5
Conclusions and Future Directions

NLRs are germ-line encoded PRRs of the innate immune system that recognize invariant molecular patterns related to damage and pathogens (DAMPs and PAMPs respectively) [Ye and Ting, 2008]. NLR regulated pathways perform controlled regulation of inflammatory and innate immune related responses to maintain cellular homeostasis [Martinon, Mayor *et al.*, 2009]. NLRs are critical to human health which is indicated by their implication in inflammation and disease including cancer [Janowski, Kolb *et al.*, 2015]. The aim of this dissertation was to 1) elucidate if amorphous nanosilica particles act as DAMPs, to induce inflammation and NLR-mediated innate immune signaling, leading to cytotoxic responses and, 2) to characterize NLR gene expression and to test our hypothesis that NLR regulated inflammation pathways could play important role in molecular stratification of LGG and GBM.

The unique physical and chemical properties of amorphous nanosilica, makes it immensely popular across various industrial and biomedical applications [Barik, Sahu *et al.*, 2008]. However, amorphous nanosilica exposure and interaction at the human-biomolecular interface remains unclear. The most common source of nanosilica exposure is human lungs through air inhalation. Our study utilizes various molecular biology assays to demonstrate the cellular and molecular effects of amorphous nanosilica exposure in human lung cell population. The findings reveal distinct time and dose dependent increase in nanosilica induced cytotoxicity for each cell type. Results highlight nanosilica induced toxicity at the nano-bio interface raising concerns over amorphous nanosilica application as stable biomedical platforms. The study provides basic mechanistic insights into amorphous nanosilica induced cytotoxicity, activation of ASC, inflammasome-associated cell death adaptor protein and distinct mode of cell death. It is clear that particle size and duration of exposure could serve as critical checkpoints for amorphous nanosilica exposure considering widespread applications, from binding industry to drug delivery and cosmetics. Amorphous nanosilica exposure-induced effects on human health and link with inflammation-associated diseases, is dependent on the cell population, relevant concentration and size of particles. Recent advances offer promising interdisciplinary applications of amorphous nanosilica, but could also lead to unforeseen adverse inflammatory and immunological effects to exposed humans. We should be concerned about possibility of nanosilica interactions with the immune system, altered immune responses and chances of exposure leading to a spectrum of inflammatory clinical manifestations. Further research needs to address particle-cell interactions, inflammasome signaling and cell death pathways in response to amorphous silica and therapeutic interventions post toxicity.

Emerging research evidences have brought commercialization of nanosilica under scrutiny; therefore, finding solutions to reduce harmful effects of amorphous nanosilica on human health holds great promise. In this direction, we have identified fenugreek seed extract mediated cytoprotection in amorphous nanosilica-treated fibroblasts via a reactive oxygen species independent pathway. *Trigonella foenum-graecum* or fenugreek is a well known anti-inflammatory agent with cytoprotective effects [Djerassi, 1992]. However, the underlying cellular and molecular mechanisms of fenugreek-mediated cytoprotection are largely unknown. We have used experimental biology and molecular dynamics for investigating the effects of fenugreek extract on nanosilica mediated cytotoxicity and LPS mediated inflammation [Yazdi, Guarda *et al.*; Shi, Zhao *et al.*, 2014] (Figure 5.1).

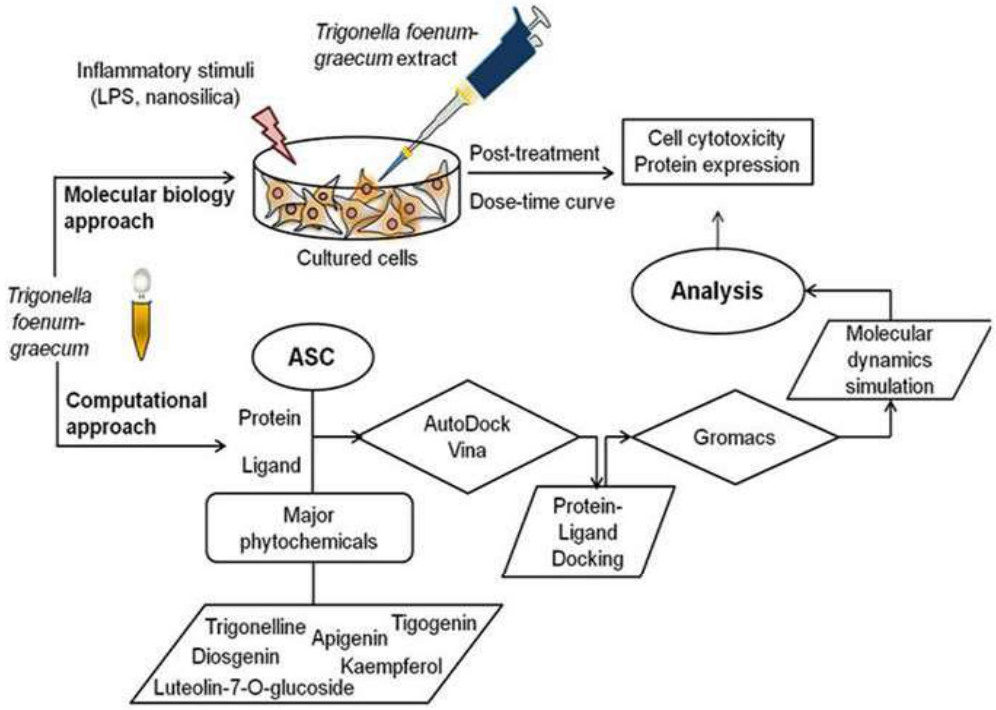

Figure 5.1 : *Analytic workflow for study of fenugreek-induced anti-inflammatory effects and possible interactions with ASC protein targeting in vitro and in silico approaches.*

Using an interdisciplinary approach allowed us to understand cellular as well as molecular effects of fenugreek on inflammation. ASC is central to inflammatory and cell death pathways associated with innate and adaptive immunity [Srinivasula, 2002]. All atom molecular dynamics simulations of ASC-Phytochemical complex reveal that individual phytochemicals in fenugreek bind to ASC via specific non-covalent interactions. The phytochemical groups chosen for molecular simulation studies of fenugreek-ASC complex are listed in Table 5.1. Fenugreek ameliorates amorphous nanosilica mediated cytotoxicity in a dose-dependent manner (Figure 5.2). Nanosilica treated cells undergo rapid cell death, and fenugreek addition significantly reduced nanosilica induced cell death. Immunofluorescence shows nuclear condensation and presence of cytoplasmic as well as perinuclear ASC specks in nanosilica treated fibroblasts. Strikingly, cells treated with both fenugreek and nanosilica remains viable, show absence of nuclear condensation and increased ASC expression with several cytoplasmic and perinuclear specks (Figure 5.3).

Table 5.1 : *Major phytochemicals present in the fenugreek seed extract*

Major Nutrients	**Phytochemicals**
Alkaloids	Trigonelline
Saponins	Tigogenin
	Diosgenin
Flavonoids	Apigenin
	Kaempferol
	Luteolin

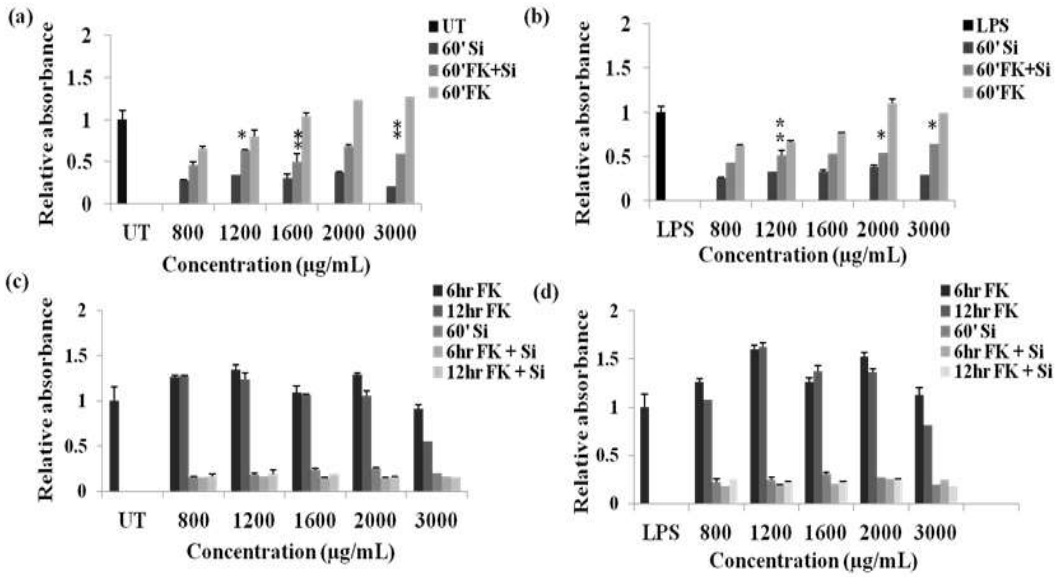

Figure 5.2 : *Fenugreek seed extract inhibits nanosilica-induced cytotoxicity.* Dose-dependent decrease in cytotoxicity caused by nanosilica (200 µg/mL) was observed in presence of fenugreek both in untreated fibroblasts (a) and LPS (0.5. µg/mL) -primed fibroblasts (b). Pretreatment of fibroblasts with fenugreek for 6h or 12h prior to nanosilica exposure did not offer any cytoprotection both in the absence (c) and presence of LPS priming (d). Data is presented as mean ± SD; *p value < 0.0.5., **p value < 0.0.0.5. by T-test analysis

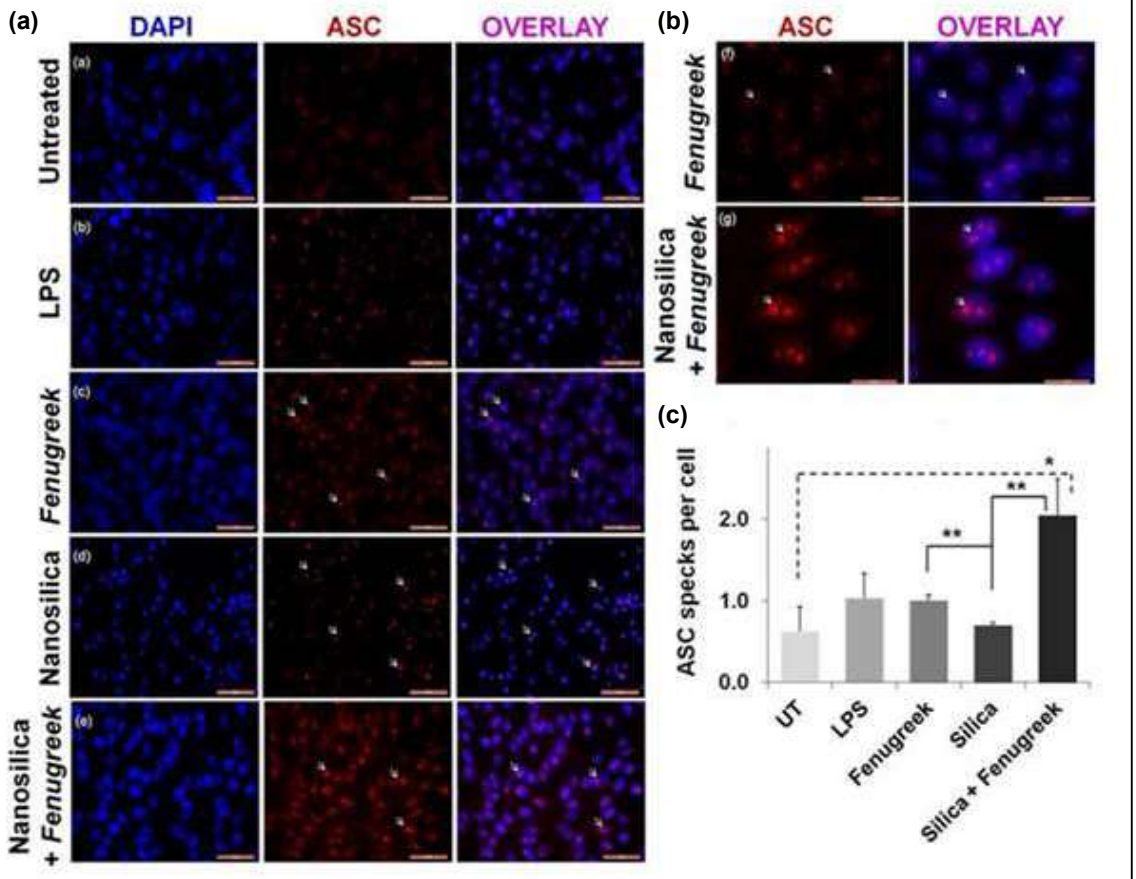

Figure 5.3 : *Fenugreek increases ASC speck formation in nanosilica treated cells.* The above panel shows reduced cytotoxicity and cell death in LPS-primed fibroblast cells upon exposure to nanosilica and fenugreek (a-c). ASC expression visualized in fibroblast cells exposed to fenugreek along with nanosilica. Right panel shows ASC expression in control, LPS treated, fenugreek treated, nanosilica treated and, nanosilica and fenugreek treated fibroblast cells are visualized by using immunofluorescence microscopy for ASC (red) and DAPI to stain nuclei (blue), Scale bar-50µm. The left panel shows inset images of fenugreek treated and both nanosilica and fenugreek treated (b) fibroblasts respectively at higher magnification. Scale bar-20µm. Graph shows ASC speck quantification in LPS-primed, silica and fenugreek treated cells (c). Data is presented as mean ± SD; *p value < 0.0.5., **p value < 0.0.0.5. by T-test analysis.

Our preliminary findings provide evidence that fenugreek regulates nanosilica mediated cytotoxicity via the interaction of the constituent phytochemicals with the inflammasome adaptor protein, ASC. While the cellular studies provide insight into the amelioration of cell death by fenugreek, the molecular dynamics analyses reveal specific residues and nature of interactions underlying the same allowing for the use of fenugreek in development of targeted therapeutics for inflammation. The interaction of ASC PYRIN domain with the phytochemicals raises possibility of fenugreek components affecting ASC activity by modulating its structure or function. These interactions might also introduce regulatory changes in ASC associated inflammation and cell death pathways. In this respect, our present study lays the foundation for a future investigation of synergetic effect of fenugreek-mediated regulation of ASC and restrained nanosilica-induced cytotoxic responses, which may offer an opportunity for development of therapeutic interventions for many inflammatory diseases by establishing their structure-function relationship.

The sensing and activation mechanisms of NLRs and their divergent roles in inflammation-induced tumorigenesis, is an extensive field of research. Recent advances highlight both beneficial and detrimental roles of NLRs during cancer [Janowski, Kolb *et al.*, 2015; Zhu and Cao, 2017], however; the role of NLRs in gliomas (most common type of primary brain tumor) remains unknown. Our study employs a two-dimensional approach, involving integrated molecular cancer data analysis and molecular biology experiments to characterize the regulation of NLRs and NLR-associated genes in LGG and GBM. Network analysis revealed major inflammatory and cell death-associated gene alterations in addition to that seen in NLRs, in case of gliomas. Differentially expressed genes show strong inverse correlation between expression and methylation levels, for GBM with respect to LGG. Genes including *AIM2*, *ATN1*, *BCL2L1*, *CASP1*, *EGFR*, *MSR1*, *NLRC3*, *NLRC4*, *NLRP3*, *NLRP12*, *NLRX1*, *NOD1*, *NOD2*, *PYCARD*, *CDK11B* and PSEN1 exhibited strong inverse correlation between expression and methylation levels in GBM. Survival analysis revealed *NLRC4*, *NLRP6*, *NOD1* and *CASP1* as genes with high prognostic value specifically for grade 2 and grade 3 gliomas. The study reports *NLRP12* as a novel prognostic marker for GBM. Immunofluorescence studies, further confirm high expression of NLR-associated genes in glioma cell lines. Our study characterizes the novel regulation of NLR family genes in different grades of glioma, using both computational and molecular biology analyses. Adding to the remarkable roles played by NLRs in inflammation, immunity and cancer, our findings highlight functional significance and regulation of NLRs in glioma pathology. The preliminary results need further investigations focused on demystifying the functional implications associated with NLR mediated innate immune signaling in LGG and GBM. Future studies should focus over underpinning cellular and molecular mechanisms regulating NLRs and NLR-associated pathways during glioma pathogenesis. The emerging role of NLR regulation in glioma, will aid significantly for devising new therapeutic strategies including innate immunity targeted prognostic markers for early glioma diagnosis and high treatment efficacy in glioma patients.