Chapter 6

Summary & Conclusions
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6.1 Summary

Despite our efforts to curb the increase and spread of antimicrobial resistance, bacteria continue to become less susceptible to antimicrobial drugs over time, and rates of discovery for new antibiotics are declining. Thus, it is essential to explore new paradigms for anti-infective therapy. One promising approach involves host-directed immune-modulatory therapies, whereby natural mechanisms in the host are exploited to enhance therapeutic benefit. In the current study, our objective is to initiate or enhance protective antimicrobial immunity while limiting inflammation-induced tissue injury.

We have tested the immune-modulatory effects of select HDPs, such as indolicidin and LL37. Our whole genome expression data following indolicidin and LL37 treatment of macrophages are in accordance with the previously published partial data sets. We further tried to enhance the immune modulatory efficacy of HDPs by covalently conjugating them to CNT and GNP. Because of conjugation with nanomaterials, efficacy of indolicidin and LL37 was increased by 1000 fold. The HDP primed mice macrophage cell line Raw264.7 and human monocytic cell line THP-1 were protected from *Salmonella typhimurium* induced cytotoxicity for 16 hours post challenge. Activation of cytokines like IL6, IL8, IL12 and several type I and type II interferons, but no expression of TNFs in macrophages indicated the inflammation proof priming of the innate immune system through indolicidin and LL37. Our systematic study on HDP induced immune priming of macrophages and its protection against ST, strengthened the concept of
modulating innate mucosal immunity as an alternative to antibiotics to combat infectious diseases in an evolution proof manner.

Probiotics are the living micro-organisms which were consumed through food unknowingly by human civilization from Neolithic era itself in the form of fermented foods. It was only after the discovery of modern fermentation technology in the 19th century; people industrialized the fermentation process and consumed fermented foods containing probiotics and prebiotics. It was observed that people consuming yoghurt habitually have longer life span and better combating capacity to infectious diseases. It was only recently in the 21st century when the menace of antibiotics resistance bugs went out of hand, people began to think about probiotics as an alternative to antibiotics. However the mechanisms through which probiotics work remained unknown until recently when people started looking at their immune modulatory properties and its ability to modify the gut mucosal microbiota. We have screened in vitro 4 highly prescribed probiotics species, but of different strains; with murine and human macrophage cell lines with respect to their immune-modulatory property and protection against ST induced cytotoxicity. *Saccharomyces boulardii*, and *Bifidobacterium bifidum* could not induce expression of select innate immune genes in macrophages which in turn were sensitive to ST induced cytotoxicity. However *Lactobacillus acidophilus* MTCC10307 and *Bacillus clausii* MTCC8326 could activate the monocytes into macrophages and modulate several cytokines, chemokines and defensins. Macrophages primed by LA and BC were found to be through NFkB and p38 MAPK pathways. The primed macrophages were protected against ST induced cytotoxicity for 16 hours post challenge. We orally gavaged these two probiotics to Th1 biased C57BL/6 mice and Th2 biased BALB/c mice with or without ST and gave a booster dose of probiotics 24 hours after
first gavage. We found that there was severe inflammation and colitis in the group of mice treated with ST after 3 days of infection and they eventually died after 6 days in the case of BALB/c and 5 days in the case of C57BL/6. We have seen that mice treated with BC and ST also have severe inflammation and colitis. However, LA and ST treated mice have no inflammation and colitis. Although both these mice strains are inbred and have little genetic difference; their response to ST, STBC and STLA were different. This differential response might be because of the different composition and abundance of gut microbiota. We profiled the gut microbiota using 16s rRNA V3 sequencing. While BALB/c microbiota was mainly dominated by firmicutes, C57BL/6 microbiota was composed of firmicutes and bacteroides in equal percentage. While major SCFA producers in BALB/c are lachnospira, that of C57BL/6 are morella. We confirmed the differential species through qRT-PCR and found that Butyricoccus pullicaecorum and Faecalibacter prausnitzii numbers increased drastically in STLA treated BALB/c mice. These are the two key bacteria found to be depleted in IBD, IBS and CD patients because of severe colitis occur. These are the two key bacteria which produce butyrate and propionate from complex carbohydrates which has anti-inflammatory property. In the case of C57BL/6 mice Clostridium lepum, Clostridium polysaccharolyticum, Prevotella dentalis and Herbinix hemicellulosilytica are the SCFA producers which increased with STLA treatment. LA might have reversed the inflammation caused by ST in this group of mice by inducing the growth of SCFA producers. But BC could not modulate the microbiome of the mouse gut in a way which could induce anti-inflammation. The inflammatory conditions in the ST and STBC treated mice are further confirmed through inflammatory gene expression in gut wall tissue and lesions in the gut evaluated through histopathological studies.
6.2 Conclusions

LL-37 or indolicidin primed THP-1 cells can efficiently protect themselves against ST induced cytotoxicity for 16 h post challenge. The genome wide gene expression study shows that pro-inflammatory and anti-apoptotic signaling in THP-1 cells treated with indolicidin was mediated through TNFRSF1A, followed by activation of NFkB and c-JUN. However, LL-37 treatment was mediated through IL1R, followed by activation of NFkB and NFAT2. Though immune modulation by LL-37 and indolicidin was partly known before, our data established the complete gene expression and signaling mechanism. The conjugation strategy enhanced the immune modulating efficacy of these two peptides by 1000 fold, which will reduce the cost of these peptides for antimicrobial treatment, thereby increasing treatment access to a wider population of developing countries.

The use of Lactobacillus and Bacillus species as probiotic dietary supplements is increasing. Each year new strains of these two genera are studied and added to the probiotics list based on their immune modulatory and antimicrobial activities. We screened two new strains of LA and BC, which displayed significant effects on modulating innate immune responses in murine macrophages and protected their cells from ST induced cytotoxicity. These two strains adhere to the surface of macrophages and have moderate cytotoxic activity. We tested LA and BC in mice as probiotics with respect to their ability to modify gut microbiota and inducing innate mucosal immunity. Microbial diversity was reported to be depleted in CD, UC, IBD, IBS, CDAD and AAD. Here we report the microbiota is also depleted in ST induced diarrhea accompanied with severe gut inflammation. However the LA induces several SCFA producing bacteria in the gut
which maintained the gut homoeostasis. But BC failed to induce such bacteria, rather induced proteobacteria which enhanced inflammation even further. So we propose that depletion of microbial diversity can be regarded as a marker for several gut related diseases. While probiotics was supplemented with many antibiotic medications to maintain the gut microbiota, it should be kept in mind that few probiotics species like BC may trigger more inflammation during ST infection. Thus each species and each strain of probiotics should be checked for their compatibility with a particular type of infection. Our comparative analysis of gene expression dynamics in macrophages following treatment with LA and further confirmed *in-vivo* in mice in maintaining gut microbial homoeostasis confirms the potential of LA MTCC10307 as a probiotic. Immune modulation by LL-37, indolicidin, and LA MTCC10307 followed by protection against ST infection, have shown the promise of the idea to combat infection by modulating natural mechanisms in the host through evolution proof manner.

### 6.3 Key Findings from the study

- Efficacy of Indolicidin and LL-37 was increased by 1000 fold by conjugation with CNT and GNP.
- Immune-modulation by Indolicidin was signaled through TNFRSF1A followed by activation of NFkB and c-JUN.
- Immune-modulation by LL-37 was mediated through IL1R, followed by activation of NFkB and NFAT2.
- LA (MTCC-10307) and BC (MTCC-8326) modulated immune genes in macrophages (Raw264.7 and THP-1) without inducing inflammation and protected them from ST (MTCC-3232) induced cytotoxicity.
BALB/c microbiota is composed of 83.7% firmicutes, 11% bacteroides, and 3.3% proteobacteria. The major genera in BALB/c microbiota were moryella (39.7%) and lachnospiracea (14.4%).

C57BL/6 microbiota consists of 49.5% firmicutes, 46.2% bacteroides, and 3.4% spirochaetes. The major genera in C57BL/6 microbiota were 54.9% prevotella, 16.7% oscillospira.

BALB/c microbiota is more diverse than C57BL/6 microbiota. This might be the cause why BALB/c microbiota is less perturbed by ST than that of C57BL/6.

- Butyrivibrio pullicaecorum, Clostridium scindens, Candidatus arthromitus, Clostridium tertium, Clostridium xylanolyticum and Faecalibacterium prausnitzii are the SCFA producing species induced in STLA treated BALB/c mice for countering inflammation.
- Clostridium lepum, Clostridium polysaccharolyticum, Prevotella dentalis and Herbinix hemicellulosilytic are the SCFA producing species induced in STLA treated C57BL/6 mice for countering inflammation.
- BC prescribed with antibiotics to maintain gut microbiota in typhoid cases may induce severe dysbiosis and inflammation in gut.

6.4 Methods developed during the course of this project

- Extraction of gDNA from gut contents (Details in Methods section).
- Designing species specific primers against 16s rRNA gene (Details in Methods section and Annexure 8).
- Profiling microbial composition through qRT-PCR, qualitatively and quantitatively. (Details in Annexure 8).
6.5 Future Prospective

- CNT and GNP conjugated indolicidin and LL-37 has 1000 fold better efficacy *in-vitro*, than the free peptides with respect to immune modulation in macrophage. However their efficacy needs to be proved *in-vivo*.

- High microbial diversity can be considered as a marker of good gut health. We have seen that ST and STBC decreased microbial diversity in BALB/c and C57BL/6 mice, which in turn increased inflammation. Also it was reported that microbial diversity was reduced drastically in CD, IBD and IBS patients who have severe inflammation in the gastrointestinal tract.

- Probiotics and prebiotics which can induce higher microbial diversity in the host can have significant implications in clearing the infectious microbes out of the gut, by depleting the substrates which sustain infectious microbes in gut.

- Morella, lachnospira, and prevetolla are the genera which contains many species producing SCFA from complex carbohydrates. Probiotics which can induce these genera can be used to treat the gut inflammatory disorders like CD, IBD and IBS.