Utilization of Transcriptomics in Evaluation of Type I Interferon Response in Shaping the Protective Immunity Induced by Live Attenuated *Leishmania* Parasites

Telly Sepahpour, FDA/CBER/OBRR; Jalal Alshaweesh, Nagasaki University, Japan; Nazli Azodi, FDA/CBER/OBRR; Komudi Singh, NIH/NHLBI; Derek D. C. Ireland, FDA/CDER/OPQ; Valanezhad Farzaneh, Nagasaki University, Japan; Risa Nakamura, Nagasaki University, Japan; Abhay R. Satoskar, Department of Pathology, Ohio State University; Ranadhir Dey, FDA/CBER/OBRR; Shinjiro Hamano, Nagasaki University, Japan; Hira L Nakhasi, FDA/CBER/OBRR; Sreenivas Gannavaram, FDA/CBER/OBRR.

Our contributions are an informal communication and represent my own best judgement. These comments do not bind or obligate FDA

Abstract

Background: Leishmaniasis is a vector borne disease caused by the various strains of *Leishmania* parasites and is endemic across the world including the United States. The disease is transmitted to humans through the bite of infected sandfly and blood transfusions. Currently, there are no FDA-approved anti-leishmania vaccines or donor screening assays, while treatment options are limited.

Purpose: We aim to elucidate the role of type I IFN response in protection following immunization with genetically modified live attenuated *leishmania* parasites. Previous studies in parasitic and viral pathogenesis established that type I interferons (IFNs) adversely impact adaptive immunity.

Methods: RNA isolated from the inoculation site in C57BL/6 mice and Human PBMCs (n=4) following infection with wildtype *L. major (LmWT)* or centrin gene deleted *L. major (LmCen$^{-/-}$)* parasites was analyzed by the NanoString Platform 4.0 to identify differentially expressed genes. Flow Cytometry analysis was conducted on cells isolated from lymph nodes and spleens of C57BL/6 mice that were immunized with *LmCen$^{-/-}$* parasites and challenged with virulent *L. donovani* parasites. Cytokines (IFN-α/β, IL-2, IL-4, IFN-γ, IL-10) from the sera or splenocytes were detected via ELISA or flow cytometry respectively.

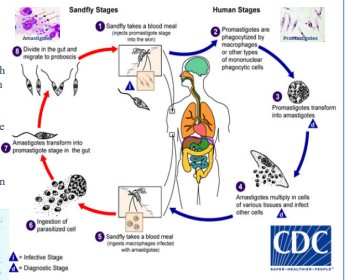
Results: Our results show that immunization with *LmCen$^{-/-}$* induces distinct type-I IFN genes such as IRF-7 compared to *LmWT* infection. The induction of a protective Th1 response as indicated by a strong IFN-γ, IL-2 and TNF production and a reduced IL-10 was coincident with a decline in the type-I IFN response as measured by IFN-α/β in the *LmCen$^{-/-}$* immunized mice indicating that type-I IFN and Th1 responses are negatively correlated.

Conclusion: Application of transcriptomic analysis to *LmCen$^{-/-}$* vaccine candidate identified signature transcriptomic patterns between the immunization and the infection environments specifically IRF-7 and IRF-3 that regulate type-I IFN responses. The adaptive immune response to immunization showed an upregulation of IL-2, TNF and INF-γ, hallmarks of a Th1 response with a concomitant reduction in the type-I IFN response suggesting that downregulation of type-I IFN response is critical to inducing protective immunity.

Introduction

Leishmaniasis

- The parasite Leishmania – blood borne pathogen
- Transmission mainly through sand fly bites as well as with blood transfusions
- There is no vaccine available currently
- With the vaccination approach, disease burden can be reduced.



Leishmaniasis, an insect vector-borne disease caused by the various strains of *Leishmania* parasites is endemic across the world including the United States. The disease is transmitted to humans through the bite of infected sandfly and blood transfusions. Currently, there are no FDA-approved anti-leishmania vaccines or donor screening assays, while treatment options are limited. Our laboratory developed a *Leishmania major centrin* gene deleted strain as a live attenuated dermotropic vaccine candidate for Leishmaniasis. Previous studies in parasitic and viral pathogenesis established that type I interferons (IFNs) are instrumental in the development of both innate and adaptive immune responses. However, the relevance of type-I IFN response in the development of protective immunity following immunization is not understood.

In this project, we aim to elucidate the role of IRF-7 mediated type I IFN response in protection following immunization with live attenuated *leishmania* parasites (*LmCen$^{-/-}$*).

Materials and Methods

Mouse Infection and Immunization: Female 6- 8-week-old C57BL/6 and IRF-7$^{-/-}$ mice were immunized with 1 × 10^6 laboratory or GLP grade total stationary phase *LmCen$^{-/-}$* parasites, by i.d. injection in the ear dermis.

Tissue Collection and Single Cell Suspension: Mouse sera were analyzed for cytokines and chemokines at the 21 days post-immunization and 14 days post-challenge experiments. Mouse ear tissue were analyzed for differential gene expression at 2 days and 7 days post-immunization with *LmCen$^{-/-}$*.

Flow Cytometry Analysis: Single cell suspensions of mouse Spleen and draining lymph nodes were prepared. Cells were counted, stained and analyzed by flow cytometry using cytometer Aurora (Cytek Biosciences, Fremont, CA)

Enzyme linked immunosorbent assays (ELISAs): ELISA was used to measure the kinetics of type I interferon (IFN-α/β) and IRF7 expression in sera of C57BL/6 mice at 2days, 7days, and 21days post immunization with *LmCen$^{-/-}$* and infection with *LmWT*.

NanoString: Extracted RNA from mouse ear tissue was analyzed on Mouse Immunology V2 panel using NanoString technology. (www.nanostring.com). and analyzed using ROSALIND platform (www.rosalind.bio).

Statistical Analysis: Statistical analysis of differences between means of groups was determined either by unpaired two-tailed Student t test or one- way ANOVA (with a post-test correction for type II error) or two-way ANOVA, using GraphPad Prism software. A p value < 0.05 was considered significant.

Results and Discussion

LmCen$^{-/-}$ immunized mice show distinct differential gene expression compared to *LmWT* infection

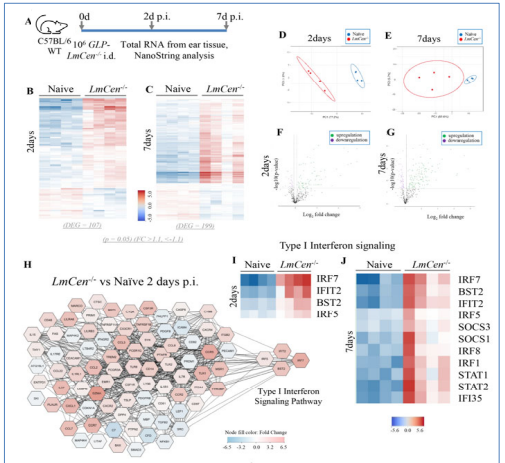
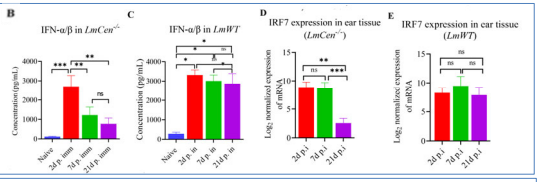


Figure 1: Distinct transcriptional profiles in tissue of *LmCen$^{-/-}$* immunized C57BL/6 mice. (A) Schematic representation of the study to identify differential expressed genes following immunization with *LmCen$^{-/-}$* parasites using the NanoString platform. **(B-C)** Heatmaps showing distinct clustering of the genes indicated by red-blue colors representing the log2 (fold change) in *LmCen$^{-/-}$* vs Naïve at 2-days p.i. **(B)** and 7-days p.i. **(C)**. **(D-E)** Principal component analysis scatterplot of normalized data at 2 days **(D)** and 7 days **(E)** p.i. Data from naïve set are shown in blue and data from *LmCen$^{-/-}$* set are shown in red. Values for each group are bound by an eclipse. **(F-G)** Volcano plots of *LmCen$^{-/-}$* vs Naïve at 2-days **(F)** and 7-days **(G)**, representing genes detected above background levels under each condition, are shown in black. Significantly upregulated genes are green, and significantly downregulated genes are purple. The x-axis represents the log ratio of the fold change, and the y-axis is the negative log of p-adj/p-value. p.i. post immunization. **(H)** The gene network through GO enrichment analysis of *LmCen$^{-/-}$* immunized vs Naïve at 2 days post immunization showing the upregulation of transcripts associated with the IRF-7 mediated type-I IFN response in the ear tissue of *LmCen$^{-/-}$* immunized mice. Gene Set Analysis (GSA) derived from heatmap clustering showing the expression of type-I IFN associated elements on **(I)** 2 days and **(J)** 7 days post-immunization.

Results and Discussion

LmCen$^{-/-}$ immunized mice show transient induction of IRF7 mediated Interferon α/β

Fig 2: (A) Schematic to measure kinetics of type-I Interferon protein expression in sera & IRF-7 expression in ear tissue following immunization or infection. (B) IFN-α/β levels at 2,7, & 21 days p.i (C) and LmWT at the identical time points. (D) Log2 normalized expression of IRF-7 expression in ear IRF7 in the ear by NanoString analysis at 2,7, & 21 days p.i with LmCen-/- (E) and infection with LmWT parasites.



IRF7$^{-/-}$ mice show enhanced Th1 protective response following immunization with *LmCen$^{-/-}$*

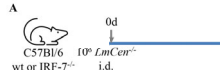
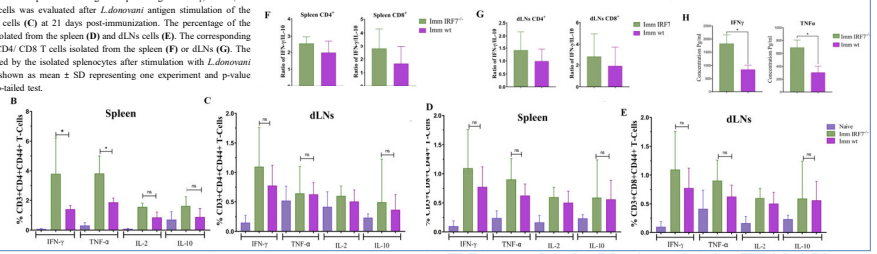


Fig 3: (A) Schematic representation of the experimental design. The percentage of IFN-γ, TNF-α, IL-2, and IL-10 producing CD4+ T cells was evaluated after *L.donovani* antigen stimulation of the isolated splenocytes **(B)** and dLNs cells **(C)** at 21 days post-immunization. The percentage of the cytokine-producing CD8+ T cells isolated from the spleen **(D)** and dLNs cells **(E)**. The corresponding ratio of IFN-γ/IL-10 produced by CD4/ CD8 T cells isolated from the spleen **(F)** or dLNs **(G)**. The levels of IFN-γ and TNF-α produced by the isolated splenocytes after stimulation with *L.donovani* antigen for 72hr **(H)**. Results are shown as mean ± SD representing one experiment and p-value determined with Mann-Whitney two-tailed test.



Development of *LmCen$^{-/-}$* induced protective immunity is correlated with IRF7 mediated downregulation of type-I IFN response

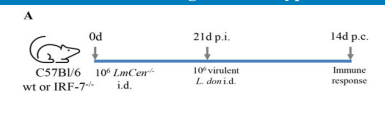
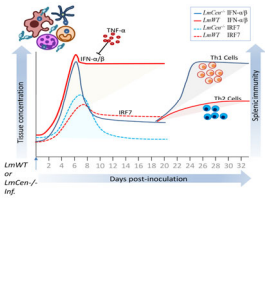


Fig 4: (A) Schematic representation of the experimental study. Percentage of IFN-γ, TNF-α, IL-2, and IL-10 producing T cells; activated CD4$^+$T cells were isolated from the spleen **(B)** or draining lymph nodes **(C)** and simulated with *Leishmania donovani* (FTAg) at 14 days post-challenge. Similar responses from the activated CD8$^+$ T cells isolated from the spleen **(D)** or draining lymph nodes **(E).** The ratio of IFN-γ/IL-10 produced by activated CD4$^+$ or CD8$^+$ T cells from the spleen **(F)** and draining lymph nodes **(G)**. Results are shown as mean ± SD and represent one experiment with the p-values determined by Mann-Whitney two-tailed test.



Conclusion

IRF-7 is induced in mice immunized with *LmCen$^{-/-}$* parasites that is downregulated within 7 days post-immunization. IRF-7$^{-/-}$ mice induce higher protective Th1 response than C57Bl/6 wild-type mice following immunization with *LmCen$^{-/-}$* parasites. Lack of IRF7 signaling enhances the protective Th1 response following challenge with virulent *L. donovani* in *LmCen$^{-/-}$* immunized C57Bl/6 mice. Reduction in IRF-7 mediated type-I IFN response is critical in the induction of Th1 immune response. IRF7 thus is the master regulator of type-I IFN response, and could be a biomarker of protective immunity.

References

1. Karmakar, S., Ismail, N., Oliveira, F., Oristian, J., Zhang, W. W., Kaviraj, S., . . . Nakhasi, H. L. (2021). Preclinical validation of a live attenuated dermotropic Leishmania vaccine against vector transmitted fatal visceral leishmaniasis. *Commun Biol, 4*(1), 929. doi:10.1038/s42003-021-02446-x
2. Zhang, W. W., Karmakar, S., Gannavaram, S., Dey, R., Lypaczewski, P., Ismail, N., . . . Nakhasi, H. L. (2020). A second generation leishmanization vaccine with a markerless attenuated Leishmania major strain using CRISPR gene editing. *Nat Commun, 11*(1), 3461. doi:10.1038/s41467-020-17154-z.
3. Haque, A., et al., *Type I IFN signaling in CD8- DCs impairs Th1-dependent malaria immunity.* J Clin Invest, 2014. **124**(6): p. 2483-96.
4. Kumar, R., et al., *Type I Interferons Suppress Anti-parasitic Immunity and Can Be Targeted to Improve Treatment of Visceral Leishmaniasis.* Cell Rep, 2020. **30**(8): p. 2512-2525 e9.
5. NanoString Technologies (www.nanostring.com)