The enormously complex yet equally fascinating process of inflammation, in its general sense is defined as a protective measure taken by the body against a variety of noxious stimuli. It is defined as pervasive phenomenon acting against perturbations of homeostatic state. These perturbations or stimuli are broadly categorized under exogenous and endogenous inducers. The exogenous forms may be microbial i.e. bacterial/ viral pathogenic species etc., or non-microbial i.e. allergens, irritants, toxic compounds etc. The endogenous components include anomalies in tissues in the form of stress, malfunctioning or damage. Upon sensing of these inducers by sensor molecules, a set of mediator molecules is elicited in response. These mediators then act upon affected cells and tissues and lastly the effectors come into play for resolution. Inflammation as documented by Celsus and Galen in as early as 1st century is characterized by the following quintet of cardinal signs: redness (rubor), heat (calor), swelling (tumor), pain (dolor) and dysfunction of the organs involved (functio laesa). Such an acute inflammatory response against infection/ injury has a unidirectional beneficial objective towards resolution of the body homeostatic phase by eliminating the underlying source of disturbance. Thus, in its normal essence, it is a beneficial, non-specific form of immune system.

But this beneficial physiological response can turn to a harmful pathogenic state, if persists in an unregulated manner. The so called, chronic inflammation that results due to continuously prevailing infection/ injury is detrimental as it lays down the foundation opportunity to various diseases including cancer. The only differences between acute and chronic inflammation known so far, are strength and duration of the inflammatory response. An acute response represents rapid, strong and short duration response compared to chronic one. But what are the mechanistic differences in terms of mediator molecules that result in either acute or chronic response remains unexplored. As discussed above, out of the categories of the molecules that comprises an inflammatory event (i.e. inducers, sensors, mediators and effectors), mediators seem to occupy the central position in deciding the acuteness or chronicity of the process. Thus the expression of these mediators e.g. cytokines, chemokines, lipid mediator molecules etc. is suspected to differ in these
two states. What are the operating mechanisms that lead to spatio-temporal difference in terms of expression, besides the genetic information being same! Partly the answer may lie in the realm of epigenetics.

Also, at the gene level, an inflammatory event seems to be interplay between pro-inflammatory and anti-inflammatory branches with respect to time and expression. In simple words, a normal inflammation starts with expression of pro-inflammatory genes (active transcription/ euchromatin at promoter site) followed by its subsidence (by repression/ heterochromatinization) and upregulation of anti-inflammatory gene expression and their repression thereafter. Thus it seems to depend on chromatin status of inflammatory genes with respect to euchromatin or heterochromatin, whether the inflammation will be acute or chronic. Hence epigenetic mechanisms (specifically histone modifications) were selected to study to further dissect the standard views of inflammation.

Epigenetics as defined by heritable changes in gene expression that are DNA independent comprises of chromatin remodeling, histone modifications, histone variants, DNA Methylation and non-coding RNA. Chromatin, i.e. association of DNA and proteins (histones and non-histones); serves the physiological function to package comparatively high amount of DNA in nucleus. On the other side, this compactness has to be relieved for vital DNA-dependent processes i.e. transcription and replication for access by enzymatic machinery involved in these processes. This need based conflict of compact and open chromatin is resolved by these epigenetic mechanisms. Out of these our interest was in histone post-translational modifications.

A number of covalent modifications on histone tails like acetylation, methylation, phosphorylation, ADP-ribosylation etc. are known along with the molecules that write, read and erase them. For example, histone acetyltransferase (HAT) adds acetyl group on specific lysine residue of a particular histone type which is read by bromodomain and later on removed by histone deacetylase (HDAC) enzyme. We chose a set of specific antibodies against these modifications (listed below in Table 1) to understand the epigenetic-inflammation link.
Considering the importance of epigenetic mechanisms in deciding the strength and duration of inflammatory process as hypothesized above, the present study was conceived with prime objective of studying epigenetic modifications of histones during inflammation. In order to dissect the inflammatory process, a flexible model was required to generate that could lead to varied degree of inflammation as per the modulation adopted. To achieve this, a murine surgical model for poly-microbial sepsis generation using ‘cecal ligation and puncture’ was selected.

Sepsis is a major health problem comprising of integrated, concomitant and often antagonistic events involving both exaggerated hyper-inflammation and immune-suppression. The malfunctioning of regulatory mechanisms to control inflammatory process leads to host damage during sepsis. Sepsis, in general starts with systemic inflammatory response syndrome (SIRS) demarcated with clinical features of Hyper/ hypothermia, tachycardia, tachypnea and leukocytosis/ leucopenia. SIRS along with infection represents ‘sepsis’ whereas sepsis in addition to organ failure is called ‘severe sepsis’. In order to understand the complex problem of sepsis, various animal models have been developed with an ever ongoing debate as to which one is most suitable in terms of mimicking the human sepsis symptoms. Out of the available injectable (i.e. endotoxemia, bacterial inoculums models) and surgical models [i.e. cecal ligation and puncture (CLP) and colon ascendens stent peritonitis (CASP)] major work has been done using injectable models. But they all have been a failure in terms of representing a true clinical situation of human sepsis specifically cytokine profile. CLP though first reported almost 30 years ago by Wichterman, did not gain much importance due to its variable responses, until 2009 when Rittirsch and Peter Ward group reported an improvised CLP technique with reduced variations. Considering its suitability in terms of mimicking the human sepsis most closely as compared to others, CLP is being well accepted as a gold standard for sepsis research in scientific community. We adopted the CLP model to address our objective as no reports were found elaborating epigenetic-inflammation link in this model, also because of added liberty of modulating the system as per the required grade of severity of infection.
Establishment and characterization of a murine surgical model of polymicrobial sepsis using ‘cecal ligation and puncture (CLP)’:

The surgical model of sepsis i.e. CLP was optimized in male Swiss albino mice within the range of 30-40 grams of weight and 3-4 months of age considering the sex, age and obesity associated variations. All the surgical procedures were performed in first six hours of the daylight to avoid the circadian rhythm linked variation in results. Mice were starved overnight to avoid pulmonary aspiration from stomach during surgery. Ketamine/ xylazine combination was used as anesthetic and analgesic. Major optimization in surgical procedure involved 90% cecum ligation with four times puncturing using 24G needle to obtain the defined severity grade i.e. mortality after 34-36 hours of surgical intervention.

The system was characterized for inflammation/ sepsis markers in terms of physiological, behavioral, histological, immunological and biochemical parameters. 100% mortality was found at 36 hours after surgery in CLP operated mice as opposed to no mortality in sham and ‘only cecal ligated (CL)’ controls. Hypothermia (Core body temperature below 32°C) was found in CLP group which never reached the normal range (i.e. 36.7°C as observed in untreated control mice). An overall decrease in total locomotory activity (ambulatory and rearing) was observed in CLP. Though within initial time points sham and CL groups also showed a decline which then re-gained the normal range. This might be because of surgical stress and associated pain. Hematoxylin-eosin stained liver sections showed cellular vacuolization and mononuclear cell infiltration in CLP. Serum aspartate transaminase (AST), serum alanine transaminase (ALT) levels and lipid peroxidation in liver was found to increase with progression of sepsis/ inflammation. Among the cytokines, pro-inflammatory cytokines i.e. TNF-α and IL-6, were observed to be dramatically increasing throughout. On the other side, anti-inflammatory cytokines i.e. IL-10 and TGF-β showed a peak at 24 hours and also at 4 hours for IL-10. The overall peritoneal macrophage activation was found to be higher in CLP group compared to controls which is well obvious because of presence of microbes in peritoneal lavage. Among all other organs (heart, lung,
spleen), liver was selected for further studies considering its indispensable role as immune organ and sufficient tissue amount for histone analyses.

**Epigenetic modifications of histones in systemically inflamed/ septic liver:**

Global histone modifications were studied in systemically inflamed/ septic liver using site specific histone modification antibodies. Also the kinetics of expression of anti-inflammatory gene TGF-β1 was done (using semi-quantitative RT-PCR) followed by characterization of its promoter in terms of histone modification status using Chromatin immuno-precipitation. Reason for choosing TGF-β1 was the unexplored anti-inflammatory branch in case of sepsis as major work is being done on pro-inflammatory roots (especially TNF-α) by various research groups.

**(A) Global histone modification analysis of systemically inflamed/ septic liver**

Global profiling of acid-extracted total histones from systemically inflamed/ septic liver was done using gel systems with ionic detergents [i.e. sodium dodecyl sulfate-polyacrylamide gel (SDS-PAAG)] and non-ionic detergents [acid-urea-triton-polyacrylamide gel (AUT-PAAG)]. There were no differences in terms of histones isolated at different time points from different surgical groups, resolved on SDS-PAAG. But AUT-PAAG showed some modified/ variant histones but due to difficulty observed in transferring them onto nitrocellulose membrane for immuno-blotting, we moved forward with SDS-PAAG system only.

Various site specific histone modification antibodies, representing active/ repressed transcription state, were used to immuno-stain the histones resolved on SDS-PAAG and transferred onto nitrocellulose membrane. Since continuous sampling of liver from same mouse to study kinetics is not feasible, each sample was collected from a different mouse at specific time point. As a pre-requisite to observe individual-to-individual variation in terms of histone modification, a group of three mice was tested with each antibody. There was no significant difference in terms of histone modification status in these mice confirming there was no individual-to-individual difference at significant level. H3acK9 (Active transcription mark) showed an increase within 4 hours of surgery followed by progressive
decrease with time whereas opposite scenario was seen for H3me²K9 (Transcription repression mark). The competitive nature of both these modifications was well observed. It might be correlated with expression of pro-inflammatory genes that are actively transcribed in initial time points. H3phS10 indicates mitosis and was found to have no signals either in controls or experimental samples. It seems to be the possibility that though liver damage is associated with sepsis but the inherent capability of regeneration of liver is not elicited (as no mitotic figure is seen). H3me³K27 and H4me³K20 showed an increase in later time points i.e. 24-36 hours of CLP whereas H3me²K36 and H3me³K4 showed fluctuating levels.

Though there was not seen any direct correlation between global histone modification status and sepsis as reported in cancer, there was significant perturbations at histone levels which indicate involvement of epigenetics in inflammation/ sepsis.

(B) Analysis of mRNA level of TGF-β1 and characterization of its promoter in terms of histone modification status in systemically inflamed/ septic liver

TGF-β1, i.e. anti-inflammatory gene, expression level was analyzed in septic liver i.e. CLP) along with controls at various time points. Reason why we have chosen TGF-β1 is: initially sepsis was considered as uncontrolled hyper-inflammation only but associated immuno-suppression has also gained scientific interest in recent past. Since most of the studies have been done to elucidate the role of pro-inflammatory branch (especially pro-inflammatory cytokines like TNF-α) of immune system during sepsis, anti-inflammatory effects raised our interest (i.e. TGF-β1). The level of TGF-β1 mRNA was found to increase at later time points after surgical intervention in CLP operated mice and also in ‘cecum ligation’ operated mice up to some extent. It clearly indicates the role of anti-inflammatory events in resolving the inflammatory process but in our system since it was very acute septic condition, probably under the pro-inflammatory events the mice got died even before the anti-inflammatory branch could have shown its effect.
The histone modification status on the promoter of TGF-β1 (Between bp -154 and +63) was analyzed by chromatin immuno-precipitation. The soluble chromatin was prepared from liver nuclei, isolated at 4 hours and 36 hours after surgery (one initial and one late time point of sham, CL and CLP) using micrococcal nuclease (MNase) digestion; followed by its precipitation using histone site specific antibodies marking active or repressed transcription status and PCR thereafter. H3acK9, denoting active transcription state, was found to be enriched at TGF-β1 promoter at 36 hours in CLP with comparatively lesser amount in CL also, at the same point of time. It again indicates that anti-inflammatory cytokine TGF-β1 is under active transcription state in later time points of sepsis/systemic inflammation. On the other hand, H3me^{3}K27 (indicating repression) was not found in any of the group at any time point. The possibility cannot be avoided of its presence at some other time point or of some other modification representing repression.

Thus, a very promising model with well in conformance of the sepsis/inflammation parameters was optimized and characterized with interesting observations for further exploration. Global histone modifications represented the possible link of involvement of epigenetics in inflammatory events as hypothesized. Also the gene level studies showed that the death observed in our mice is probably the result of continuous euchromatinized state of pro-inflammatory cytokine genes that do not get transcriptionally inactive to resolve the inflammatory event. Anti-inflammatory cytokine do come into existence as a result of active transcription at later stage but before its effects are mediated, the mice die under the hyper-inflammatory state.