Cytokines play a major role in determining protection and pathology in infection and inflammatory diseases. Epigenetic modulators, signalling components and metabolic flux in conjunction may regulate a disease outcome. However, it is crucial to identify a potential target in order to develop precision bio- synthetic therapeutic molecules and moreover an accurate delivery of those bio-synthetic devices. Systems and synthetic biology aims to reconstruct robust signalling models and identify potential targets to design or assemble existing bioparts or bio-components for useful bioproperties. Bio-engineered cells equipped with bio-synthetic circuits can be employed in bio-medicine for pathway designing, fine tuning of target expression and molecular engineering. Through our work we have studied reciprocal expression of IL-10 and IL-12 in infection model and we aim to generate bio-synthetic devices for bio-engineering of synthetic cells.

International studies on IL-10 and IL-12 reciprocity:

(Uyemura, K et al; 1996) The cross-regulatory role of IL-10 was indicated by the expression of IL-10 in atherosclerotic lesions, and they demonstrated that exogenous rIL-10 inhibited LDL-induced IL-12 release. This data suggested that the balance between IL-12 and IL-10 production contributes to the level of immune-mediated tissue injury in atherosclerosis

(Miguel Aste-Amezaga et al; 1998) The effect of IL-10 on IL-12 gene regulation in human PBMCs and purified monocytes stimulated by *Staphylococcus aureus* or LPS showed that IL-10 exerts its suppressive effect on *IL-12 p40*and *p35* as well as *TNF-α* gene expression mainly at the transcriptional level by a mechanism that requires de novo protein synthesis.

(Antonio Sica et al; 2000) Defective IL-12 expression is due to autocrine production of IL-10 and is paralleled by lack of NF-κB activation. Commitment of macrophages to high production of immunosuppressive cytokines may represent a powerful strategy used by tumors to escape immune surveillance.

(Bacellar O et al; 2000) Evaluated the role of IL-12 in the recovery of the ability to produce IFN-γ and whether or not IL-4, IL-10 and/or TGF-β could suppress IFN-γ production by PBMC from treated VL patients.

(Tiago S Medina et al; 2001) The relationship between cytokine IL-10/IFN-gamma levels, parasitaemia, and their gene polymorphisms was examined and the participation of pro-inflammatory and regulatory balance during a natural immune response in *Plasmodium vivax*-infected individuals was observed.

(Ma, Xiaojing et al; 2005) IL-10 and IL-12 play very important immunoregulatory roles in host defense and immune homeostasis. Being anti- and pro-inflammatory in nature, respectively, their functions are antagonistically opposing. Hence, more caution is needed in interpreting data derived from studies of individual cytokine or receptor chains.

(Kevin M Dennehy et al; 2009) A collaborative response mediated by Dectin-1 and TLR2 showed enhanced IL-23, IL-6 and IL-10 production in Dendritic cells, while down-regulating IL-12 relative to the levels produced by TLR ligation alone.

India is one of the countries which have reported several cases of Leishmaniasis; 1.14 to 8.76 per 10,000 populations, with an average incidence rate of 3.05. It is majorly reported in Uttar Pradesh, Bihar, Jharkhand, and West Bengal and in few parts of Gujarat and Maharashtra. Hence it becomes a prime importance that studies on cytokines should be centered on India.

Nation-wide studies on IL-12-IL-10 reciprocity:

(Varadhachary AS et al; 2001) Immunomodulatory role of LDLs offer a potential means to upregulate IL-10 production which inhibits IL-12 production and prevent arterial inflammation.

(Ram Kumar Mathur et al; 2004) *Leishmania* skews CD40 signaling toward ERK-1/2, inducing IL-10, which inhibits activation of CD40-induced p38MAPK and expression of inducible nitric oxide synthase-2 (iNOS-2) and IL-12. Whereby, *Leishmania* differentially modulates CD40-engaged, reciprocally functioning signaling modules, and provide a new conceptual framework for immune homeostasis.

(Dey P et al; 2016) Showed the impact of IFN and IL-10 pathways on Rac2-iNOS interaction leads to over-production of NO and thereby causes chronic inflammation in the RA synovium. The interplay between STAT1 and RAC2 in the regulation of NO could have implications for the identification of therapeutic targets for RA.

(Sweta Karan et al; 2016) Cytokines are widely used as adjuvants in mammals. The dimeric alpha helical structure and function of IL-10 of *L. rohita* as a key regulator of anti-inflammatory response have remained conserved during evolution; application of the r*Lr*IL-10 to control excessive immune response in fish can be studied.

(Vishakha Bhurani and Sarat Kumar Dalai; 2018) A balance between IL-10 and IL-12 secretion by host cells helps in maintaining homeostatic state. A slight imbalance in any of the processes of their function can lead to immunopathology, cancer, autoimmunity and chronic established infections. Targeting therapeutics towards maintaining the balance could help the host restore the equilibrium.

(Alti Dayakar et al; 2019) The understanding of wide-spectrum of cytokines and their interaction with immune cells that determine the clinical outcome of *Leishmaniasis* is helpful for pathology of any infectious disease. There are key set of cytokines that are involved in the disease progression namely IL-10, TGF-β, and IL-4 and host protection namely IFN-γ, IL-12, TNF-α, and IL-2 during VL. Measuring the ratio of cytokines is a promising approach. For example, IFN-γ/IL-10 ratio is predictive of disease severity in VL.

(Waghmare et al; 2019) showed that serum levels of inflammatory TNF-α IFN-γ and anti-inflammatory cytokines IL-10 and IL-8 among different TB groups showed that TNF-alpha and IFN-gamma have important roles in immune response and might be considered as indicators for response to ATT. However, high levels of IL-10 with low IL-8 appear to be associated with poor outcome and possibility of relapse.

Work done so far:

Reconstruction of healthy state model (IL-12 regulating IL-10) and diseased state model (IL-10 regulating IL-12) identified reciprocal expression of both cytokines at 6 h time point through simulations. PCA and flux analysis revealed NFAT5, SHP-1, IL-12, IL-10, IFN-γ and NO as key components in regulating model robustness. IL-12, NFAT-5, SHP-1 were identified as cross talk points in both models which was later validated using western blot analysis at different time points of infection in RAW264.7 cells with *L.major*. Post 6 h expression of IL-10, IL-12 and SHP-1 was observed to be significantly altered (Figure 1). Using single cell ATAC sequencing, we have identified a distinctive subset of macrophages at 6 h infection time point sample, termed as "sleepy macrophages." These macrophages exhibit downregulated housekeeping genes while expressing a unique set of variable features which included M1 and M2 associated regulatory genes such as Mef2c, Isl1, Fosl2, Rxa, Jun, Pparg, Ascl1, Onecut2 and Gata1. These sleepy macrophages exhibited similar expression pattern of IL-10 and IL-12 in peritoneal macrophages infected with *L.major* as observed in RAW264.7 cells. These macrophages had reduced expression of MHC-II and were G0/G1 phase of cell cycle (Figure 2). As NFAT5 was particularly associated for regulation of IL-10 and IL-12 expression, we developed high salt diet Balb/c mice model. NFAT5 expression is associated with hypertonicity; hence we used HSD mice to study infection and inflammation pattern associated with NFAT5. In Figure 3 we observed expression of NFAT5 in HSD mice infected with *L.major* is higher as compared to infection model. Expression of SHP-1 was significantly downregulated in HSD model.

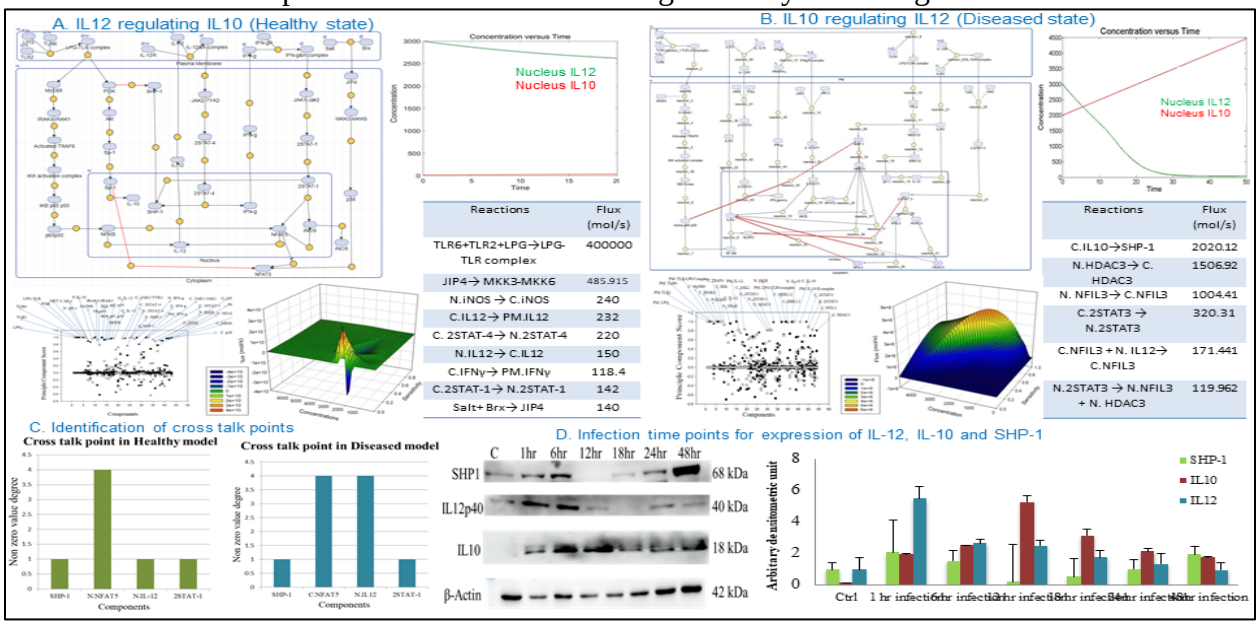


Figure 1: Reconstruction of mathematical models with its analysis and validation

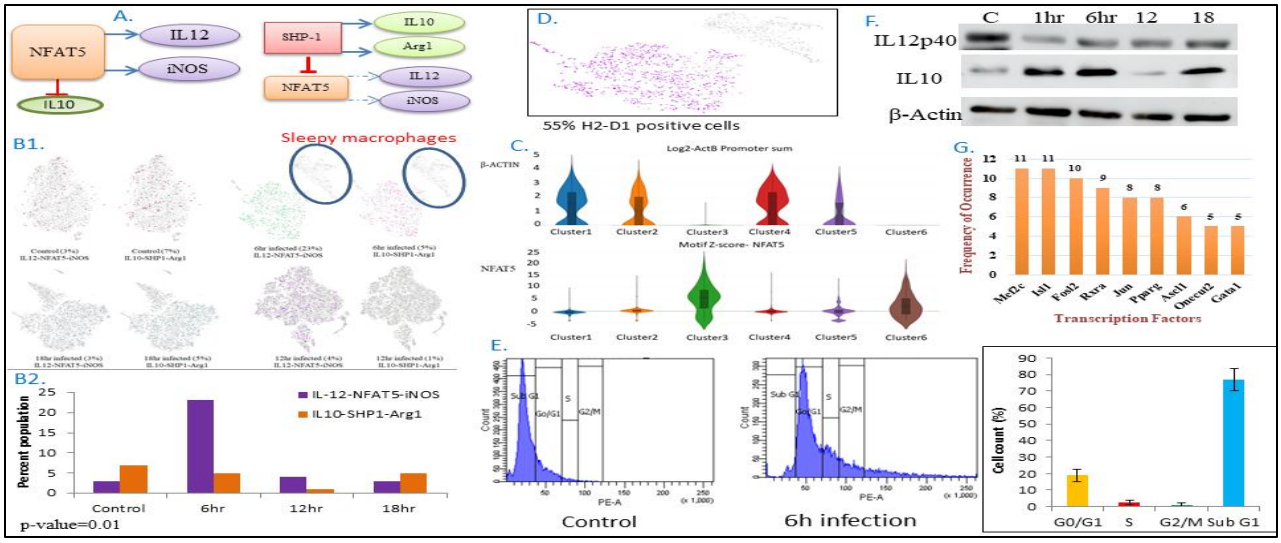


Figure 2: Single cell ATAC sequencing to identify sleepy macrophages and its features.

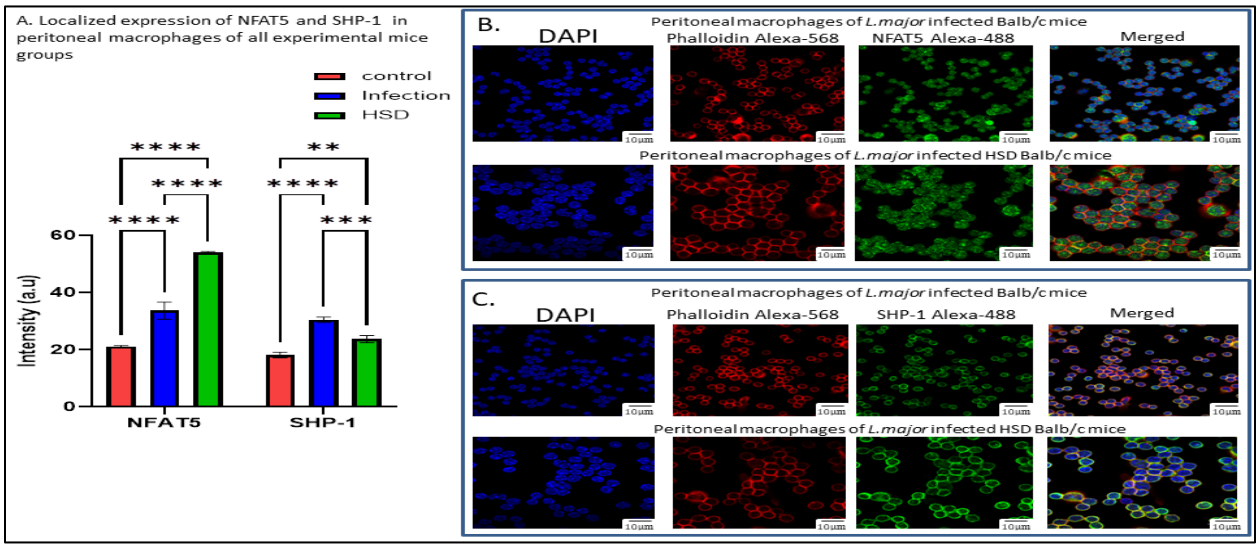


Figure 3: Localized expression of NFAT5 and SHP-1 in infection and HSD model.

The host response to CL induced by *L.major* was examined in both infected and HSD mice groups. Notably, there was no significant difference in the weight of mice in the HSD group. However, a marked reduction in lesion size was identified in the infected HSD group compared to other groups. Analysis of NFAT5 expression in peritoneal macrophages revealed higher levels in the HSD group compared to the other groups. Furthermore, the cutaneous lesions in HSD mice exhibited an intact skin barrier, reduced immune cell infiltration, and smaller vacuole sizes compared to the infected group. Interestingly, the lesions in HSD mice showed increased NFAT5 expression and decreased SHP-1 expression, in contrast to the infected group, which exhibited high SHP-1 and low NFAT5 expression. These findings suggest distinct immunological responses and skin barrier characteristics in HSD mice during *L.major*-induced CL (Figure 4). To investigate the inflammatory response to infection in both infected and HSD mice, we

employed FACS to identify M1 and M2 macrophages expressing CD80 and CD163 cell surface markers, respectively, in the context of CL. Our observations revealed a significant decrease in CD80 positive (CD80+) population and increase in CD163 positive (CD163+) population in infected mice lesion. Conversely, the CD80+ population was higher in HSD mice and CD163+ cell population was notably lower in the HSD group compared to infected mice. Additionally, nitrite level estimation, serving as an indicator of NO in the sera, indicated that HSD mice may exhibit elevated NO levels compared to the control and infection groups. Furthermore, cytokine levels of IL-12 and IL-10 were assessed using ELISA. Notably, IL-12 levels were significantly lowest in the infected mice group, whereas HSD mice exhibited a higher concentration of IL-12. Results showed lower levels of IL-10 in HSD mice compared to other experimental groups, with the infection group displaying a higher concentration of IL-10. These findings shed light on the immune responses and cytokine profiles in the context of CL (Figure 5).

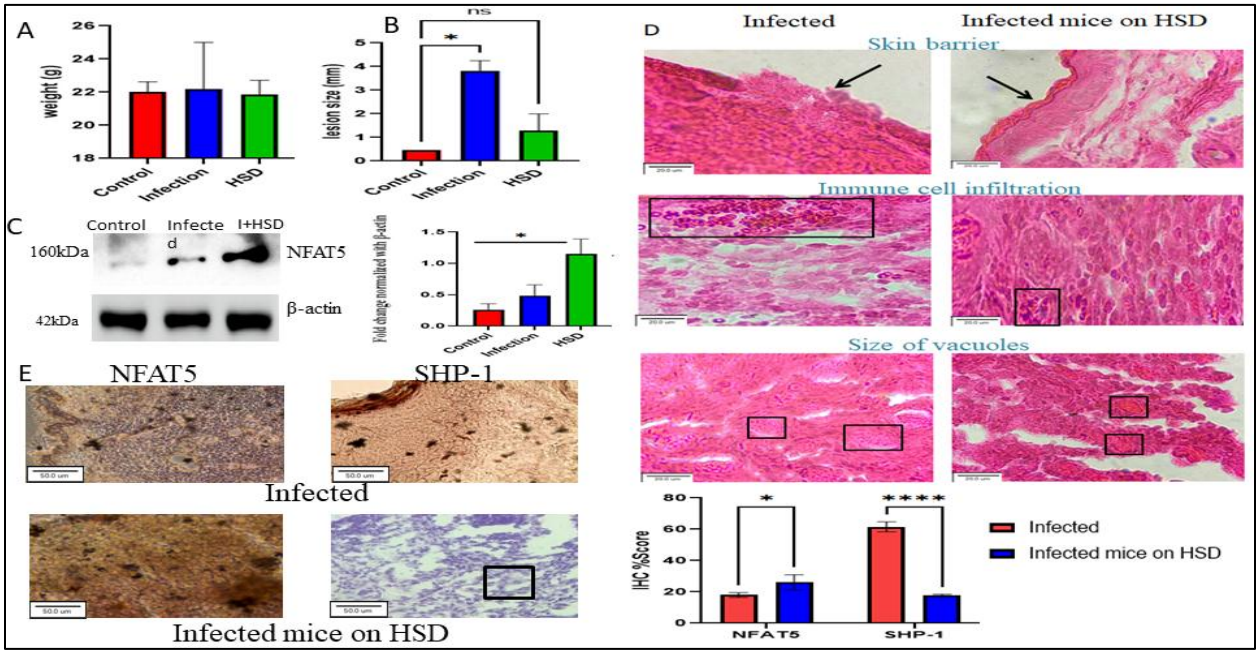


Figure 4: Analysis of NFAT5 and SHP-1 expression in infection and HSD mice.

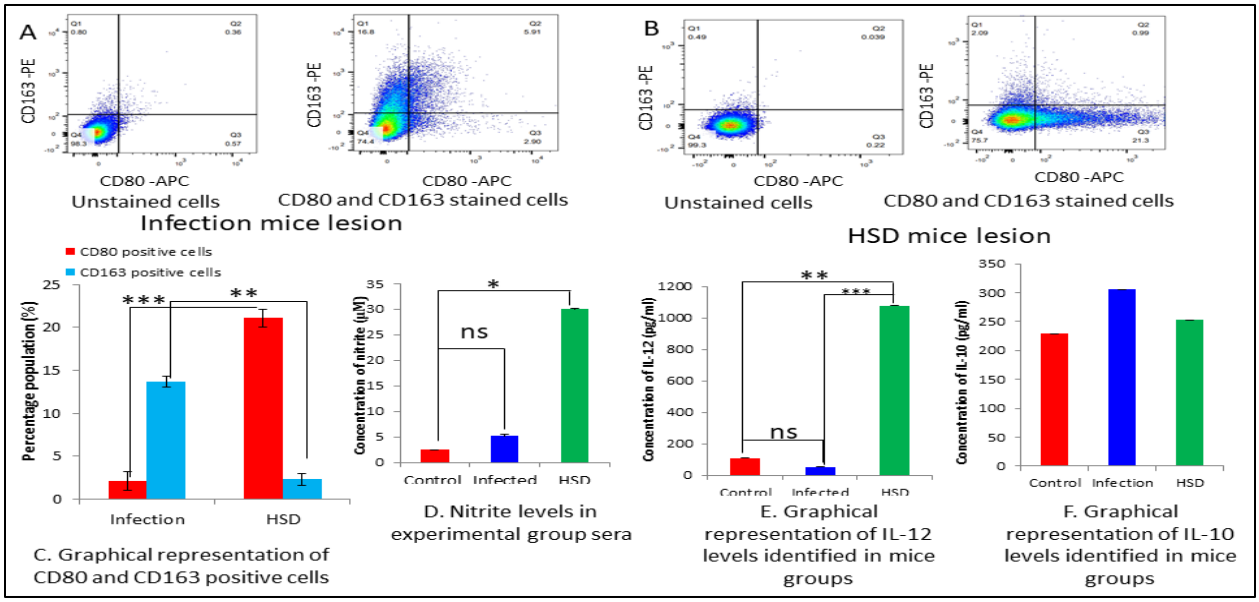
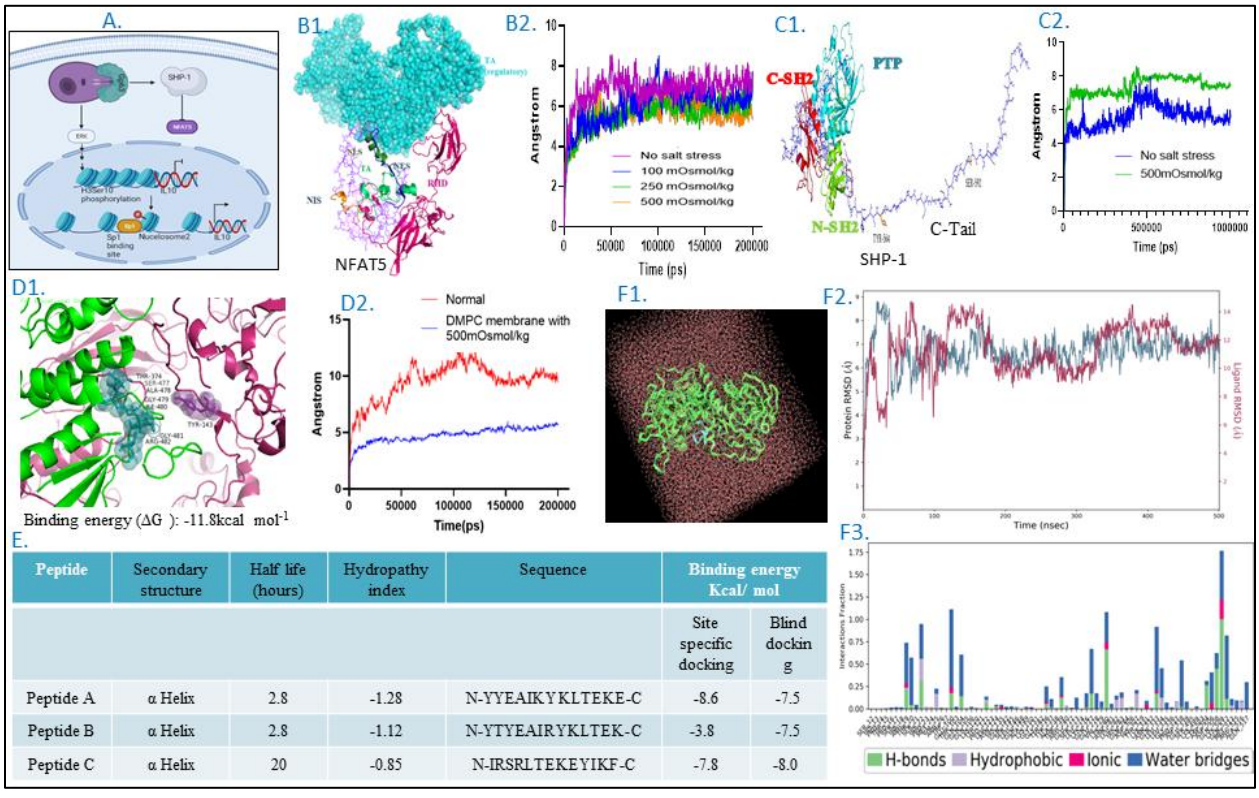


Figure 5: Analysis of immune response to *L.major* infection in all experimental groups.

To inhibit SHP-1 phosphatase activity against nuclear localization signal of NFAT5, we predicted the structure of both proteins and studied their interactions in infection and inflammatory hypertonic conditions. We predicted the binding residues of SHP-1 and designed inhibitory peptides against those residues. We studied membrane stability and peptide SHP-1 interactions in infection and hypertonic condition (Figure 6).



E.

Peptide	Secondary structure	Half life (hours)	Hydropathy index	Sequence	Binding energy Kcal/ mol	
					Site specific docking	Blind docking
Peptide A	α Helix	2.8	-1.28	N-YYEAIKYKLTEKE-C	-8.6	-7.5
Peptide B	α Helix	2.8	-1.12	N-YTYEAIRYKLTEK-C	-3.8	-7.5
Peptide C	α Helix	20	-0.85	N-IRSRLTEKEYIKF-C	-7.8	-8.0

Figure 6: Structural and interaction studies of NFAT5 and SHP-1 with peptide designing, screening and its validation.

The concentration for peptides to significantly eliminate intracellular parasite was observed to be 250µg/ml which was estimated through parasite load assay on peritoneal macrophages. These peptides were introduced intra-lesionally in infected ears of mice they should non significant effect on their weight however, the lesion size significantly reduced. IL-10-/- and IL-12 -/- mice were taken as cytokine control. qRT-PCR and Griess assay revealed that Peptide A showed significant NO production. Also, higher number of CD80 positive cells was observed in ear lesion of PepA mice. ELISA from sera isolated from mice groups highlighted PepA to have high IL-12 expression as compared to IL-10. Western blot analysis showed expression of SHP-1 and P-SHP-1 was lower in PepA mice peritoneal macrophages. Nuclear localization of NFAT5 was highest in case of PepA mice (Figure 7). Nuclear localization of NFAT5 was higher in IL10-/- as compared to IL-12-/-. Cytokine profiling of both knockouts highlighted that IL-10-/- have higher pro-inflammatory cytokine expression and higher Nos2 expression (Figure 8).

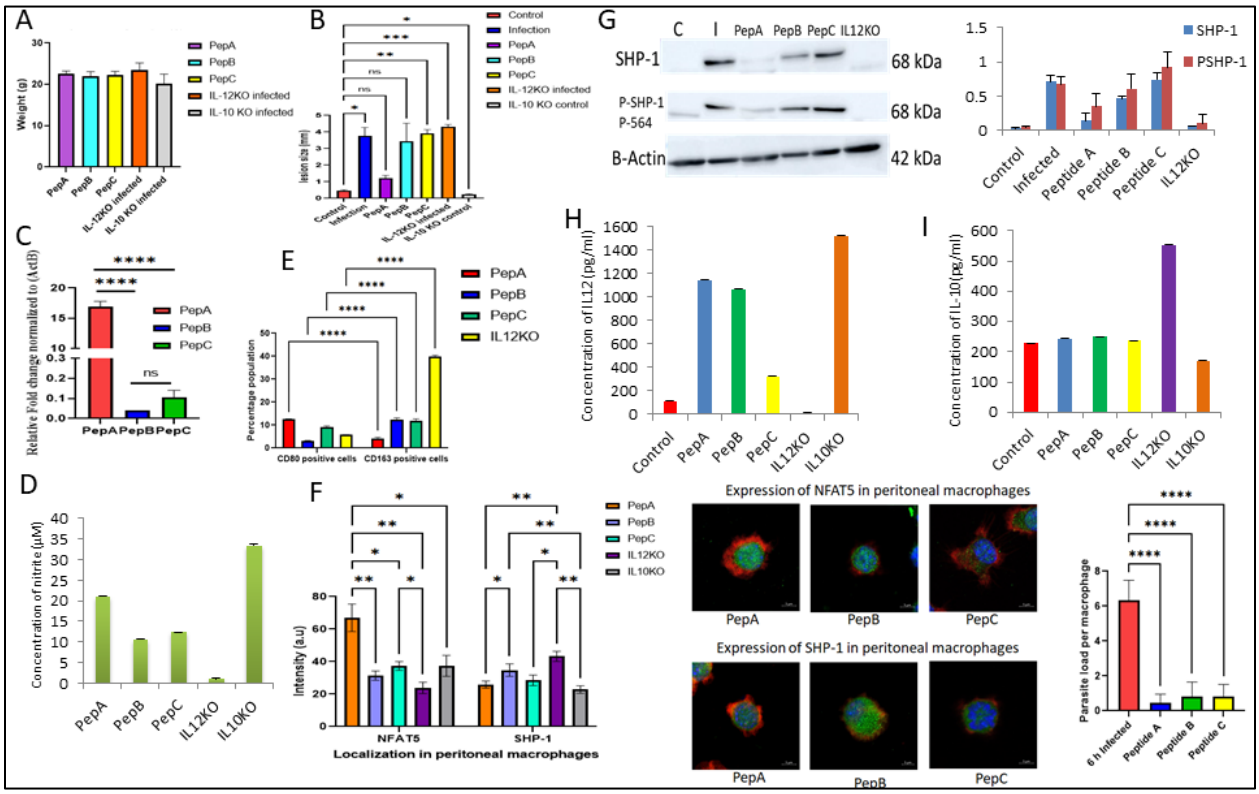


Figure7 : Effect of peptides on host immune response with its effect on NFAT5 and SHP-1.

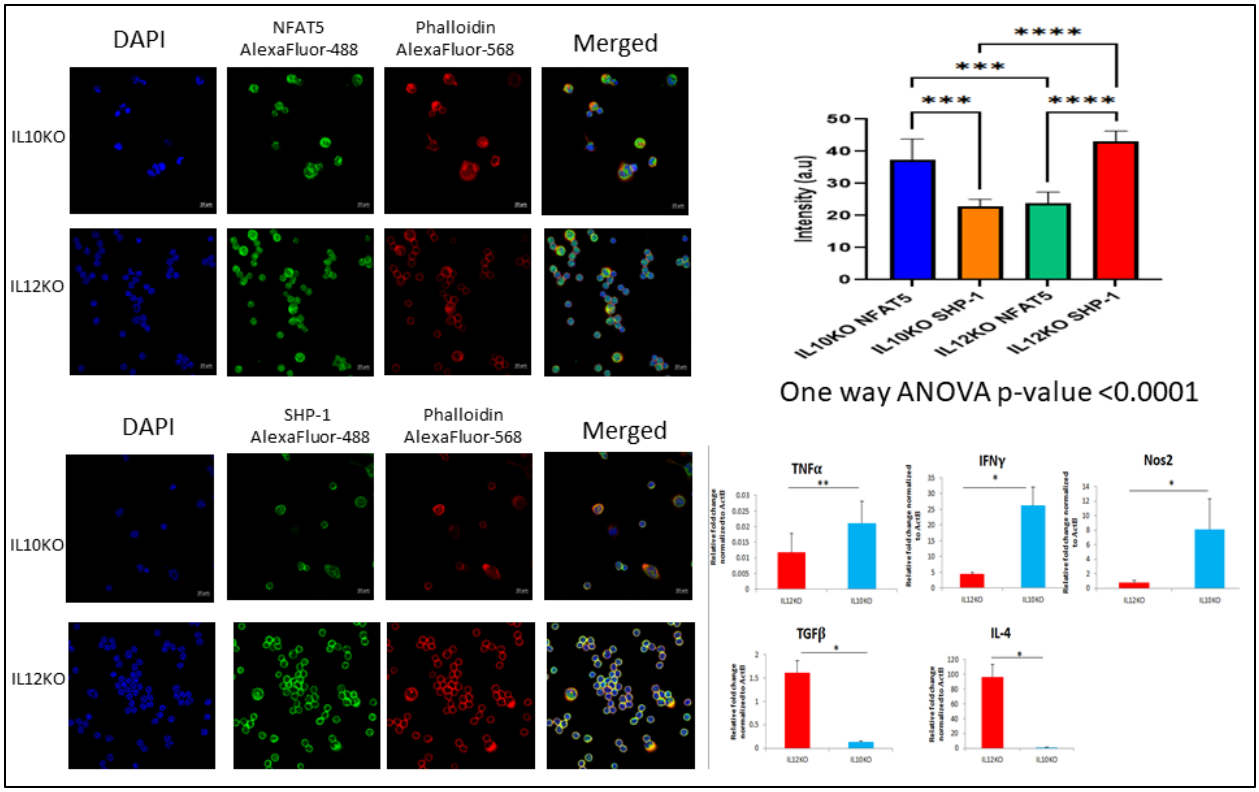
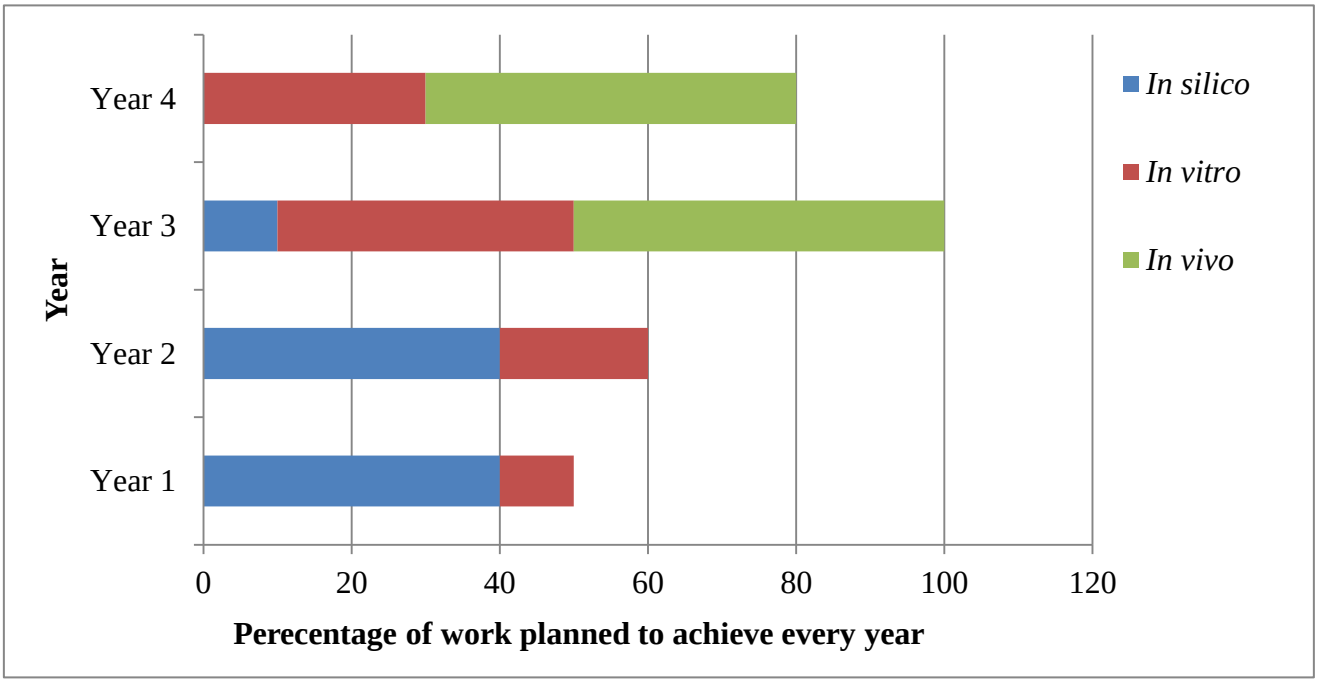


Figure 8: Cytokine profiling and expression analysis of NFAT5 and SHP-1 in IL-10-/- and IL-12-/-.

Work plan



	Year 1	Year 2	Year 3	Year 4
In silico	Reconstruction of mathematical models for infection and inflammation model.	Domain analysis of the target.	Designing of Tetracycline operon (TetON) based inducible biosynthetic device.	
	Simulations, local sensitivity analysis, Principle component analysis, model reduction	Motif identification for bio-synthetic device development	Deterministic simulation of iGEM assembled parts for 100 time unit.	
	Identification of cross talk points and validation *in vitro*	Generation of virtual library of bio-synthetic devices against the targeted motif	Molecular dynamics simulation of biosynthetic molecule with target protein	
	Phylogeny and Molecular clock analysis of identified target.	Artificial intelligence based position-specific scoring matrix employing screening of biosynthetic molecules.	Analysis of single cell ATAC sequencing data to identify cell population having unique phenotype.	
	Structure prediction of the Identified target.	Blind docking and information driven docking of biosynthetic devices against the target.	Validation of biosynthetic device using boolean networks and multi-objective genetic algorithm	
In vitro	Cell culture: RAW 264.7 can be cultured as per: RAW 264.7 (A TCC® TIB71™).	Infection of RAW264.7 cells with *L.major* stationary phase promastigotes and western blot analysis for the target.	Introduction of biosynthetic device in plasmid vector.	Transfection of RAW264.7 cells and peritoneal macrophages with biosynthetic device to generate bio-engineered cells.
	L.major (MHOM/Su73/5AS KH) can be grown in RPMI+ 20% FBS at 27ºC in T25 culture flask.	Single cell ATAC sequencing of infection time points samples.	Transformation of *E.coli* DH5α competent strain with biosynthetic device.	Sorting of cells expressing biosynthetic device.
			Isolation of bio-synthetic device using	Localized expression analysis of target upon

			mini-prep method.	expression of biosynthetic device through confocal microscopy
			Agarose gel electrophoresis of isolated biosynthetic device. Restriction digestion and ligation of biosynthetic device.	Parasite load assay post infection in transfected cells.
In vivo			Infecting Balb/c mice with *L.major* promastigotes.	Adoptive cell transfer of biosynthetic devices positive cells at site of infection in Balb/c mice
			Immuno-histochemistry of lesion for expression analysis of target	Flow cytometry analysis to identify immune cell sub populations in biosynthetic device recipient mice and knock out mice.
			Flow cytometry analysis to identify immune cell sub populations	Cytokine profiling of biosynthetic device recipient mice and knock out mice.
			Cytokine profiling of infected mice using qRT-PCR	ELISA of cytokines using sera samples of biosynthetic device recipient mice and knock out mice.
			ELISA of cytokines using sera samples of mice.	
			Localized expression analysis of target on peritoneal macrophages.	

Expected outcomes of every year

Year 1: Mathematical model will be reconstructed and reduced to understand infection and inflammation associated signalling components with Systems perspective. Key components and potential therapeutic target will be identified. Validation of potential therapeutic target using

signalling inducers and inhibitors will validate cross talk point. Sequence conservation from phylogenetic analysis will identify potential isoforms and family members of therapeutic target.

Year 2: Domain analysis and motif identification will be performed in order to identify conserved regions of the potential targets against which biosynthetic device will be designed using synthetic biology. Genetic parts will be cloned into plasmids to get a regulatory gene construct. Single cell ATAC sequencing will identify open chromatin and closed chromatin regions in samples which will shed insights into immune cell phenotypes present in sample.

Year 3: Validation of biosynthetic device at spatio-temporal level will be done in order to see parasite clearance and long term effect of biosynthetic molecule in cytokine homeostasis. MDS studies between peptide and target will be performed in order to understand how it might sustain *in vivo* system. *Balb/c* mice will be infected with *L.major* to develop lesion. Analysis of innate immune response and effect of parasite in mice through cytokine expression will be studied.

Year 4: Bio-synthetic device will be transfected to generate bio-engineered immune cells which can specifically target intracellular parasite. Bio-engineered cells transfected with biosynthetic device will be injected into *Balb/c* mice to see its effect on parasite clearance and host immune response.

Future outcome of bio-synthetic devices in inflammation/ infection models for developing bioengineered synthetic therapeutics

- The bio-engineered cells expressing biosynthetic device against the target will bind to the target protein and act like a therapeutic to inhibit its activity in the host.
- Infection and inflammation will be reduced in the recipients to achieve disease resolving effect.
- Bio-synthetic device can be given in the form of vaccine and its effect can be checked by healing of the lesion.
- Bio-engineered cells can be explored as an output module for cytokine expression, surface markers and for immuno-metabolic flux.
- Bio-synthetic devices can be engineered to generate an array of immuno-engineered cells for precision targeting such as T-cell therapy, B-cell directed CAR-T cell therapy, biomolecule sensors, generation of bio-synthetic proliferator cells.

26/8/2024