancers	- Maintains intestinal epithelial barrier permeability and balanced inflammatory responses - Nod1 polymorphisms	[Zaki, 2013] [Kutikhin, 2011; Correa, Milutinovic *et al.*, 2012]
NOD2	CARD15;CLR16.3; NLRC2	Transmissible colitis and colorectal tumorigenesis Gastric, breast, prostate, lung and other cancers	- Nod2 deficiency induces dysbiosis - Nod2 mutations and polymorphisms	[Couturier-Maillard, Secher *et al.*, 2013] [Maeda, Hsu *et al.*, 2005; Kutikhin, 2011]
ASC	PYCARD; TMS; TMS1; CARD5; TMS-1	Hepatocellular carcinoma, Medulloblastoma, Breast, Ovarian and Colorectal cancer, Glioblastoma (GBM) Melanoma	- Hypermethylation of TMS1 gene promoter Hypermethylation of ASC highly correlated with late stage tumor Dual role in melanoma-Inhibit tumorigenesis in primary melanoma by inhibiting NF-κB and enhance NF-κB activation to induce tumorigenic pathways	[Levine, Stimson-Crider *et al.*, 2003; Stone, Bobo *et al.*, 2004; Machida, 2006; Knight, Patel *et al.*, 2015] [Liu, Luo *et al.*, 2012]

2.4 INFLAMMASOME FORMING NLRs

NLRs upon activation form a multimeric protein platform known as the "inflammasome" (Table 2.1) [Schroder and Tschopp, 2010]. The inflammasome complex consists of an NLR protein, an adaptor protein: ASC) and a caspase [Franchi, Eigenbrod *et al.,* 2009]. ASC utilizes its CARD domain to recruit the inactive pro form of Caspase-1 [Srinivasula, 2002]. Inflammasome activation results in auto-proteolytic cleavage of Caspase-1 to form the mature active Caspase-1 within the inflammasome complex (Figure 2.3). Caspase-1 can cleave and activate up to 70 different substrates including pro-IL-1β and pro-IL-18 into active IL-1β

and IL-18 [Shao, Yeretssian *et al.*, 2007; Keller, Rüegg *et al.*, 2008]. IL-1β and IL-18 function as proinflammatory cytokines and mediate activation of various signaling pathways including NF-κB, JNK and p38 MAPK signaling pathways. Activated immune cells release IL-1β and IL-18 giving rise to an inflammatory microenvironment (Figure 2.3). Inflammasome forming NLRs protect the host by modulating cytokine production, damage repair and resolution of inflammation.

2.4.1 NLRP3

NLRP3 is the most well studied NLR so far. The *Nlrp3* gene encodes an N-terminal pyrin domain, a central NBD domain and a C-terminal LRR. NLRP3 lacks a CARD domain and therefore, interacts with ASC to recruit Procaspase-1 (Table 2.1). NLRP3 inflammasome comprises of NLRP3, the adaptor protein ASC and pro-Caspase-1. NLRP3 inflammasome activation has been observed in response to a number of PAMPs, DAMPs and irritants [Schroder and Tschopp, 2010]. Mutation in human *Nlrp3* is associated with cold induced auto-inflammatory syndromes- FCAS (familial cold-induced auto-inflammatory syndrome), MWS (Muckle-Wells syndrome), and NOMID/CINCA (neonatal onset multisystem inflammatory disorder or chronic infantile neurologic cutaneous and articular syndrome) [Hoffman, Mueller *et al.*, 2001; Feldmann, Prieur *et al.*, 2002]. *Nlrp3* mutations and single-nucleotide polymorphisms (SNPs) have been associated with many inflammation-associated diseases [Conforti-Andreoni, Ricciardi-Castagnoli *et al.*, 2011]. Genetic alterations in genes encoding NLRP3 and CARD8 lead to complex inflammatory diseases such as; inflammatory bowel disease, cardiovascular disease, rheumatoid arthritis and type 1 diabetes [Verma, Lerm *et al.*, 2008; Paramel, Sirsjö *et al.*, 2015]. The role of NLRP3 inflammasome in metabolic diseases is well established [De Nardo and Latz, 2011]. Caspase recruitment domain-containing protein8 (CARD8; also known as TUCAN/CARDINAL) is a negative regulator of NLRP3 inflammasome activation [Ito, Hara *et al.*, 2014]. While some studies have also shown that CARD8 knockdown did not affect IL-1β protein expression release[Fransén, 2012]. Ito, *et al.*, showed that CARD8 can interact with Wild Type (WT) NLRP3 and reduce IL-1β release but not with CAPS associated mutant NLRP3. NLRP3 inflammasomes can also associate with Caspase-8 to mediate IL-1β production and apoptosis in Caspase-1-deficient dendritic cells and facilitate pyroptosis in wild-type dendritic cells [Antonopoulos, Russo *et al.*, 2015]. Regulation of T_H2 differentiation in CD4$^+$ T cells by NLRP3 expression shows specific inflammasome-independent role of NLRP3 mediated via STAT5 and IL-2 signaling pathway [Bruchard, Rebé *et al.*, 2015]. Further *in-vitro* analyses have confirmed specific involvement of NLRP3 in T_H2 and T_H17 polarization and nuclear localization of NLRP3 in T_H2 cells.

2.4.2 NLRP3 and Cancer

Very few studies have characterized the role of the NLRP3 inflammasome in cancer. Using a dextran sulfate sodium (DSS)-induced colitis model of human ulcerative colitis [Wirtz, Neufert *et al.*, 2007; Chassaing, Aitken *et al.*, 2014], Allen *et al.* first demonstrated the hypersusceptibility of *Nlrp3* and *Casp1* deficient mice to DSS-induced colitis. This chemically induced colitis model shares pathology similar to human ulcerative colitis. Defective inflammasome activation leads to loss of epithelial integrity, massive leukocyte infiltration and increased chemokine expression in *Nlrp3$^{-/-}$* and *Casp1$^{-/-}$* mice, leading to high mortality rates [Allen, TeKippe *et al.*, 2010]. These results were supported by parallel findings that showed NLRP3 inflammasome activation as a negative regulator of tumorigenesis during colitis-associated cancer [Zaki, Boyd *et al.*, 2010], with NLRP3 inflammasome dependent IL-18 production protecting against colorectal tumorigenesis. *Nlrp3*-deficient mice are highly susceptible to colitis-associated colorectal tumor formation and show severe inflammation, hyperplasia and tumor burden. Dysregulated IL-18 expression is followed by increased chemokine expression suggestive of higher macrophage infiltration in the colon of *Nlrp3* and *Casp1*-deficient mice. Macrophages release various tumorigenic factors in the tumor microenvironment, this is reflected in the significantly higher expression of COX-2 mRNA in colon tissue of *Nlrp3$^{-/-}$* and *Casp1$^{-/-}$* mice [Zaki, Vogel *et al.*, 2010]. Hu *et al.*, showed no differences in tumor formation between the *Nlrp3$^{-/-}$* and WT mice. Further experiments proved that

increased tumorigenesis in *Casp1-/-* mice, was not mediated by *Nlrp3*. Moreover, NLRP3 expression was primarily restricted to the hematopoietic compartment [Hu, Elinav *et al.*, 2010]. Surprisingly, study of DSS-induced ulcerative colitis in *Casp11-/-* mice indicate Caspase-11-mediated non-canonical inflammasome activation, an additional pathway for production and release of pro-inflammatory cytokines, IL-1β and IL-18 [Oficjalska, Raverdeau *et al.*, 2015]. *Casp11-/-* mice exhibit enhanced susceptibility towards DSS and impaired IL-18 production. *Casp11-/-* mice show decreased intestinal epithelial cell proliferation and disrupted epithelial cell barrier suggesting protective role of Caspase-11 in maintaining intestinal epithelial cell integrity. During acute DSS-induced intestinal inflammation, *Casp11-/-* mice show a phenotype similar to that of the *Casp1/11-/-* mice.

NLRP3 inflammasome plays regulatory role in the pathology of melanomas, gastric cancer and hepatocellular carcinoma; in addition NLRP3 inflammasome activation also regulates adaptive immune response to cancer vaccines (Table 2.2). The NLRP3 inflammasome is constitutively expressed and activated in human melanoma cells [Verma, Bivik *et al.*, 2012]. Paracrine IL-1 signaling via secreted IL-1β enhances NF-κB-dependent pro-IL-1β synthesis, thereby forming a positive IL-1 feedback loop in metastatic melanoma cells. In case of gastric cancer, *Mycoplasma hyorhinis* promotes tumor development via NLRP3 inflammasome activation, accompanied with increase in cell migration and invasion [Xu, Li *et al.*, 2013]. In this regard, NLRP3 activation was not cell type restricted and there was increased IL-1β and IL-18 production in *M. hyorhinis* infected human monocytic cells. IL-1β but not IL-18 was found to be involved in gastric cancer cell migration and invasion.

Hepatocellular carcinoma (HCC) is the third leading cause of cancer deaths worldwide. The expression of NLRP3 inflammasome components is down-regulated in hepatic parenchymal cells in HCC. Complete loss of NLRP3 inflammasome activation positively correlates with a higher HCC pathological grade suggesting protective role of NLRP3 against HCC cancer development and progression [Wei, Mu *et al.*, 2013]. NLRP3 inflammasome is activated during anticancer chemotherapy (oxaliplatin). Autophagic tumor (EL4 or EG7 thymoma) cells release ATP sensed by the P_2X_7 receptors of dendritic cells leading to NLRP3 inflammasome activation [Ghiringhelli, Apetoh *et al.*, 2009]. Antigen presenting dendritic cells prime CD8+ T lymphocytes generating IFN-γ-dependent antitumor responses. Functional P_2X_7 receptor dependent activation of NLRP3 inflammasome and IL-1β secretion, are necessary for T-cell priming. Notably, this study conflicts with recent results that NLRP3 dampens anti-tumor responses elicited by dendritic cells vaccination. NLRP3 expression was found to be upregulated in tumor-associated myeloid derived suppressor cells (MDSCs). *Nlrp3-/-* mice had fewer MDSCs accumulating at the tumor site and increased survival rate upon DC vaccination [van Deventer, Burgents *et al.*, 2010]. Increased survival curves were obtained for both metastatic melanoma (B16-F10) and lymphoma (EG7-OVA) tumor cells. *In-vitro* analysis showed that *Nlrp3-/-* MDSCs are morphologically and functionally equivalent to the WT MDSCs. However, the WT dendritic cells generated significant amount of IL-12. In summary, NLRP3 expression in the presence of IL-12, dampens antitumor immunity and reduces the efficacy of dendritic cell vaccine by excessive infiltration of MDSCs to the tumor site. Both groups focused on different immune cells associated with adaptive immunity and therefore differences in findings may be attributed to difference in tumor cell types, stimuli for NLRP3 inflammasome activation and experimental protocols.

Several studies have implicated increased IL-1β secretion in the tumor microenvironment promoting inflammation, early angiogenic response, tumor induction and progression [Carmi, Dotan *et al.*, 2013; Tarassishin, Casper *et al.*, 2014]. In a study utilizing U87, U251 and patient derived- GBM cell lines, IL-1 acted as a primary signal stimulating IL-1β expression and NLRP3 inflammasome activation. IL-1 induced glioma cells showed activated STAT3 expression, increased angiogenesis and release of neurotoxic substances leading to tumor progression [Tarassishin, Casper *et al.*, 2014]. IL-1β microenvironment significantly increased migration and invasion in the human glioma U87MG and U251MG cell lines [Fathima Hurmath, Ramaswamy *et al.*, 2014]. These findings need validation in mouse models and patient data; however, they suggest possible novel strategies to increase the anti-tumor activity

of cancer drugs and vaccines by strategic modulation of either inflammasome components or their products, such as IL-1β and IL-18.

In summary, the contrasting roles of NLRP3 in cancer, hints at the complexity of function of the NLRP3 inflammasome, its multi-faceted functions and regulation of inflammatory pathways across multiple stages of carcinogenesis.

2.4.3 NLRC4

The *Nlrc4* gene encodes an N-terminal CARD domain, a central NACHT domain, and C-terminal LRRs containing protein (Table 2.1). *Nlrc4* is predominantly expressed in hematopoietic tissues and cells [Poyet, 2001]. Recently reported crystal structure of mouse NLRC4 revealed an auto inhibitory mechanism of NLRC4 wherein the LRR sequesters mNLRC4 in a monomeric state. Disruption in the ADP-mediated NBD-WHD (winged-helix domain) or NBD-HD2/NBD-LRR interactions resulted in constitutive activation of NLRC4 [Hu, Yan *et al.*, 2013].The LRR domain of NLRC4 interacts directly with the chaperone heat-shock protein HSP90, and an ubiquitin ligase-associated protein SGT1 to form an inhibited but receptive oligomeric state. The interaction leads to LRR deletion, resulting in NLRC4 activation [Mayor, Martinon *et al.*, 2007]. Phosphorylation of NLRC4 at a single, evolutionarily conserved residue, Ser 533, by PKCδ kinase is required for NLRC4 inflammasome assembly formation [Qu, Misaghi *et al.*, 2012]. NLRC4 inflammasome is activated in response to Gram-negative bacteria such as *Salmonella typhimurium*, *Shigella flexneri*, *Legionella pneumophila* and *Pseudomonas aeruginosa* [Lage, Longo *et al.*, 2014]. Activation in response to intracellular bacteria is critical for Caspase-1 mediated pyroptosis as well as maturation and extracellular release of IL-1β and IL-18 [Miao, Leaf *et al.*, 2010]. NLRC4 senses cytoplasmic flagellin and bacterial type III secretion apparatus [Zhao, Yang *et al.*, 2011]. NLRC4 inflammasome activation in response to *S.flexneri* requires bacterial type III secretion system and is independent of flagellin [Suzuki, Franchi *et al.*, 2007]. Both NLRP3 and NLRC4 inflammasome activation occur in response to *S.typhimurium* infection [Broz, Newton *et al.*, 2010]. Inflammasome activation in macrophages infected with *S.typhimurium* causes recruitment of both NLRC4 and NLRP3 to the same inflammasome complex. ASC is present in the outer ring structure while NLRs and caspases remain internally localized. NLRP3, NLRC4, Caspase-1 and Caspase-8 are present in the same ASC speck for robust IL-1β production [Man, Hopkins *et al.*, 2014]. NLRC4 dependent IL-1β production helps in discriminating pathogenic bacteria from commensal bacteria. Therefore, NLRC4 provides protection against enteric pathogens in intestinal epithelial cells [Nordlander, Pott *et al.*, 2013].

2.4.4 NLRC4 and Cancer

Azoxymethane (AOM)-DSS is a model for inflammation induced colorectal cancer (CAC) [Wirtz, Neufert *et al.*, 2007]. This model has been used for studying function of inflammasomes in colitis and CAC. AOM (a potent carcinogen) and DSS (chronic colitis inducing agent) are systemically administered to induce inflammation-associated colon tumorigenesis in mice. Allen *et al.*, found that *Nlrc4-/-* mice showed no significant difference in tumorigenesis as compared to similarly treated WT mice. NLRP3 expression and function in hematopoietic cells but not in intestinal epithelial or stromal cells is responsible for protection against increased tumorigenesis [Allen, TeKippe *et al.*, 2010]. In contrast, Hu *et al.*, showed that regulation of inflammation-induced tumorigenesis is mediated by NLRC4 and Caspase-1 [Hu, Elinav *et al.*, 2010]. No difference in inflammation between WT and *Casp1-/-* mice, was observed during acute DSS-colitis. In the AOM-DSS inflammation-induced colorectal cancer model, *Casp1-/-* and *Nlrc4-/-* mice exhibited increased tumor load and tumor number per mice. In addition, enhanced colon epithelial and tumor cell proliferation was observed in the *Casp1-/-* mice. Caspase-1 and NLRC4 were relatively highly expressed in both colonic epithelial cells and CD45+ hematopoietic cells in the colon. These results conclude that an intrinsic epithelial cell effect exacerbates tumorigenesis in the absence of Caspase-1 or NLRC4 activity. While results from both the groups are conflicting and present contradictory findings, the differences may have arisen from the experiment models, colon tissue and protocols resulting in differences in susceptibility to infection and epithelial integrity. The most significant conclusion from these

studies is the protective role of NLRs against increased tumorigenesis in colitis and colitis-associated cancer.

2.4.5 NAIP/BIRC1/NLRB1

NAIPs are a family of NLRs with seven paralogs in mice (NAIP1–7) and one member in humans (*hNaip*). The human *Naip* gene encodes the Neuronal apoptosis and inhibitor protein (NAIP) also termed as BIRC1 baculoviral IAP repeat-containing protein. NAIP consists of 3 domains; an N-terminal BIR domain, a central NACHT domain and a C-terminal LRR domain (Table 2.1). NAIP lacks the RING zinc finger domain present in other baculovirus and human IAPs. In 1995, Roy *et al.*, first reported partial deletion of *Naip* gene in patients with spinal muscular atrophy (SMA) [Roy, Mahadevan *et al.*, 1995]. Years later, deletion in *Naip* gene was positively correlated with the clinical severity of spinal muscular atrophy patients [Watihayati, Fatemeh *et al.*, 2009]. NAIP protein is expressed in brain, placenta, kidney, spleen, and heart tissues. NAIP expression is highest in the placenta and lowest in the heart. NAIP expression is also found in peripheral blood mononuclear cells while differentiating into macrophages [Maier, Balabanian *et al.*, 2007]. NAIP mediates suppression of apoptosis and increased cell survival in the Chinese hamster ovary (CHO) cell line after serum deprivation or menadione induced oxidative injury [Listen, Roy *et al.*, 1996]. *Naip* gene expression inhibits caspase-3 and caspase-7 mediated apoptotic pathways [Maier, Lahoua *et al.*, 2002]. NAIP directly interacts via its BIR3 domain with procaspase-9. Association of NAIP with procaspase-9 prevents the autoproteolysis of caspase-9 in the apoptosome complex, putting a brake to caspase-9-mediated cell death pathways [Davoodi, Ghahremani *et al.*, 2010]. Integrity of both NOD (nucleotide-binding oligomerization domain) and BIR domains of *Naip* gene are necessary for effective inhibition of procaspase-9 auto-proteolysis. NAIP also forms NOD-NOD domain interactions with the APAF-1 and BIR3 mediated interaction with procaspase-9 [Karimpour, Davoodi *et al.*, 2011]. *hNaip* gene is involved in bacterial sensing and inducing pyroptosis in human macrophages and epithelial cells [Vinzing, Eitel *et al.*, 2008]. NLRC4 recognizes the T3SS needle protein with the help of hNAIP and forms hNAIP-NLRC4 inflammasome complex [Yang, Zhao *et al.*, 2013].

In the brain, NAIP interaction with hippocalcin mediates protection to neurons from calcium induced cell death via caspase-3-dependent and -independent pathways[Mercer, Korhonen *et al.*, 2000]. Upregulation of *Naip* expression at 6 hours post traumatic brain injury inhibits apoptosis of neurons and oligodendrocytes. While 24 hours post- traumatic brain injury, NAIP and procaspase-3 expression is decreased and PARP1 (poly(ADP-ribose) polymerase) cleavage increases, leading to maximum apoptosis [Hutchison, Derrane *et al.*, 2001]. NAIP plays critical role in generating innate immune inflammatory response via Caspase-1, 4, 5 activation [Martinon and Tschopp, 2006].

2.4.6 NAIP and Cancer

Cancer triggers molecular events that result in inhibition of apoptotic pathways [Bai and Wang, 2014]. Many anti-apoptotic proteins are overexpressed during cancer [Evan and Vousden, 2001]. Upregulation of these proteins results in increased tumor cell survival and tumor progression. NAIP expression is significantly elevated in malignantly transformed oral squamous cell carcinomas as compared to the corresponding oral potentially malignant disorders. In support of this data, *Naip* allele was found to be methylated in normal oral mucosa tissues suggesting, *Naip* expression could be one of the early events in carcinogenesis [Chen, Huse *et al.*, 2010]. *Naip* is not expressed in normal breast tissue but is expressed at high levels in breast cancer with other unfavorable clinical features such as tumor stage and size [Choi, Hwang *et al.*, 2007]. These results offer strong possibility of using NAIP as a biomarker for early diagnosis of squamous cell carcinomas, breast cancer and other malignancies. These studies need to be replicated with large data sets. Mazrouei *et al.*, observed no significant change in NAIP expression in lymphoma tissues and controls. NAIP expression is significantly different in reactive lymphoid hyperplasia (RLH) lymph nodes and normal lymph nodes indicating that *Naip* function is not restricted to apoptosis regulation but is actively involved in inflammatory responses as well [Mazrouei, Ziaei *et al.*, 2012]. Overexpression of IAP family members

excluding NAIP has been detected in prostate cancer [Krajewska, Krajewski *et al.*, 2003]. Later, Chiu *et al.*, observed increased NAIP expression in response to androgen deprivation in prostate cancer [Chiu, Yong *et al.*, 2010]. A link between increased NF-kB DNA-binding activity and *Naip* expression was observed but the mechanism behind NF-kB activation and increased *Naip* transcription remains unclear. In well differentiated adenocarcinomas younger patients exhibit higher NAIP expression in comparison to elderly patients [Endo, Abe *et al.*, 2004]. Expression was similar for both age-groups in case of normal colonic mucosa. NAIP expression was reported to be significantly more in normal tissues than in well differentiated adenocarcinoma, however, the small sample size and relatively broad age group demand further exploration in this direction. Stronger NAIP expression has been seen in esophageal cancer, though the results have not been statistically significant [Nemoto, Kitagawa *et al.*, 2004]. This may be related to the number of tissue samples available for the study.

2.4.7 NLRP6

NLRP6 consists of an N-terminal PYRIN , a central NBD and C-terminal LRRs domain as shown in Table 2.1 [Grenier, Wang *et al.*, 2002]. *Nlrp6* is highly expressed in the intestine and helps in maintaining intestinal homeostasis by regulating gut microflora [Chen, 2014]. *Nlrp6* deficient mice are highly resistant to infection with the bacterial pathogens *Listeria monocytogenes*, *S.typhimurium* and *Escherichia coli*. NLRP6 acts as negative regulator of canonical NF-κB and MAPK- dependent inflammatory signaling. Increased release of canonical NF-κB and MAPK-dependent cytokines and chemokines was found in *Nlrp6*^{-/-} mice. Both haematopoietic and non-haematopoietic cells contribute to NLRP6-mediated inhibition of bacterial clearance [Anand, Malireddi *et al.*, 2012]. NLRP6 inflammasome deficiency leads to altered colonic microbiota and increased risk for colitis occurrence. NLRP6 is recruited to the "specks" formed by ASC oligomerization present in the cytoplasm, leading to Procaspase-1 activation and release of active IL-1β and IL-18. Decreased IL-18 levels were present in colonic epithelial cells of *Nlrp6*-deficient mice, along with characteristics of spontaneous intestinal hyperplasia, inflammatory cell recruitment and exacerbation of DSS-induced colitis via induction of CCL5 chemokine release. Bacterial components were found to be responsible for transferrable colitis phenotype in the *Nlrp6*-deficient mice [Elinav, Strowig *et al.*, 2011].

2.4.8 NLRP6 and Cancer

The association of NLRP6 with colon cancer was established in 2011, when Chen *et al.* reported Nlrp6 deficient mice were more susceptible to DSS- induced colitis and colitis associated colon tumorigenesis as compared to WT controls. *Nlrp6*-deficient mice were unable to efficiently resolve inflammation and repair damaged tissue, resulting in excessive epithelial tissue proliferation and tumor induction [Chen, Liu *et al.*, 2011]. Nlrp6 function in hematopoietic-derived cells is significantly important in conferring protection against colitis-induced tumorigenesis. IL-18 serum levels are decreased in *Nlrp6*-deficient mice both before and after DSS-treatment. Although, there is an increase in other pro-inflammatory mediators (MIP2, TNF- α, IL-6, IL-1β, IFN-ɣ) in *Nlrp6*-deficient mice [Normand, Delanoye-Crespin *et al.*, 2011]. NLRP6 controls epithelial self-renewal and colorectal carcinogenesis upon injury (DSS-induced colitis). There is enhanced colitis-associated tumor growth in the *Nlrp6*-deficient mice. Lack of NLRP6 inflammasome renders injury-induced mice prone to developing relapsing colitis. Taken together, all these results provide evidence for the protective role of NLRP6 in regulation and maintenance of intestinal inflammation [Hu, Elinav *et al.*, 2013]. Recently, role of aberrant inflammasome induced microbiota in colorectal cancer development was investigated. In *Asc*^{-/-} mice, there was enhanced tendency for inflammation induced tumorigenesis, driven by their altered microflora. As expected, microflora transferred from the *Nlrp6*^{-/-} mice played critical role in the enhanced susceptibility to inflammation-induced tumorigenesis. Further experimental analysis revealed enhanced tumorigenesis in co-housed WT mice was driven by the microbiota, mediated by IL-18 and CCL5. Colitogenic microflora-induced NLRP6 inflammasome promotes excessive epithelial cell proliferation via IL-6 signaling in transmissible cancer [Martinon, Burns *et al.*, 2002]. These studies reveal the critically important

role of NLRP6 in maintaining the gut microflora homeostasis (Table 1.2). Notably, NLRP6 regulates specific host immune response towards different microflora components.

2.4.9 NLRP1

NLRP1 was the first member of the NLR family to be characterized with inflammasome assembly and Caspase-1 activation [Martinon, Burns *et al.*, 2002]. NLRP1 is a sensor for muramyl dipeptide, *Toxoplasma gondii* and *Bacillus anthracis* lethal toxin [Hsu, Ali *et al.*, 2008; Chavarría-Smith and Vance, 2013; Ewald, Chavarria-Smith *et al.*, 2014; Chavarría-Smith and Vance, 2015]. NLRP1 consists of both PYRIN and CARD domains along with the function to find domain (FIIND domain). Unlike other NLRs, NLRP1 directly recruits Procaspase-1 through CARD. The auto-proteolytic processing within the FIIND/ZU5-UPA domains is essential for NLRP1 inflammasome activation [Finger, Lich *et al.*, 2012]. The anti-apoptotic proteins Bcl-2 or Bcl-XL and HHV8 are cellular and viral regulators of NLRP1 that bind and suppress NLRP1 activation [Bruey, Bruey-Sedano *et al.*, 2007; Gregory, Davis *et al.*, 2011]. NLRP1 functions include forming inflammasome complex with ASC and Caspase-1, binding to the patched receptor via DRAL adaptor protein and regulating caspase-9 mediated apoptosis by binding to Apaf-1 [Faustin, Lartigue *et al.*, 2007; Mille, Thibert *et al.*, 2009]. NLRP1 polymorphisms have been associated with various pathologies like vitiligo, rheumatoid arthritis, Crohn's disease, Alzheimer's and melanoma [Pontillo, Catamo *et al.*, 2012; Sui, Li *et al.*, 2012; Verma, Bivik *et al.*, 2012]. *In-vitro* analyses show increased NLRP1-mediated Caspase-1-dependent pyroptosis in cortical neurons in response to amyloid-β in models of Alzheimer's disease [Tan, Tan *et al.*, 2014]. *Nlrp1* and *Caspase-1*-deficient mice exhibit significantly reduced neuronal pyroptosis and reversed cognitive impairments. The neuronal NLRP1 inflammasome consists of Caspase-1, Caspase-11, NLRP1, ASC, the inhibitor of apoptosis protein XIAP, and pannexin-1 [de Rivero Vaccari, Lotocki *et al.*, 2009]. Therapeutic neutralization of ASC interferes with NLRP1 inflammasome signaling and significantly reduces Caspase-1 activation. Therefore, NLRP1 is an important part of innate immunity in the brain reducing damage caused by post-traumatic brain inflammation. NLRP1 also acts as a cellular sentinel to activate Caspase-1 in response to haematopoietic and infectious stress [Masters, Gerlic *et al.*, 2012]. NLRP1a generates Caspase-1-containing inflammasome *in-vivo*, independently of Caspase-11 and ASC. The NLRP1 inflammasome formation drives IL-1β-dependent inflammatory disease, which is negatively regulated by IL-18. NLRP1a-induced pyroptosis was observed in the haematopoietic progenitor cells, leading to leucopenia and anemia. Kovarova *et al.*, have shown that NLRP1 inflammasome-induced pyroptosis, mediated by Caspase-1 causes acute lung injury and morbidity in mice [Kovarova, Hesker *et al.*].

2.4.10 NLRP1 and Cancer

NLR polymorphisms have been investigated for association with certain cancers. NLRP1 allele was found to be significantly more frequent in malignant mesothelioma suggesting NLRP1 as a novel factor possibly involved in the development of mesothelioma [Girardelli, Maestri *et al.*, 2012]. Williams *et al.*, have shown significant dysregulation of NLRP1, inflammasome forming NLR in inflammatory bowel disease and colitis-associated cancer [Williams, Leeth *et al.*, 2015]. *Nlrp1b⁻/⁻* mice models of experimental colitis were studied using DSS and AOM-DSS-mediated inflammation induced tumorigenesis. NLRP1 inflammasome attenuated gastrointestinal inflammation and progression of colitis-induced tumorigenesis. Enhanced tumorigenesis observed in the *Nlrp1b⁻/⁻* mice is correlated with the attenuated levels of IL-1β and IL-18 in the colon. The *Nlrp1b⁻/⁻* mice exhibited increased cell death, epithelial barrier disruption and disease severity. Bone marrow reconstitution experiments demonstrated association of NLRP1 inflammasome with the non-hematopoietic cells to generate host immune response in case of inflammatory bowel disease and cancer. The regulatory role of NLRP1 in colon cancer is consistent with the view that multiple NLR inflammasomes are involved in maintaining disease pathology and intestinal homeostasis.

2.5 NLR-LIKE INFLAMMASOME FORMING PROTEINS

2.5.1 AIM2

AIM2 (absent in melanoma 2) was originally identified while screening for tumor suppressor genes associated with melanoma [DeYoung, Ray *et al.*, 1997]. AIM2- an interferon-inducible gene also known as PYHIN4, is an NLR-like gene encoding cytosolic protein. The AIM2 protein consists of a N-terminal PYD, mediating homotypic interactions with ASC and a C-terminal HIN-200 domain for DNA binding [Davis, Wen *et al.*]. AIM2 recognizes cytoplasmic DNA and forms specific AIM2/ASC specks, suggesting a novel inflammasome platform inducing activation of ASC-mediated apoptotic and pyroptotic cell death pathways [Hornung, Ablasser *et al.*, 2009; Warren, Armstrong *et al.*, 2010; Sagulenko, Thygesen *et al.*, 2013]. The AIM2 pyrin domain drives filament formation and dsDNA binding, the key signal transduction mechanism responsible for the AIM2 inflammasome formation [Morrone, Matyszewski *et al.*, 2015]. Upon activation, AIM2 triggers innate immunity and provides host defense against both bacterial and viral pathogens, such as *Francisella tularensis, Listeria monocytogenes* and *Mycobacterium tuberculosis* infection [Fernandes-Alnemri, Yu *et al.*, 2010; Rathinam, Jiang *et al.*, 2010; Sauer, Witte *et al.*, 2010; Pierini, Juruj *et al.*, 2012; Saiga, Kitada *et al.*, 2012]. Mitochondrial reactive oxygen species (ROS) production, the transcription factor IRF1 and guanylate-binding proteins (GBPs) target AIM2 inflammasome activation by *Francisella tularensis* infection [Crane, Bauler *et al.*, 2014; Man, Karki *et al.*, 2015]. Innate immune signaling in response to *Porphyromonas gingivalis*-induced periodontitis, hemazoin and DNA during malaria involves activation of both NLRP3 and AIM2 inflammasomes and release of IL-1β and IL-18 cytokines and pyroptotic cell death [Kalantari, DeOliveira *et al.*, 2014; Park, Na *et al.*, 2014]. IFN-inducible IFI16 along with AIM2 acts as innate immune sensor for cytosolic double-stranded DNA in human fibroblasts [Duan, Ponomareva *et al.*, 2011]. Nuclear factor E2-related Factor-2 (Nrf2), a key transcription factor for cellular redox homeostasis regulation was found to be an essential mediator for NLRP3 and AIM2 inflammasome signaling in Myd88-dependent manner [Zhao, Gillette *et al.*, 2014]. The crystal structure of AIM2 PYD reveals a death domain fold with a short α3 helix that is buttressed by a highly conserved lysine residue at the α2 helix, stabilizing the α3 helix for potential interaction with partner domains. The structural characterization of AIM2 has provided novel insights into the PYD-HIN and PYD-PYD interactions important for AIM2 auto inhibition and inflammasome assembly [Jin, Perry *et al.*, 2013]. The microtubule-associated protein EB1 links AIM2 inflammasome activation and non-classical secretion of IL-1β induced by LC3-dependent autophagy [Wang, Huang *et al.*, 2014].

Recently, the role of AIM2 inflammasome was explored in the brain. AIM2 inflammasome mediated regulation was observed during acute *Staphylococcus aureus*-induced CNS inflammation [Hanamsagar, Aldrich *et al.*, 2014]. Distinct survival patterns and susceptibility to infection observed in the *Aim2-/-* mice, explains critical role of AIM2 in immune regulation of CNS during bacterial infection. Significantly reduced expression of IL-1β and other key inflammatory mediators, including IL-6, CXCL1, CXCL10, and CCL2 in the CNS of *Aim2-/-* and *Asc-/-* mice, implicates autocrine/paracrine actions of IL-1β in CNS inflammation. Both AIM2 and NLRC4 inflammasomes contribute in acute brain injury independently of NLRP [Denes, Coutts *et al.*, 2015]. In fact, the *Aim2-/-* and *Nlrc4-/-* mice show reduced injury and improved behavioral outcomes. The study suggests novel role of inflammasomes in brain injury, especially AIM2 and NLRC4 as key drivers for sterile inflammation responses in the brain.

2.5.2 AIM2 and Cancer

AIM2 plays a protective role against colorectal cancer progression. The lack of AIM2 or its reduced expression was positively correlated with poor survival in colorectal cancer patients [Dihlmann, Tao *et al.*, 2014]. AIM2 decreases the likelihood of colorectal cancer development by tight regulation of tumorigenic susceptibility and intestinal stem cell proliferation independently of inflammasomes [Man, Zhu *et al.*, 2015]. AIM2 interacts with and limits the activation of DNA-dependent protein kinase (DNA-PK), a PI3K-family member, to reduce Akt

phosphorylation for suppression of colon tumorigenesis [Wilson, Petrucelli *et al.*, 2015]. Therefore, AIM2 limits tumor burden and cancer progression in an inflammasome-independent fashion. The protective role of AIM2 has been identified in breast and colon cancer. AIM2 suppresses breast cancer cell growth *in-vitro* (MCF-7 cell line) and tumor formation *in-vivo*. Subsequently, AIM2 also suppresses the viability and tumorigenicity of breast cancer cells. *in-vivo* experiments show AIM2-mediated inhibition of breast cancer cell proliferation by suppression of NF-κB transcriptional activity and desensitizing TNF-α-mediated NF-κB activation [Chen, 2006]. Further, *in-vitro* analysis observed restoration of AIM2 induces G2/M arrest causing reduction in cell proliferation and confers invasive phenotype in colon cancer cells [Patsos, Germann *et al.*, 2009]. The constitutive levels of interferon- inducible AIM2 mRNA and protein were significantly lower in prostate cancer cells as compared to the benign prostate hyperplasia cells [Ponomareva, Liu *et al.*, 2013]. The microarray based gene expression profiling of AIM2-responsive target genes suggests novel role of interferon/AIM2/Interferon-stimulated genes cascade in colorectal tumors. Several interferon-stimulated genes including, TLR3 and CIITA were found to be significantly upregulated. AIM2 activation resulted in activation of both IFN-γ independent and dependent interferon stimulated genes. The authors conclude that the recently identified role of AIM2 in inflammasome-mediated cell death was not observed here in colorectal cancer cells [Lee, Li *et al.*, 2012]. Gene expression profiling by Liu et al, presents both NLRP3 and AIM2 as potential biomarkers for colorectal cancer and cancer progression [Liu, Truax *et al.*, 2015]. Extensive analysis of multiple cancer datasets revealed significantly decreased expression levels of NLRs - NLRP1, NLRP3, NLRC3, NLRC4 and AIM2 in human colorectal cancer. Together, these results link AIM2 with tumor suppression activity, and keeping in mind the well-recognized roles of NLRs in tumorigenesis, AIM2 could also function as a double-edged sword.

2.5.3 ASC/TMS1/PYCARD

ASC also known as TMS1 (target of methylation-induced silencing1) is an adaptor molecule that mediates inflammatory and apoptotic signaling. ASC contains the PYRIN/PAAD death domain in addition to the CARD protein-protein interaction domain (Table 2.1) [Hasegawa, Imamura *et al.*, 2009]. *Asc* gene is predominantly expressed in monocytes and mucosal epithelial cells. The functions of ASC include; regulation of Procaspase-1 activation, maturation of the cytokines- IL-1β and IL-18 and activation of NF-kB [Stehlik, Lee *et al.*, 2003]. Members of NLR family including NLRP1, NLRP3 and NLRC4, and the adaptor protein, ASC are critical components of the inflammasome that results in Procaspase-1 activation [Franchi, Eigenbrod *et al.*, 2009]. Later, inflammasome mediated cell death activity was linked with Caspase-8.Co-expression of NLRC4 with ASC induces NF-kB activation and apoptosis mediated by IkB kinase (IKK) and Caspase-8, respectively[Masumoto, Dowds *et al.*, 2003]. ASC is central to several death pathways including p53–Bax mitochondrial apoptosis pathway, NF-kB and Caspase-8-dependent apoptosis [Ohtsuka, Ryu *et al.*, 2004]. ASC directs NF-kB activation by regulating receptor interacting protein-2 (RIP2) Caspase-1 interactions [Sarkar, Duncan *et al.*, 2006]. AIM2 and NLRP3 inflammasomes recruit and activate Caspase-8 and -1 via ASC leading to both apoptotic and pyroptotic cell death [Sagulenko, Thygesen *et al.*, 2013]. Though, *in-vitro* studies suggest an axis between ASC/NLRC4/NF-κB activation, these findings require confirmation *in vivo*.

2.5.4 ASC and Cancer

Asc gene is overexpressed in several tumors, triggering apoptosis and formation of ASC specks from its intra-nuclear localization to punctate cytosolic structures. Recent studies have proved methylation-associated silencing of *Asc* across many cancer types [McConnell and Vertino, 2004]. However, the epigenetic mechanisms underlying regulation of *Asc* silencing or overexpression remains unknown. The aberrant methylation of *Asc* and gene silencing was first reported in human breast cancer cells. *Asc* is inactivated in almost 40% of the breast cancers [Levine, Stimson-Crider *et al.*, 2003]. Supporting evidence came from a study reporting *Asc* methylation in colorectal cancer tissues and cell lines lacking ASC protein expression [Yokoyama, Sagara *et al.*, 2003]. Later on, aberrant methylation was also well correlated with

loss of ASC expression in ovarian cancer. Methylation-mediated *Asc* silencing in tumor cells provides an extended survival and escape to apoptosis. In addition to methylation, histone deacetylation is also responsible for *Asc* gene silencing in ovarian cancer [Terasawa, 2004]. Machida *et al.*, found hypermethylation of *Asc* as a marker for late lung cancer cells and in sputum could predict prognosis in patients resected for early-stage disease [Machida, 2006]. Though, the ASC protein levels were reduced in all lung cancer types, the hypermethylation particularly correlated with late tumor stages being present in 60% of late-stage tumors. Methylation of *Asc* gene promoter is both a frequent and early event in prostate cancer carcinogenesis and is associated with the aggressive prostate cancer [Das, Ramachandran *et al.*, 2006]. Recent study of *Asc* methylation in glioblastoma shows reduced or absent expression of ASC in human glioblastoma cell lines and loss in expression was associated with aberrant methylation of CpG Island in the promoter of the *Asc* gene. Further analysis showed inverse correlation between the degree of methylation and level of ASC expression [Stone, Bobo *et al.*, 2004]. Another research group found hypermethylation of *Asc* gene promoter in 57.1% of long term survival glioblastoma, distinguishing it from the classic GBM having 16% of the cases with hypermethylation [Martinez, Schackert *et al.*, 2006]. Similarly, Zhang *et al.*, observed epigenetic inactivation of *Asc* in hepatocellular carcinoma cells. Here, *Asc* inactivation is regulated by promoter hypermethylation associated gene silencing, accompanied with histone H3 Lysine 9 (H3K9) hypoacetylation and trimethylation modifications in a coordinated way [Zhang, Li *et al.*, 2007]. The dual role of ASC in human melanoma tumorigenesis was identified by Liu *et al.* group. ASC expression in metastatic melanoma was found to be down-regulated as compared to primary melanoma [Liu, Luo *et al.*, 2012]. As expected, silencing of *Asc* in metastatic melanoma resulted in reduced cell viability and suppressed tumorigenesis. *Asc* knockdown resulted in inhibition of Caspase-1 activation and IL-1β secretion in both primary and metastatic melanoma. It also resulted in activation/suppression of phosphorylated IkB kinase (IKK) α/β expression and NF-kB activity in metastatic/primary melanoma. *Asc* was also found to be highly expressed in mouse models of medulloblastoma. *Asc* deficiency profoundly reduced proliferation and extended survival rate. ASC plays the role of tumor promoter in medulloblastoma [Knight, Patel *et al.*, 2014]. Interestingly, ASC has always been identified as a tumor suppressor in specific cancer models. These results reveal complex role played by ASC in regulating cell proliferation. Based on these findings, ASC represents a potential modulator of inflammatory responses that may help in coordinating the activity of NLRs and cytokine activating caspases in mammalian cells. DNA hypermethylated gene promoter sequences have always been extremely promising cancer markers. Certainly, they can be used for early diagnosis or prognosis depending on the change in gene expression during tumor induction and progression.

2.6 NON-INFLAMMASOME FORMING NLRs

Non-inflammasome forming NLRs, such as NLRX1, NLRP12, NLRC3, NOD1 and NOD2 form a significantly important subgroup of the NLR family that can both positively and negatively regulate inflammation. These NLRs do not form an inflammatory complex upon activation but regulate inflammation associated pathways by other mechanisms [Ting, Duncan *et al.*, 2010]. Non-inflammasome forming NLRs modulate NF-kB and other major inflammation regulatory pathways, which are crucial in chronic inflammation and inflammation-induced tumorigenesis [Allen, Wilson *et al.*, 2012; Allen, 2014]. NLRs modulate these pathways through interaction with a specific upstream/downstream molecule belonging to that pathway. Their known functions reveal the fact that dysregulation in NF-kB and other inflammation associated signaling pathways by these NLRs are very important and play critical role in tumor induction and progression (Table 2.2).

2.6.1 NLRP12

NLRP12 also known as Monarch-I and PYPAF7, belongs to the non-inflammasome forming subgroup of NLRs [Wang, Manji *et al.*, 2002]. It is one of the first NLR proteins to be studied. It has a tripartite domain structure, with an N-terminal PYRIN domain, a central NBD,

and a C-terminal domain composed of at least 12 LRR motifs as shown in Table 2.1 [Pinheiro, Eibl *et al.*, 2011]. In humans, *Nlrp12* is expressed in peripheral blood leukocytes, including granulocytes, eosinophils, monocytes, and dendritic cells (DCs) [Williams, Taxman *et al.*, 2003]. Recent *in-vitro* research studies have shown NLRP12 negatively regulates inflammation by attenuating both canonical (via interaction with IRAK1) and non-canonical (via interaction with TRAF3 and NF-kB inducing kinase (NIK)) NF-kB pathway [Conti, Davis *et al.*, 2005; Lich, Williams *et al.*, 2007; Ye, Lich *et al.*, 2008]. NLRP12 also down-regulates MAPK pathway by inhibiting ERK signaling [Allen, Wilson *et al.*, 2012]. *S. typhimurium* induces NLRP12-mediated dampening of host immune defenses to persist and survive inside the host. During salmonellosis, NLRP12 inhibits NF-κB and ERK activation by suppressing phosphorylation of IκBα and ERK, preventing efficient clearance of bacterial burden [Zaki, Man *et al.*, 2013].

2.6.2 NLRP12 and Cancer

Zaki *et al.*, first observed that *Nlrp12* deficient mice are hyper-susceptible to DSS-induced colitis and have increased colitis-associated colorectal tumorigenesis. NLRP12 plays a critical role in dampening the inflammatory response in myeloid cells and during DSS- induced colitis. The expression of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-17, IL-15 and chemokines (G-CSF, eotaxin, KC, IP-10, MIP-1α, MIP-1β, MIP2) were significantly higher in the colon of *Nlrp12* $^{-/-}$ mice, leading to hyperplasia and increased tumorigenesis. Therefore, *Nlrp12* deficiency leads to increased and prolonged inflammatory responses in colon tissue. Significantly higher activation levels of MAPK, NF-κB, STAT and AKT were observed, accompanied with massive infiltration of macrophages and increased proliferation of epithelial cells particularly in the hyperplastic colon regions of the AOM/DSS treated *Nlrp12* $^{-/-}$ colon tissue [Zaki, Vogel *et al.*, 2011]. Collectively, these results suggest NLRP12 signaling in the hematopoietic cells is critical for protection against colitis and CAC. Allen *et al.*, confirmed increased susceptibility of *Nlrp12* $^{-/-}$ mice towards inflammation-driven colon tumorigenesis. The *ex-vivo* contribution of NLRP12 is reflected in terms of enhanced non-canonical NF-kB signaling and MAPK activation, observed in the myeloid dendritic cells isolated from the *Nlrp12* $^{-/-}$ mice. NLRP12 functionally interacts with TRAF3, which in turn directly interacts with NIK, inducing NIK degradation. The pathogenesis of *Nlrp12* $^{-/-}$ mice was found to be derived from both the hematopoietic and non-hematopoietic compartments. Further experimental analysis, suggested an early role for NLRP12 in the AOM+DSS model through both hematopoietic and non-hematopoietic compartments. Coincidental with the polyp formation, the effect of NLRP12 was derived primarily from the non-hematopoietic compartment [Allen, Wilson *et al.*, 2012]. In summary, the study indicates how NLRP12 attenuates the development of experimental colitis and suppresses colitis-associated cancer. These studies strongly suggest that functions of NLRP12 might be cell/tissue-specific for specific stage of inflammation/tumorigenesis. Both *ex-vivo* and *in-vivo* studies have proved a critical role played by NLRP12 in regulation of major signaling pathways associated with inflammation and inflammation-associated tumorigenesis (Table 2.2).

2.6.3 NLRX1

NLRX1 is the first non-cytoplasmic NLR protein discovered. NLRX1 is localized in mitochondria however; the exact localization within mitochondria remains to be characterized [Moore, Bergstralh *et al.*, 2008; Xiao and Ting, 2012]. Its structure constitutes a highly conserved NBD and LRRs domain as seen in Table 2.1. *Nlrx1* expression is highest in mitochondria-rich tissues such as muscle and heart. Major functions of *Nlrx1* includes negative regulation of anti-viral inflammatory response via MAVS- RIG1 signaling pathway or TLR- induced NF-kB signaling by targeting TRAF6 and IKK signaling pathway [Allen, Moore *et al.*, 2011; Xia, Cui *et al.*, 2011]. NLRX1 inhibits NF-kB activation by inhibition of IKKα and IKKβ phosphorylation. These results show specific knockdown of *Nlrx1* resulted in increased gene expression of cytokines; TNF- α and IL-6 and chemokines; CCL2 and CXCL10 in response to LPS treatment [Xia, Cui *et al.*, 2011]. NLRX1 also exerts positive control of NF-kB and JNK signaling pathway for active production of ROS in response to TNF-α, poly (I:C) and pathogens [Tattoli, Carneiro *et al.*, 2008]. NLRX1 binds to the ssRNA, dsRNA, poly (I:C) but not with DNA [Hong, Yoon *et*

al., 2012]. A study by Soares *et al.* discovered that NLRX1 is not involved in inhibition of MAVS-dependent anti-viral signaling. NLRX1 interacts with a mitochondrial matrix protein, UQCRC2 to induce ROS production but exact association between the two is yet to be shown [Soares, Tattoli *et al.*, 2012]. NLRX1 also forms complex with mitochondrial Tu translation elongation factor protein, TUFM, that dually regulates IFN-ϒ production and promotes autophagy during viral infection [Lei, Wen *et al.*, 2012]. Recently, crystal structure of NLRX1 C-terminal was elucidated, describing association of LRR and neighboring helical domains to form a hexameric platform which promotes the interaction/binding of NLRX1 with its target proteins. It's only recently that researchers have started exploring NLRX1, therefore, its major regulatory functions, RNA recognition by NLRX1 and NLRX1 –RNA interaction mechanism are still under investigation.

2.6.4 NLRX1 and Cancer

Nlrx1 plays an important role in regulating the balance between intrinsic and extrinsic apoptosis in cancer cells. *Nlrx1* positively regulates apoptosis in response to intrinsic apoptosis signals and that may be this is why *Nlrx1* expression is down regulated in cancer cells. *Nlrx1*-/- mice develop fewer tumors than WT mice in the AOM-induced colorectal cancer murine model. *Nlrx1*-deficiency reduced cancer progression in this cancer model. In contrast, in a AOM/DSS treated colitis-associated cancer model, *Nlrx1*-/- mice developed a more severe pathology, showing increased sensitivity to DSS colitis. NLRX1 protects against DSS-induced damage and AOM/DSS-triggered colorectal cancer. This differential role of NLRX1 might be consequence of an exacerbated rate of apoptotic cell death in response to DSS treatment followed by increased epithelial proliferation as part of repair of damaged tissue [Soares, Tattoli *et al.*, 2014]. Recent data indicates NLRX1 attenuates tumorigenesis through the negative regulation of AKT and NF-κB signaling [Allen, Moore *et al.*, 2011]. NLRX1 sensitizes cells to TNF-α induced cell death by activating Caspase-8. *In vitro* studies suggest that NLRX1 expression suppresses clonogenic ability, anchorage-independent growth and migration of cancer cells. Interestingly, NLRX1 may also contribute to the metabolic switch toward glycolysis in these tumor cells. Study extended to *in-vivo* models has shown suppression of tumorigenicity in nude mice by NLRX1 [Singh, Poteryakhina *et al.*, 2015]. Given the current understanding of NLRX1 and its role in innate immune responses, NLRX1 might serve as a promising target for manipulating immune response in inflammation-associated diseases and cancer pathology.

2.6.5 NOD1 and NOD2

NOD1 and NOD2 (nucleotide-binding oligomerization domain-containing protein 1 and 2, also known as NLRC1 and NLRC2, respectively) were the first NLRs to be characterized. The NOD-proteins are amongst the most prominent members of the NLR (NOD-LRR) family of proteins that contain nucleotide-binding NACHT domains, receptor-like LRR domains and CARD domains. NOD1 contains a single amino-terminal CARD, and NOD2 contains tandem N-terminal CARD domains that mediate interactions with the CARD domains of RIP2 [Correa, Milutinovic *et al.*, 2012]. While *Nod1* is ubiquitously expressed, *Nod2* is expressed more in hematopoietic cells [Philpott, Sorbara *et al.*, 2013]. Several E3 ubiquitin ligases have been suggested to be involved in the activation of NOD1 and NOD2 signaling, including cellular inhibitor of apoptosis protein 1 (CIAP1), CIAP2, and TRAF6 [Bertrand, Doiron *et al.*, 2009]. Interestingly, both NOD1 and NOD2 can be recruited to the plasma membrane during infection and ligand recognition. NOD2 promotes the membrane recruitment of RICK, a serine-threonine kinase involved in NF-κB activation downstream of NOD2 [Lecine, Esmiol *et al.*, 2007]. Ubiquitin is also able to bind to the caspase recruitment domains of NOD1 and NOD2, competing with RIP2 for binding and reducing activation. Furthermore, polyubiquitylated RIP2 is a substrate for the deubiquitylating enzyme, A20, which negatively regulates NOD2 signaling.

Nod1 and *Nod2* recognize muropeptides derived from cell walls of Gram-positive and Gram-negative bacteria, such as *Listeria monocytogenes* and *Shigella flexineri*. *Nod1* and *Nod2* are important for microbial recognition and host defense after TLR stimulation [Kim, Park *et al.*, 2008]. NOD1 mediates selective recognition of bacteria through detection of iE-DAP-containing

peptidoglycan [Chamaillard, Hashimoto *et al.*, 2003]. NOD1-dependent responses play a critical role in host resistance against *Trypanosoma cruzi, Pseudomonas aeruginosa,* and *Helicobacter pylori* infections [Travassos, 2005; Silva, Gutierrez *et al.*, 2009; Grubman, Kaparakis *et al.*, 2010]. NOD1 also participates in the induction of a noninfectious pancreatitis via its response to commensal organisms [Tsuji, Watanabe *et al.*, 2012]. NOD1 and NOD2 proteins contribute to the maintenance of mucosal homeostasis and the induction of mucosal inflammation [Strober, Murray *et al.*, 2005]. *Nod2* acts as an intracellular receptor for sensing of muramyl dipeptide [Mo, Boyle *et al.*, 2012]. *Card8* negatively regulates *Nod2*-mediated signaling, displaying a novel molecular switch involved in the endogenous regulation of *Nod2*-dependent inflammatory processes [von Kampen, Lipinski *et al.*, 2010]. The molecular chaperone protein, HSP70 binds to and stabilizes NOD2 mutants, providing an effective therapy for Crohn's disease [Mohanan and Grimes, 2014].

2.6.6 NOD1 and NOD2 in Cancer

The activation of NOD1 and NOD2 triggers recruitment of the adaptor proteins RICK and CARD9, resulting in K63-linked ubiquitylation and activation of NF-κB and the MAP kinase signaling cascades [Kanneganti, Lamkanfi *et al.*, 2007]. Critical role of NOD1 signaling in maintaining intestinal epithelial barrier permeability and balancing inflammatory responses has been shown using AOM/DSS-induced colon-associated tumorigenesis [Chen, Shaw *et al.*, 2008]. NOD1, RIP2 and Caspase12 have been described as potentially novel biomarkers for oral squamous cell carcinoma development and progression[Wang, Jiang *et al.*, 2014].

The role of NOD2 is being studied extensively in colitis and colorectal tumorigenesis because of its well-recognized genetic association with inflammatory bowel disease. *Nod2* mutations and polymorphisms have been linked with increased to colorectal cancer [Zaki, 2013]. In addition to its well-characterized role in NF-κB and MAPK activation, NOD2 is also involved in the regulation of autophagy, which plays a crucial role in intestinal homeostasis and tumorigenesis. *Nod2* deficiency-induced dysbiosis gives rise to a reversible transmissible colitis and colitis-associated carcinogenesis in mice. Results show that *Nod2/Rip2* deficiency confers a maternally transmissible colitis risk on immunocompetent host [Couturier-Maillard, Secher *et al.*, 2013]. Additionally, fecal dysbiosis in *Nod2*-deficient mice also sensitized the colonic mucosa to DSS-induced chemical injury. Researchers have shown that the NOD2 3020insC mutation may be a genetic predisposing factor for breast, lung cancer and colorectal cancer [Kurzawski, Suchy *et al.*, 2004]. *Nod1/Nlrc1* and *Nod2/Nlrc2* gene polymorphisms have been identified in cancer etiology. Several *Nod1/Nod2* polymorphisms may be associated with altered risk of gastric, colorectal, breast, ovarian, prostate, testicular, lung, skin cancer and various other cancers [Kutikhin, 2011].

2.7 CURRENT CANCER THERAPEUTICS

Cancer cells overcome the host innate immune surveillance system to promote tumorigenesis and constitute a tumor-promoting microenvironment. The inflammatory tumor microenvironment plays major role in cancer development and progression as depicted in Figure 2.2. The microenvironment consists of increased expression of pro-inflammatory cytokines, chemokines and other major inflammatory molecules facilitating interaction between normal and cancer cells. Given the major role played by cytokines during chronic inflammation, tumor induction and tumor development, cytokines became a common target of therapeutics for auto-inflammatory diseases. IL-1 dysregulation has been reported in several auto-inflammatory diseases [Gabay, Lamacchia *et al.*, 2010]. Therefore, IL-1 signaling pathway inhibitors are being used for effective treatment of cold induced periodic syndromes including NOMID, MWS, and FCAS syndromes associated with various *Nlrp3* mutations. The first such IL-1 inhibitor was anakinra followed by rilonacept and canakinumab [Dhimolea, 2011]. The direct blockade of important immunoregulators like IL-1β compromises the host immune response to infectious agents or damage- associated molecules and limits the anticancer immune response elicited by immunogenic cell death inducers. Apart from IL-1β, IL-18

signaling can also be blocked by neutralizing antibodies or recombinant IL-18BP (Interleukin-18 binding protein) (Figure 2.4). The inhibition of inflammasomes or therapeutic neutralization of their products would exert profound effects on carcinogenesis and tumor progression [Zitvogel, Kepp *et al.*, 2012].

Over the years, multiple therapeutic strategies have been utilized for targeting cancer cells. Recently, liposomes packaged siRNA targeting the *Pkn3* gene (protein kinase N3) for lung metastasis inhibition has been approved for evaluation in a human clinical trial [Shen, Sun *et al.*, 2012]. siRNAs are potent activators of innate immunity but do have some major off-target effects on gene expression, antibody response against delivery vehicle and immune-toxicities due to excessive cytokine release. Re-expressing tumor-suppressive miRNAs holds great promise for cancer therapy. It is well accepted that aberrant miRNA expression is linked to cancer, hence delivering tumor-suppressive miRNAs and silencing oncogenic miRNAs has been successful in various mouse models [Kasinski and Slack, 2011]. Reprogrammed viruses have also been utilized as oncolytic vectors for developing virus based therapeutics for cancer. Currently, DNA viruses in clinical trials include adenovirus (AD), herpes simplex virus 1 (HSV1) and vaccinia virus. The selection of HSV to treat GBM utilizing this approach gives great hope [Friedman, 2006]. The only engineered oncolytic RNA virus undergoing clinical trials is the measles virus. Oncolytic viruses hold great potential as self-amplifying cancer therapeutics and can be applied in combination with radiation and chemotherapy. Desirable protease targets (for example- matrix metalloproteinases) for oncolytic viruses are either expressed preferentially or at high levels by cancer cells. In fact, Ad H101 is the first reprogrammed virus to be approved as a cancer drug and has been administered to hundreds of patients with head and neck carcinoma in China [Cattaneo, Miest *et al.*, 2008]. Today, peptide-vaccination targeting the epidermal growth factor receptor (EGFR) mutation III (vIII) is one of the most prominent examples of immunotherapy for glioblastoma [Hegi, Rajakannu *et al.*, 2012]. Treatment of glioblastoma patients with cediranib, pan-VEGF receptor tyrosine kinase inhibitor resulted in increased tumor perfusion, and improved survival rate because of the vascular normalization [Sorensen, Emblem *et al.*, 2012].

The pivotal role of IKKβ/NF-κB signaling pathway in inhibition of programmed cell death and constitutive expression in various malignancies, strongly suggest the use of IKKβ and/or NF-κB inhibitors for cancer treatment. Currently available drugs are non-specific Ikkβ or NF-kB inhibitors inclusive of anti-inflammatory agents, NSAIDs, cyclopentenone prostaglandins, proteasome inhibitors, and glucocorticoids. IKKβ/NF-κB inhibitors block NF-κB activation in infiltrating inflammatory cells, which are important source of tumor growth and survival factors [Luo, 2005]. Targeting specific NF-kB-regulated ligands for regulating particular gene expression makes this a promising approach for cancer therapy.

The critical role played by NLRs in major inflammatory pathways associated with cancer has provided a new direction to cancer therapeutics [Kent and Blander, 2014]. Recently, the crystal structures for NLR domains such as PYRIN, CARD and NBD have been elucidated. The focus is on developing drugs that directly interact with the NLR domains for selective activation or inhibition of a NLR. Distinct NLR ligands may be used as vaccine adjuvants for enhancement of innate immune response. Depending on the particular cancer and its stage, inflammasome activation or inhibition can be utilized as cancer therapeutics. The emerging role of inflammasomes in host innate immune responses suggests inflammasome components as direct drug targets for cancer (Figure 2.4). Opsona Therapeutics recently published that the cytokine release inhibitory drug 3 (CRID3, also known as CP-456,773) targets ASC oligomerization during NLRP3 and AIM2 inflammasome activation (Coll and O'Neill, 2011) [López-Castejón and Pelegrín, 2012]. Anticancer chemotherapeutics are particularly efficient when they succeed in killing tumor cells through immunogenic cell death, and thereby converting dying cells into therapeutic vaccines [Krysko, Garg *et al.*, 2012]. Earlier, immunomodulators such as thalidomide (anti-inflammatory and anti-angiogenic) were used that may exert inhibitory effects on the NLPR3 inflammasome but also have strong teratogenic activity. The anti-inflammatory activity of thalidomide is mediated via Caspase-1 in mice. Thalidomide has been approved for the treatment of inflammatory skin diseases and cancer

[López-Castejón and Pelegrín, 2012]. NLRs present promising option for novel therapeutics targets for transformation of the tumor-promoting microenvironment into an anti-tumor microenvironment (Figure 2.4).
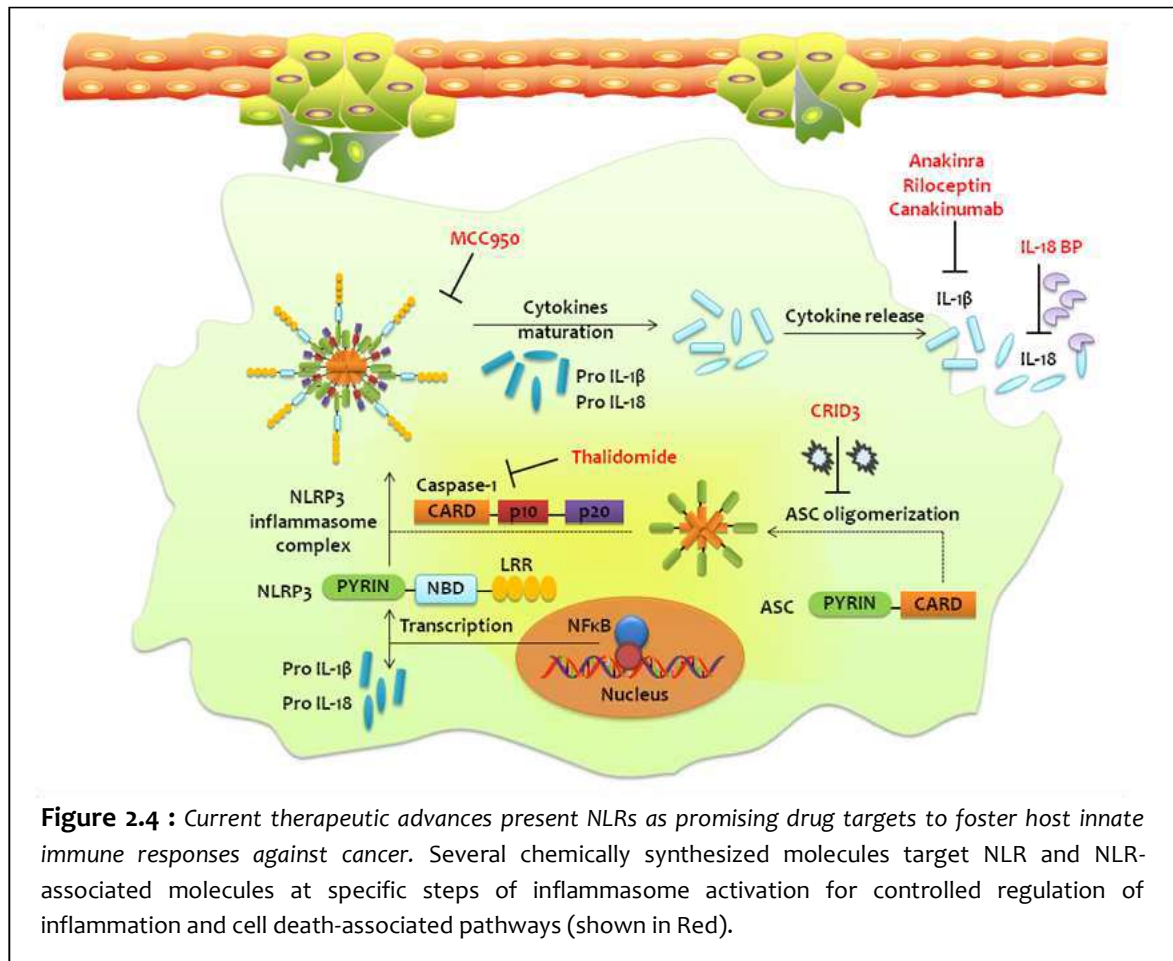

Figure 2.4 : *Current therapeutic advances present NLRs as promising drug targets to foster host innate immune responses against cancer.* Several chemically synthesized molecules target NLR and NLR-associated molecules at specific steps of inflammasome activation for controlled regulation of inflammation and cell death-associated pathways (shown in Red).

2.8 FUTURE DIRECTIONS

Inflammasomes display contrasting roles across multiple stages of tumorigenesis. Even though NLRs play important roles in innate immunity and inflammation, information related to NLR- ligand interactions, inflammasome activation and NLR-associated intracellular and extracellular signaling pathways remain largely unknown. Further insights into the upstream and downstream signaling molecules participating in NLR-associated signaling are needed. Hence, it is important to unravel the molecular mechanisms responsible for activation of anti-tumor or tumor-promoting inflammasome-associated pathways. Keeping in view, the future prospects of NLRs, inflammasomes and their products as cancer biomarkers some questions remain unanswered -

- While the association of NLRs and major inflammatory pathways with cancer has opened up a new field of cancer therapeutics, the regulatory mechanisms of NLR dependent signaling pathways remain unclear. These regulatory molecules might serve as valuable biomarkers as well as highly specific drug targets to modulate an NLR associated pathway.
- The molecular mechanism behind NLRs affecting the activation/inhibition of other NLRs remains largely unknown.
- The regulatory molecules anchoring ligands towards the cytoplasmic NLRs and the feedback loops responsible for balanced activation of the NLR inflammatory pathways need further investigation.

2.9 CONCLUDING REMARKS

The chapter highlights strong interplay of NLRs in innate immunity, inflammation and cancer. NLRs are highly conserved pattern recognition receptor sensors making significant contribution to the innate immune regulation in both healthy and diseased humans. NLRs sense specific damage and pathogen associated molecules, initiate repair mechanisms, generating controlled inflammatory and immune responses. Though major advances have been made in the field of NLRs, mechanistic insights into the sensing and activation of NLRs, NLR-regulated inflammation and innate immune signaling pathways requires further investigation. In summarizing the newly identified pro- and anti-tumorigenic roles of NLR family members in cancer, we find research evidence support cell and tissue-specific regulation of NLRs in various cancers. Interestingly, the role of NLRs and NLR-associated signaling molecules in glioma remains unknown. The review critically evaluates the role of NLRs in inflammation and tumor regulation, and suggests NLRs and NLR-associated signaling molecules as promising therapeutic targets and biomarkers for early cancer prognosis, treatment and control.