6. Discussion

The choice of a vaccine candidate has been the perplexing one in any infectious disease. Generally, the virulence factors were targeted for many vaccine-preventable diseases as these factors were proposed to be responsible for infection and pathogenesis. Life-long immunity conferred to cured cases of leishmaniasis indicated that prophylaxis-based protection is possible against this parasitic disease. Choice of a vaccine candidate that elicits Th-memory based host-protective immune response has been quite tedious, as *Leishmania* has many virulence factors, some of which were put to clinical trials for an efficacious anti-leishmanial vaccine but have not yielded enthusiastic results (264,265).

In this study, we have tried to identify the virulence factors of *L. major* based on the differences in the transcriptome profile of virulent and avirulent strains. We performed qPCR analysis of leishmanial genes based on previous pan-genome microarray and validation qPCR data of different *Leishmania* strains. We observed that genes such as NAD+ synthase, Adenylate kinase, LmjF_36_3850, PAP, TDR-1, LmjF_36_3850 are significantly down-regulated in HP-infected macrophages compared to LP-infected macrophages, with or without IFN-γ stimulation.

Based on above observations, we assessed the immunogenicity and post-challenge protective efficacy of LmjF_36_3850 in a susceptible BALB/c mice model. Increases in the levels of IgG1 as compared to IgG2a have been attributed to pro-leishmanial immune response and vice-versa in both human CL and mouse models (145,266). Herein, LmjF_36_3850 DNA vaccination increased IgG2a and IgG1 levels and elicits a mixed T-helper response. Even after *L. major* challenge infection, vaccinated mice had higher levels

of IgG1 antibodies as compared to IgG2a. In spite of a higher IgG2a/IgG1 ratio in the vaccinated mice as compared to controls, the progress of *L. major* infection was not hindered. Footpad lesions of vaccinated mice were comparable to control vector primed mice and insignificantly less than the unprimed *L. major* infected BALB/c mice.

Expression of major immuno-regulatory cytokine IL-10, Treg-transcription factor FOXP3 and IL-4 were higher in the lymphocytes of DNA vaccinated mice as compared to controls, while the expression of IFN-γ was decreased, albeit insignificantly **(Figure 10a),** corroborating the previous studies which associated IFN-γ associated Th1 response as a major anti-leishmanial factor (267) and IL-10 promotes parasite multiplication in susceptible mice (180). This increase in IL-10 expression may, in part, be responsible for higher parasite load observed in the vaccinated mice **(Fig. 10a).** On the other hand, CSA re-stimulated cells from the LmjF_36_3850 vaccinated mice produced lower IFN-γ, while the levels of IL-4 and IL-10, remained comparable to unprimed mice. The contradictory results observed in recall assay could be due to the overall cytokine profile of *Leishmania* specific T cells, rather than LmjF_36_3850 specific T cells, rapidly proliferating after CSA stimulation post-challenge infection. T cells from DNA vaccinated mice also express higher CTLA-4, which is the major co-inhibitory molecule during APC-T cell interaction in both *L. donovani* and *L. major* (268,269), suggesting immuno-regulatory, pro-parasitic conditions predominating in DNA vaccinated mice.

Flow cytometric analysis of T-helper memory subsets in study groups revealed an increase in IL-10 secreting effector and central memory Treg cells, but there were no differences in the population of IFN-γ secreting effector memory TH1 cells. Although an increase in

Central memory TH1 cells was observed, circulating effector memory T-cells are actively involved in parasite clearance rather than central memory cells. These results shed light on the fact that LmjF_36_3850 priming predominantly induces naïve T-cells to Treg subtype. One possible explanation is that LmjF_36_3850 may contain Treg and TH2 inducing epitopes in its sequence, although antigen-specific T-reg cells remain debatable (270).

Next, we tested heterologous prime-boost (HPB) vaccination strategy using LmAdeK as a vaccine candidate. LmAdeK expression was also higher in LP-infected macrophages, and the expression remained undetectable in HP-infected macrophages, with or without stimulation. Heterologous prime-boost (HPB) vaccination was found to be highly immunogenic and induced a better disease-eliminating response in many disease models (246). While DNA vaccination with LACK antigen was unable to protect mice from *L. donovani* infection (271), HPB strategy elicited higher IFN-γ and was efficacious against VL (272). HPB vaccination with LmAdeK elicited higher IgG2a levels and a higher IgG2a/IgG1 ratio **(Figure 16a)** suggesting that it induced a Th1-biased immune response. HPB-vaccinated mice maintained a higher IgG2a levels even after five weeks of challenge infection **(Figure 16b).**

Higher numbers of IFN-γ-secreting Th1 cells but fewer IL-4-/IL-10-secreting cells elicited by HPB vaccination may be responsible for reduced severity and burden of *L. major* infection in LmAdek-vaccinated mice. Moreover, CTLA-4 expression was undetectable in HPB-vaccinated group, which suggests that CTLA-4 is an important factor in *L. major* progression (273) and its inhibition may augment vaccine-induced immunity by influencing vaccination outcome (274).

Importance of central and effector memory T cells has been well established in CL as an indicator of vaccination efficacy. Mice vaccinated with LmAdeK using HPB strategy had lower population of Th2-T_{EM} and Treg-T_{EM} cells **(Figure 19e, 19i)** which are major anti-inflammatory, pro-leishmanial Th subsets. This corroborated with lower expression of IL-4 and IL-10 as observed in HPB vaccinated mice. Fewer IL-17 secreting Th17-T_{EM} cells in HPB vaccinated mice **(Figure 19g)** appeared to be important in controlling *L. major* infection, as they had been implicated in the progression of CL in BALB/c mice (190). The population of IFN-γ secreting Th1-T_{EM} cells was lower in HPB vaccinated group **(Figure 19c)**. As we observed higher levels of IFN-γ in lymphocytes of HPB vaccinated mice **(Figure 18)**, it was possible that IFN-γ secreting memory T cells might be rapidly activated and converted from memory to effector phenotype during *Leishmania* infection. Our data indicated that vaccination with LmAdeK using HPB strategy induced a higher Th1 and lower Th2/Th17 response reducing challenge *L. major* infection.