Unraveling Pregnancy-Induced Modulation of the Innate Immune Response to Mycobacterium tuberculosis Through mRNA Sequencing of Infected Monocytes

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Abstract

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This thesis investigates the dynamics of monocyte-derived macrophage gene expression in response to Mycobacterium tuberculosis (Mtb) infection during pregnancy, focusing specifically on the third trimester. Using mRNA sequencing, we found significant changes in gene expression related to the innate immune response, particularly in the JAK-STAT pathway associated with tuberculosis reactivation. Analysis revealed over 500 differently expressed genes in infected samples, primarily involved in cytokine signaling and inflammation. Comparing third trimester of pregnancy to pre-pregnancy highlighted differences in pathways such as calcium signaling, MAPK, TNF, MTORC, and hypoxia signaling. Downregulated genes like GBP5, TNFRSF4, and NIBAN1 suggest compromised immune response in late pregnancy, potentially worsening TB infection or reactivation of latent TB. Understanding these changes could improve TB prevention, diagnosis, and treatment in pregnant populations.

Introduction

Pregnancy induces profound changes in a woman's immune system to accommodate the growing fetus. These immunological adaptations are essential to protect both the mother and the fetus, balancing immune tolerance towards the fetus with the ability to mount effective responses against pathogens(Erlebacher, 2013). While fighting infection, pregnancy presents an extra burden on the immune system, complicating the body's ability to respond effectively.

Tuberculosis (TB) caused by the organism *Mycobacterium tuberculosis* (Mtb) continues to be a major global health challenge, and pregnancy increases the likelihood of TB reactivation to twice the normal rate during postpartum period (Jonsson et al., 2020). The reactivation risk is further increased in the setting of HIV co-infection(Mathad & Gupta, 2012). Furthermore, in various cohorts, indicators of Mtb exposure that rely on T cell responses are diminished during pregnancy(Aghaeepour et al., 2017; Saha et al., 2022). It is crucial to understand how pregnancy affects body's first line of defense, innate immune response to Mtb, to improve health outcomes for pregnant women at risk of or suffering from TB.

Monocyte-derived macrophages play a crucial role in for the initial defense against Mtb and are the primary replicative niche for Mtb(Cohen et al., 2022; Huang et al., 2018). These immune cells are responsible for recognizing, engulfing, and destroying Mtb, as well as orchestrating a complex network of signaling pathways to mount an effective defense. Upon infection with Mtb, macrophages become activated via TLR (toll-like receptor) signaling, which in turn activates the NF-κB signaling pathway, leading to the production of pro-inflammatory cytokines such as TNF-α, thereby controlling a positive feedback loop.(Fallahi-Sichani et al., 2012; Poladian et al., 2023). This pathway also controls apoptosis and autophagy in the process of granuloma formation containing infection. Downstream of NF-κB activation, the production of interferons (IFNs) initiates the activation of the JAK-STAT signaling pathway. This pathway plays a pivotal role in maintaining macrophage polarization towards the secretion of pro-inflammatory cytokines. Through this coordinated response, macrophages contribute effectively to the immune defense against Mtb(Schindler & Plumlee, 2008; Zhou et al., 2019). Biological agents inhibiting this Janus kinase (JAK) family leads to reactivation of latent tuberculosis(Winthrop et al., 2016). Mtb also induces shifts in cellular metabolism, including glycolysis and oxidative phosphorylation, to create a favorable environment for its survival(Vrieling et al., 2020). Furthermore, Mtb infected macrophages that are high in lipid content and promote pathways involved in ferroptosis eventually leading to necrotic death and release of mycobacteria into the extracellular space(Amaral et al., 2019; Meunier & Neyrolles, 2019). These alterations in macrophage function also lead to changes in T-cell activation and memory phenotypes(Kaech et al., 2002).

During pregnancy, however, the function and behavior of monocytes undergo significant changes to ensure fetal tolerance and survival(Schumacher et al., 2014). The changes in immune responses are dynamic over the course of pregnancy(Aghaeepour et al., 2017). Agheeapur et al. found that there is a shift towards a more pro-inflammatory state in monocytes during mid-pregnancy, characterized by modulated expression of Toll-like Receptor (TLR) Signaling Pathway, NF-κB Signaling Pathway, JAK-STAT Signaling Pathway, PI3K-Akt Signaling Pathway, and MAPK Signaling Pathway. These immune system functional changes are highly orchestrated and follow a precise timeline throughout a normal pregnancy. Other studies have found shift towards an anti-inflammatory and catabolic state towards late pregnancy (Sharma et al., 2022). This transition makes the host more vulnerable to infections and sepsis during the postpartum period. Additionally, T-cell responses are found to be impaired during late pregnancy in individuals with latent TB infection(Saha et al., 2022). All these immune modulatory changes impact the TB diagnostic test during pregnancy (Mathad et al., 2014; Mathad et al., 2022).

My thesis aims to investigate the alterations in gene transcription expression patterns in Mtb-infected monocytes during the second and third trimesters of pregnancy. To explore the molecular pathways involved in the host innate immune response in the presence of Mtb infection during pregnancy, it is essential to target monocyte-derived macrophages. I hypothesized that T-cell signaling pathways originating from macrophages differ significantly during late pregnancy, and the interaction between T cells and macrophages is altered to ensure effective immune activity during this period. This study aims to assess the global gene expression profiles of Mtb-infected macrophages longitudinally isolated from women at various stages of pregnancy. By leveraging the high throughput mRNA sequencing technology, I seek to unravel the transcriptomic landscape of monocyte derived macrophages isolated from pregnant women with latent TB infection (LTBI). By comparing these profiles with the non-pregnant state, I aim to generate a detailed gene expression dataset that reflects the impact of pregnancy on the innate immune response to Mtb.

Method

Study population

This study used a subset of cryopreserved PBMCs from the Partners PrEP Study (NCT0557245) which was a randomized clinical trial evaluating antiretroviral preexposure prophylaxis (PrEP) in HIV serodiscordant couples(Baeten et al., 2012). Conducted between 2007 and 2012, the study enrolled a total of 4,758 participants across nine sites in Kenya and Uganda. Eligibility criteria for HIV-positive partners included a CD4 cell count greater than 250, no history of AIDS-defining diagnoses, and not currently using antiretroviral therapy (ART). Persons living with HIV (PLWH) who were not already on ART and became pregnant were referred to services for the prevention of mother-to-child transmission. For this analysis, I randomly selected 10 participants who had samples representing different trimesters of pregnancy (n=2 first trimester, n=4 second trimester, n=4 third trimester) along with longitudinal paired samples from before pregnancy and postpartum.

Monocyte isolation and in-vitro Mtb infection

CD14+ Monocytes were isolated from cryopreserved peripheral blood mononuclear cells (PBMCs) using magnetic bead column isolation (Miltenyi). After isolation they werecultured with monocyte colony-stimulating factor (M-CSF) in RPMI-1640 (Gibco) supplemented with 10% fetal bovine serum (Atlas) to induce adherence, following established protocols (Simmons et al., 2021). To avoid artificial homogenization of transcriptional responses due to *ex vivo* culture conditions, all experiments were conducted within 48 hours of PBMC thawing and M-CSF treatment. In a biosafety level 3 (BSL3) laboratory, monocytes were either stimulated with H37Rv (multiplicity of infection [MOI], 1.0) or treated with a medium. RNA was extracted using miRNeasy Mini Kit (Qiagen) after 6 hours of stimulation. Samples were processed together to avoid batch effects.

RNA sequencing and data processing

After assuring RNA quality (RIN ≥ 8.0) by Qubit and ultra-low input cDNA library preparation was done in Agilent 2100 bioanalyzer. Samples with low RNA quality had concordance with low PBMC count and were discarded. mRNA sequencing and alignments were done using Illumina NovaSeq S2 V1.5 with an average sequencing read depth of ~98.6x. Read filtering, and analysis were performed in R v4.0.2 and Python 3.1. Samples were filtered with with read counts $<1.5\times10^7$. Genes were filtered with mean log2 counts of 1 or below and gene IDs were incorporated from the Ensembl database. To account for differences in sequencing depth and RNA composition across samples DESeq2 normalization was done followed by LFC (log fold change) shrinkage to filter genes with low counts and high variability(Muzellec et al., 2023).

Trimester specific analysis was done using multifactor DESeq analysis tool(*PyDESeq2 documentation*).

Differential gene expressions and gene set enrichment analyses (GSEA)

To identify the trimester specific differential expression of the gene multifactor DESeq analysis was done using infection status (Mtb vs control) and pregnancy time points as design factors. Genes were filtered again with DESeq statistical summary p value<0.05 and absolute log fold change >1. To understand the biological pathways, network analysis was performed using ShinyGO 8.0(Ge et al., 2020). Pathway enrichment analysis was done using KEGG database.

Results

In vitro Mtb infection upregulated genes associated with host TB immune response

To ensure the integrity of the mRNA sequencing, I conducted a comparative Wald test of differentially expressed genes (DEGs) between (Mtb-infected samples and their paired controls. This analysis was aimed at identifying significant changes in gene expression due to Mtb infection. After normalization and rigorous statistical filtering, I identified 551 DEGs (supplementary table 1). The selection criteria for these genes included a log2 fold change (log2FC) greater than 1 and an adjusted p-value (q-value) less than 0.05 (Figure 1a). This stringent threshold ensures that the identified DEGs are both biologically significant and statistically robust, minimizing the likelihood of identifying genes of interest falsely. Among these 551 DEGs, the top twenty upregulated genes showed the most pronounced increase in expression levels in response to Mtb infection. Functional annotation and pathway analysis revealed that these top twenty DEGsare predominantly involved in cytokine signaling pathways and the regulation of inflammatory responses in human macrophages (Figure 1b).

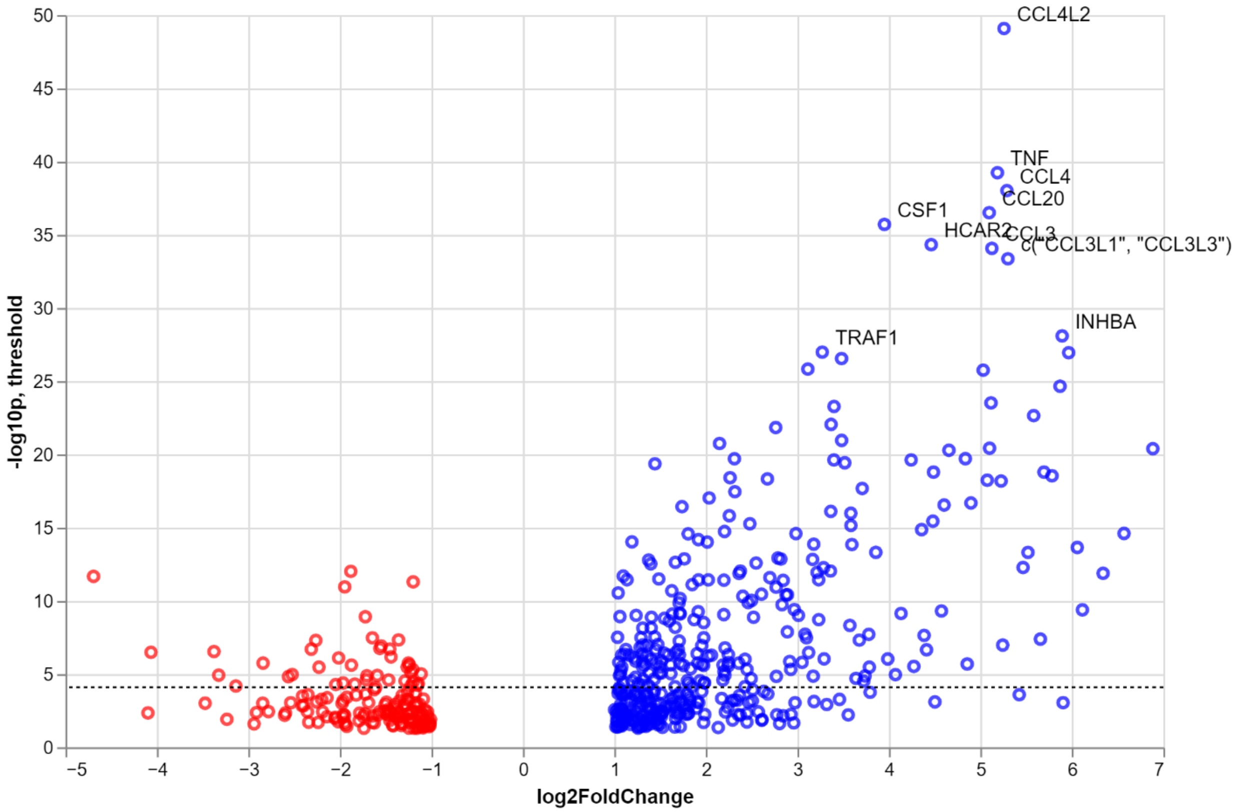

Figure 1a: Volcano plot showing 551 differentially expressed genes after PyDESeq2 normalization and LFC (log fold change) shrinkage with absolute value of log2 fold-change >1. Genes are color coded based on their fold change direction. The threshold line was drawn at -log10 of Bonferroni corrected p values [-log10(0.00009)=4.1]. Top 10 DEGs are highlighted.

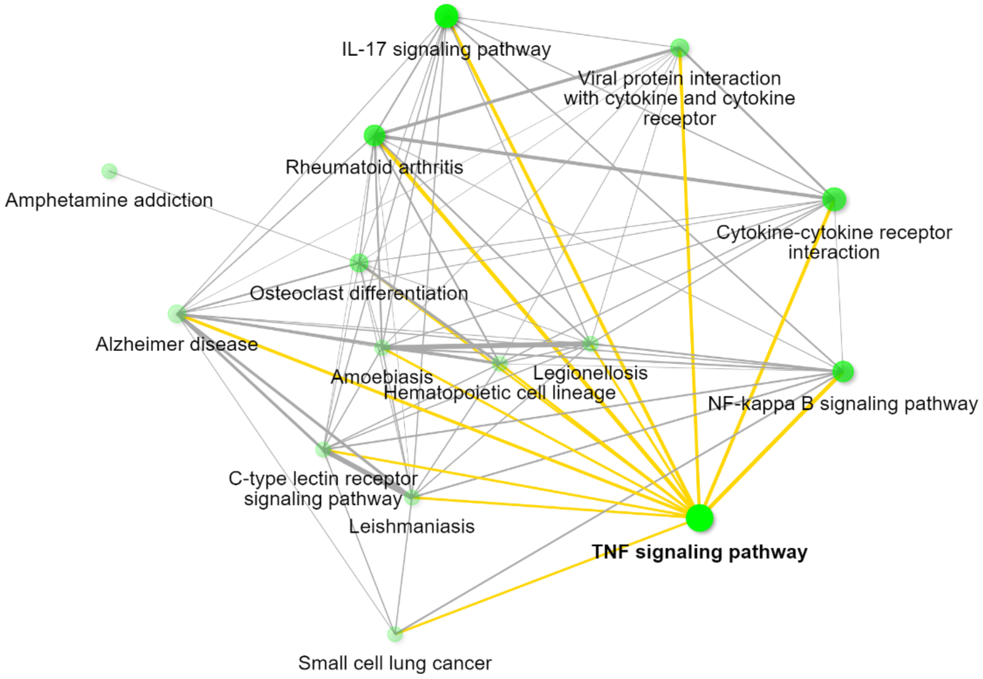

Figure 1b: Pathway enrichment analysis conducted on 551 differentially expressed genes, employing a stringent False Discovery Rate (FDR) cutoff of 0.01. Pathways were curated from the KEGG database. Within the visualization, larger nodes denote a greater abundance of associated genes, while darker edges signify more robust connections between adjacent pathways. This analysis was executed utilizing ShinyGO 0.080.

Hierarchical clustering analysis revealed gene modules associated with the host immune response to Mtb and highlighted outlier samples

Hierarchical clustering analysis of the 551 differentially expressed genes (DEGs) was performed to group genes with similar expression patterns and link them to relevant biological pathways. This analysis revealed that most immune regulatory genes were upregulated in Mtb-infected samples compared to controls (Figure 2a). 127 genes were associated with Ferroptosis (exhibiting the highest fold enrichment) and other host immune regulatory pathways against Mtb (Figure 2b). The sample dendrogram broadly indicated sample grouping based on their infectious status (Figure 2c). Control and Mtb-infected samples were positioned at opposite corners of the dendrogram, with a few outliers observed. Samples not clustering with their expected groups may suggest unique biological responses or potential sample misclassification, highlighting areas that warrant further investigation to understand these deviations.

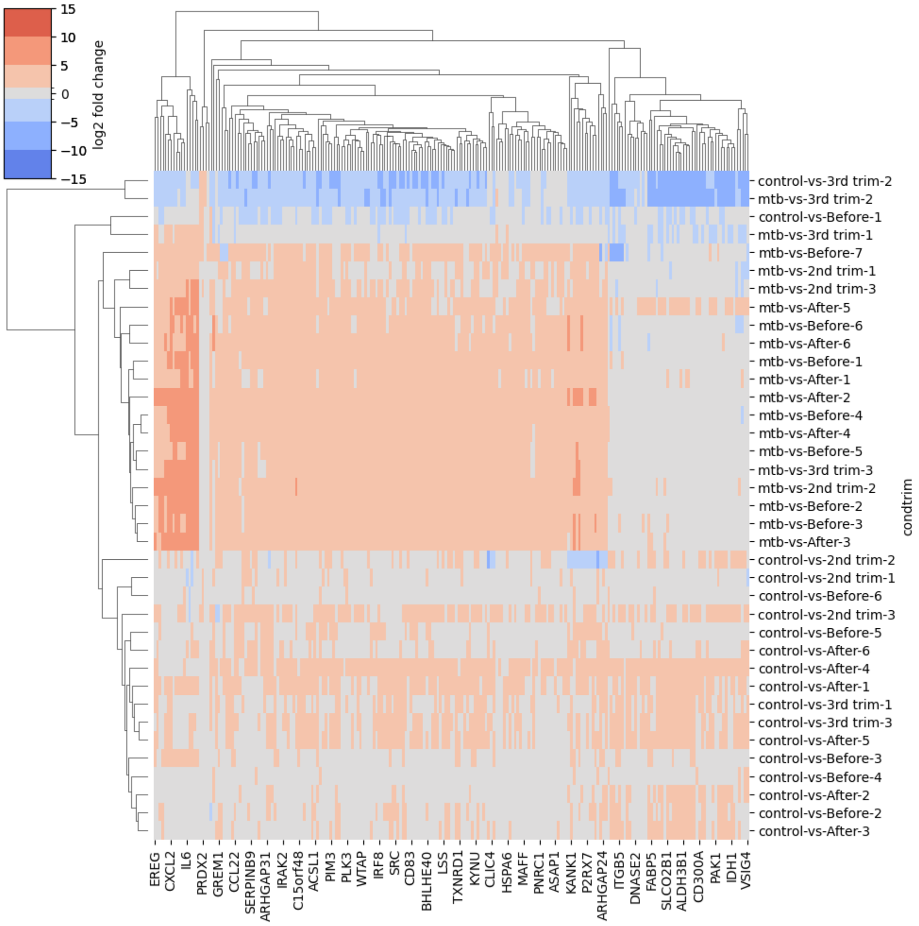

Figure 2a: Hierarchical cluster map using the “seaborn” package in Python showing gene hits in Mtb infected and uninfected samples. Genes were color coded based on their log2 fold change value. Red denotes upregulated genes in the mtb clustered samples and blue denotes downregulation of genes in control. The biggest cluster consists of 127 genes. Control and Mtb infected samples were clustered separately.

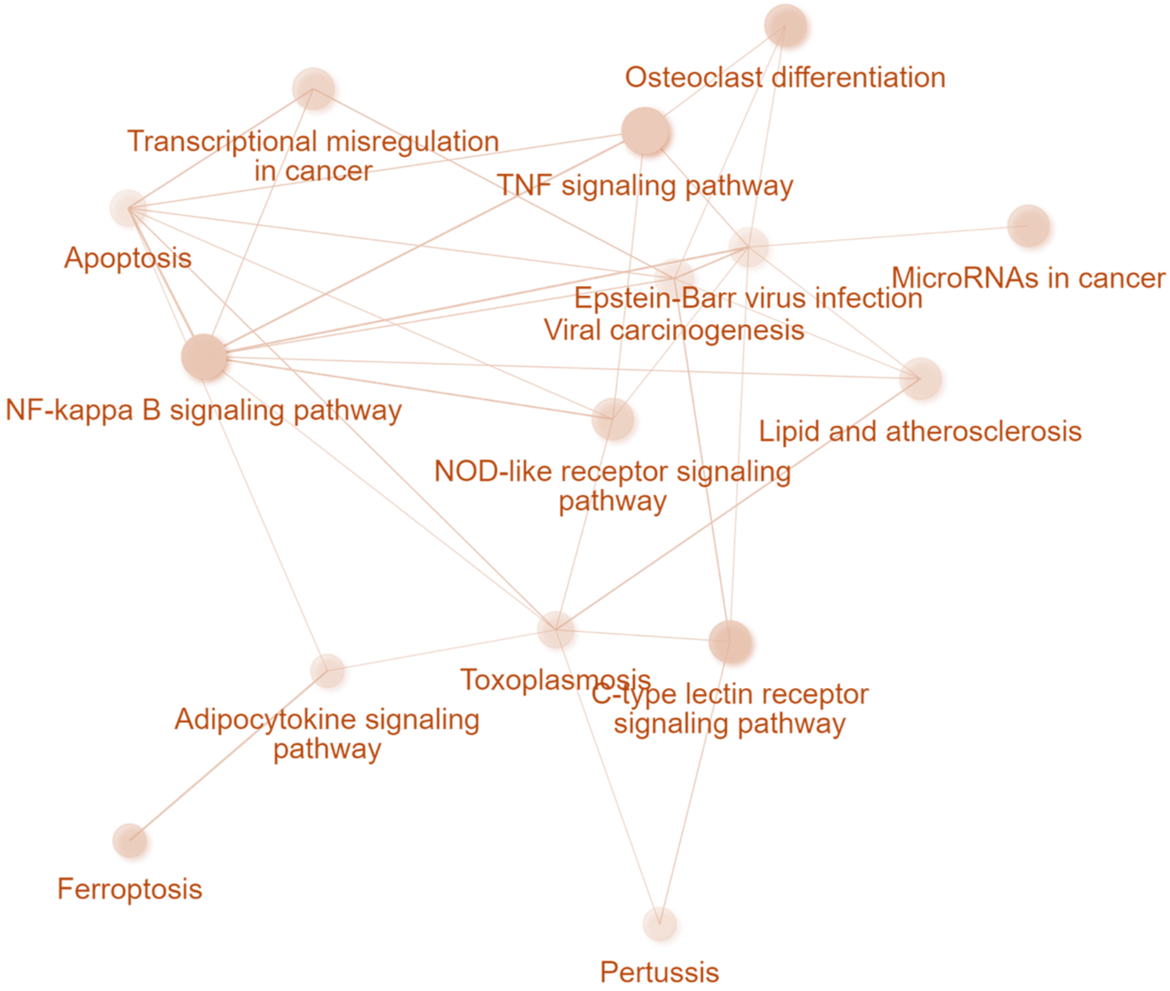

Figure 2b: Pathway enrichment analysis conducted on 127 clustered genes, employing a stringent False Discovery Rate (FDR) cutoff of 0.01. Pathways were curated from the KEGG database. Within the visualization, larger nodes denote a greater abundance of associated genes, while darker edges signify more robust connections between adjacent pathways. This analysis was executed utilizing ShinyGO 0.080.

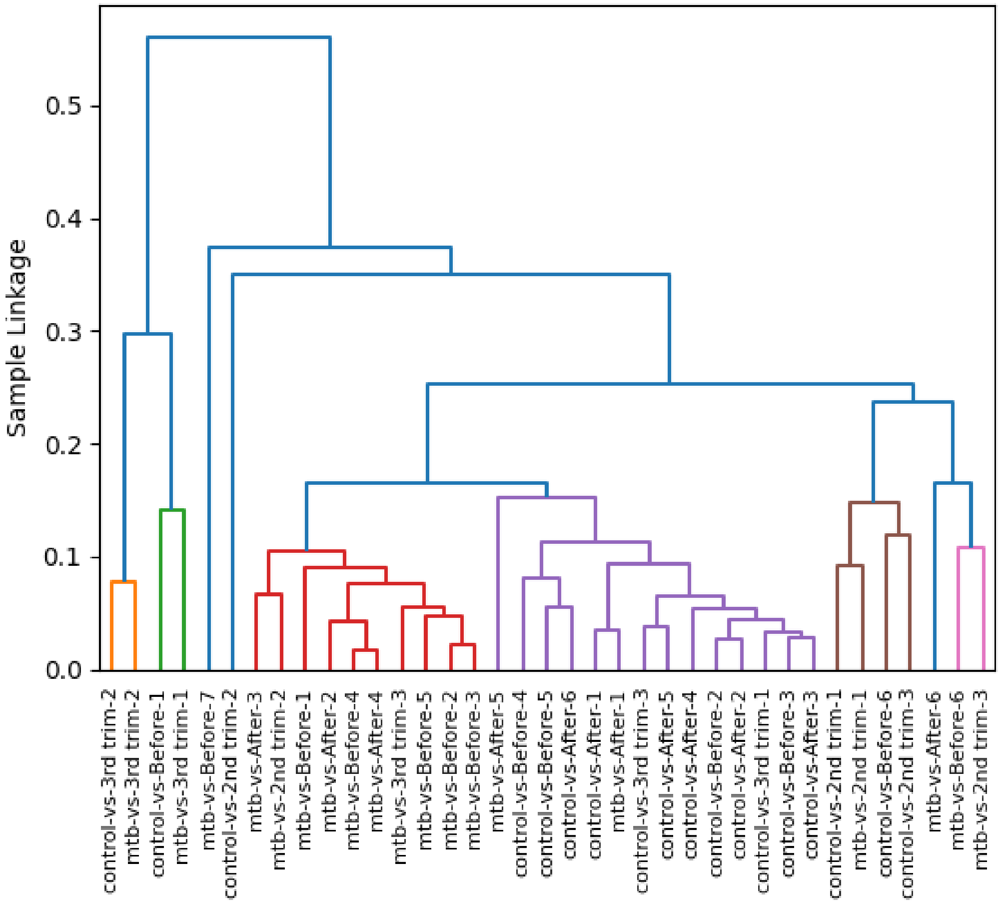

Figure 2c: Dendogram showing sample correlation. The threshold was set at 0.16 to visualize different clusters. The greatest cluster consists of 14 samples 12 of which represents the uninfected group.

Comprehensive analysis of gene expression in Mtb-Infected samples considering pregnancy timeline revealed distinct monocyte transcriptomic signature during third trimester

Incorporating a pregnancy timeline design factor in DESeq2 statistics alongside Mtb infectious status enabled us to account for the potential effects of pregnancy trimester on gene expression. This comprehensive analysis revealed more than 200 genes that were differentially expressed (both up and down regulated) due to Mtb infection considering the natural changes in gene expression profile across different stages of pregnancy and non-pregnant states. Pathway enrichment analysis with a stringent FDR cut-off <0.01 indicated that these DEGs were associated with host immune regulatory pathways, mostly similar to those identified in previous analyses except JAK-STAT signaling pathway (Figures 3a). No significant differences were observed between pre-pregnancy vs. postpartum periods and second trimester vs. postpartum. However, comparison between the second trimester vs. the pre-pregnancy timeline revealed two distinct genes, “COL1A1” and “MYEOV,” with significantly different expression patterns. I found 32 DEGS (supplementary table 2) when comparing the third trimester to the pre-pregnancy timeline. With an FDR cut off <0.05, pathway enrichment analysis revealed these genes were linked to the “calcium signaling pathway” and the “MAPK signaling pathway” according to the KEGG database. Additionally, using the Hallmark MSig database, we connected these genes to the “TNF and MTORC signaling pathways” and “hypoxia” (Figure 3b, 3c).

As this is an exploratory analysis, I adopted a less conservative FDR cutoff (0.1) to further investigate the pathways involved in unique immune modulation during third trimester. The analysis identified additional hallmark immune regulatory pathways (Figures 3d, 3e). Pathways such as the unfolded protein response activated by endoplasmic reticulum (ER) stress and the PI3K/AKT Signaling Pathway, pivotal in regulating cell metabolism, emerged. These pathways are involved in stress response, apoptosis and autophagy regulation which could be manipulated by Mtb to enhance its own survival and replication. Additionally, the inclusion of fluid shear stress and atherosclerosis pathways may suggest a potential connection between endothelial dysfunction leading to atherosclerosis and impaired macrophage function and worsening TB outcome. This broader approach highlighted the complex interplay between Mtb infection and the dynamic gene expression changes during late pregnancy with a potential downside of the association being less reliable.

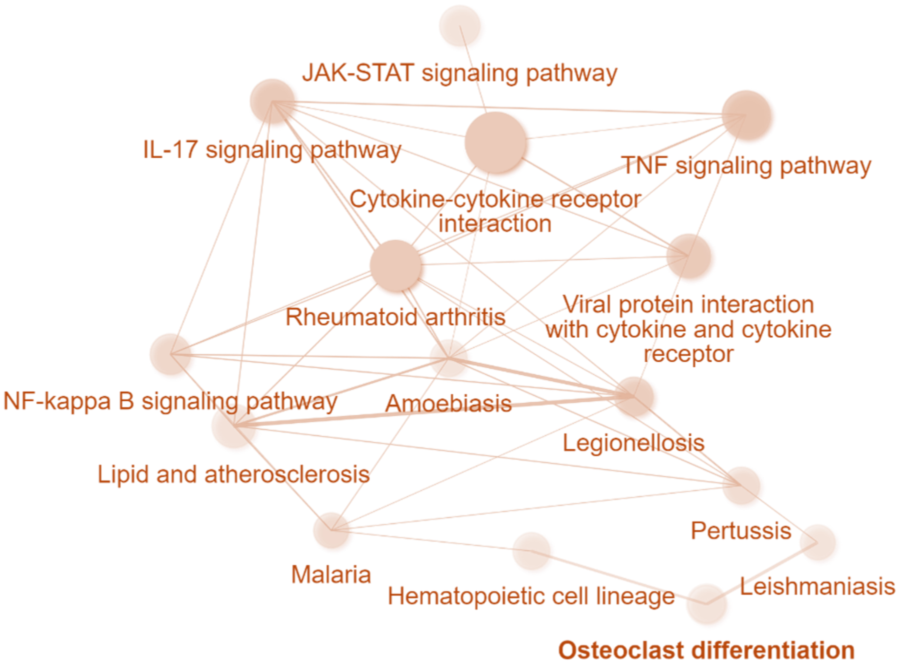

Figure 3a: Pathway enrichment analysis conducted on ~270 DEGs after accounting for changes in before, during and after pregnancy, employing a stringent False Discovery Rate (FDR) cutoff of 0.01. Pathways were curated from the KEGG database. Within the visualization, larger nodes denote a greater abundance of associated genes, while darker edges signify more robust connections between adjacent pathways. This analysis was executed utilizing ShinyGO 0.080.

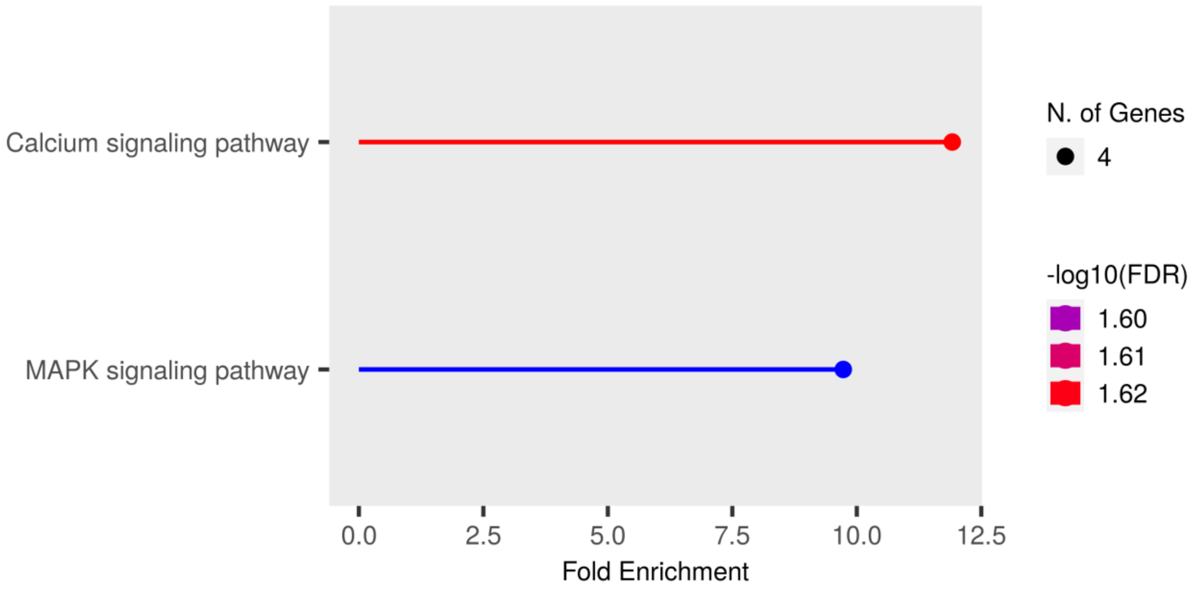

Figure 3b: Lollipop chart showing 32 DEGs after comparing third trimester of pregnancy with before pregnancy timeline, employing a stringent False Discovery Rate (FDR) cutoff of 0.05. Pathways were curated from the **KEGG database.** Within the visualization, bigger nodes denote a greater abundance of associated genes while darker edge denotes degree of fold change along X-axis. This analysis was executed utilizing SinyGO 0.080.

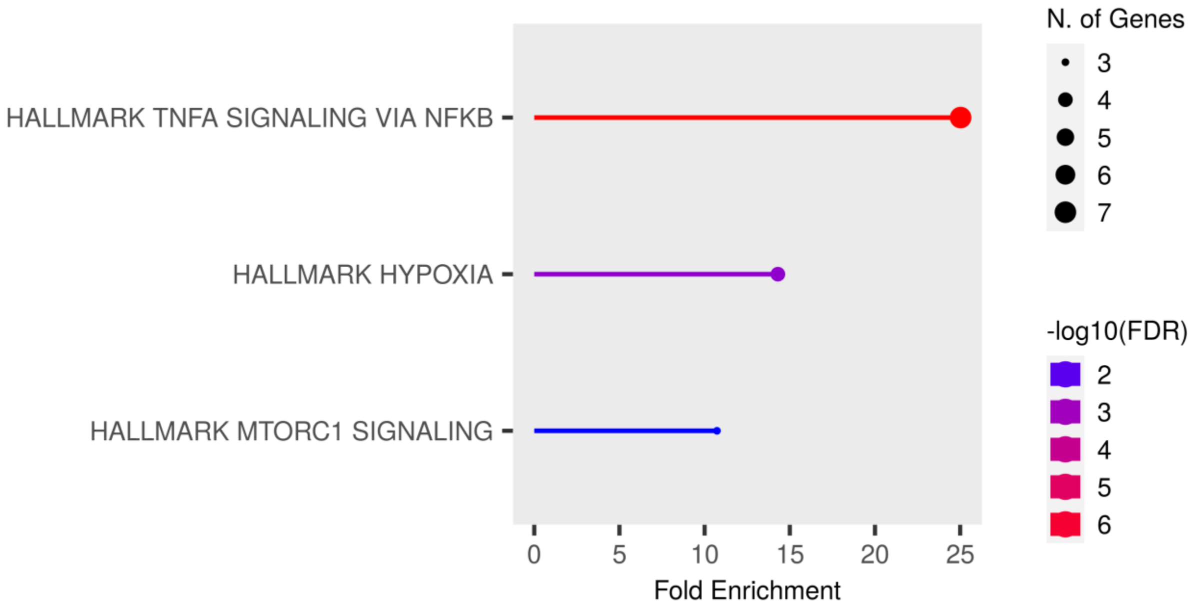

Figure 3c: Lollipop chart showing 32 DEGs after comparing third trimester of pregnancy with before pregnancy timeline, employing a stringent False Discovery Rate (FDR) cutoff of 0.05. Pathways were curated from the **Hallmark MSig database**. Within the visualization, bigger nodes denote a greater abundance of associated genes while length of the edge denotes degree of fold change along X-axis. This analysis was executed utilizing SinyGO 0.080.

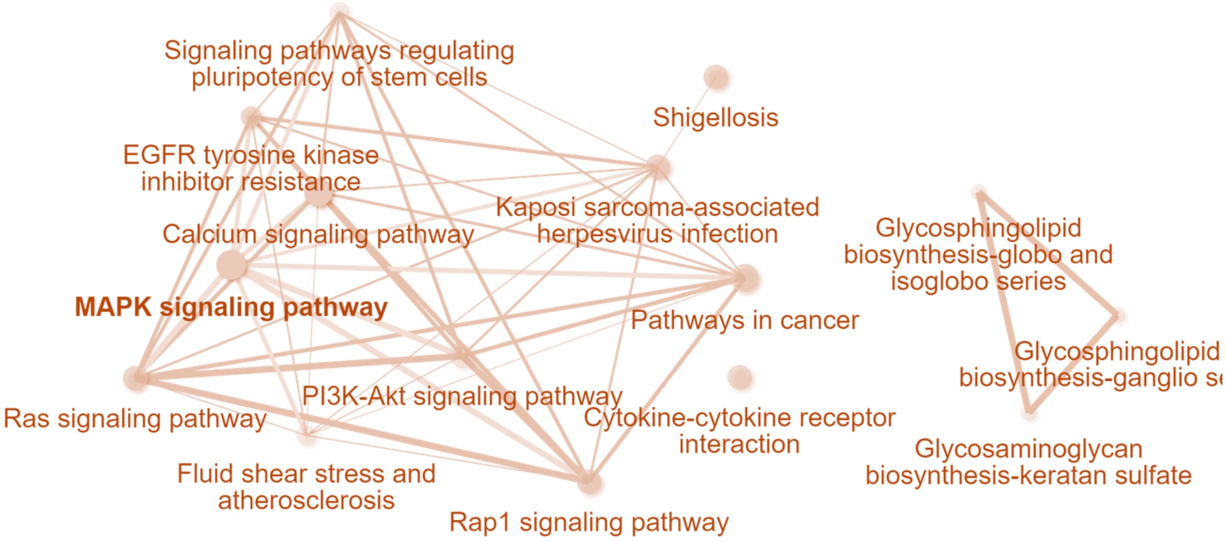

Figure 3d: Pathway enrichment analysis conducted on 32 DEGs after accounting for changes in different pregnancy timeline, employing a less conservative False Discovery Rate (FDR) cutoff of 0.1. Pathways were curated from the **KEGG database**. Within the visualization, larger nodes denote a greater abundance of associated genes, while darker edges signify more robust connections between adjacent pathways. This analysis was executed utilizing SinyGO 0.080.

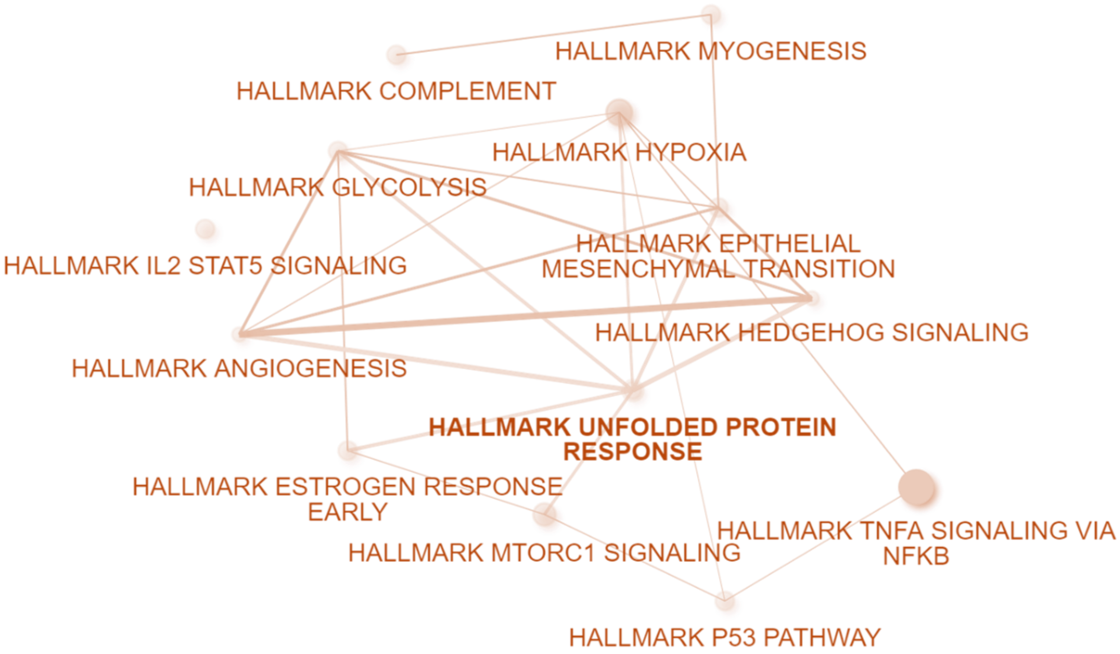

Figure 3e: Pathway enrichment analysis conducted on 32 DEGs after accounting for changes in different pregnancy timeline, employing a less conservative False Discovery Rate (FDR) cutoff of 0.1. Pathways were curated from the **Hallmark MSig database**. Within the visualization, larger nodes denote a greater abundance of associated genes, while darker edges signify more robust connections between adjacent pathways. This analysis was executed utilizing ShinyGO 0.080.

Discussion

My thesis aimed to elucidate the intricate dynamics of monocyte derived macrophage gene expression in response to Mtb infection in the context of pregnancy. Through a thorough exploration employing high-throughput mRNA sequencing, changes in gene expression profile associated with the host innate immune response to Mtb were identified exclusively during third trimester compared to non-pregnant state. It was interesting to find that Mtb-infected monocytes demonstrated altered expression of genes in the JAK-STAT pathway while comparing pregnancy and non-pregnant times. This pathway plays a crucial role in maintaining immune tolerance between the mother and fetus and has historically been associated with the reactivation of latent tuberculosis(Li et al., 2023). These findings underscore the complex interplay between pregnancy and host-pathogen interactions emphasizing the need for personalized approaches in understanding disease outcomes in pregnant population.

The initial analysis revealed over five hundred DEGs in Mtb-infected samples compared to controls. Among these, the top twenty upregulated genes were primarily involved in cytokine signaling pathways and the regulation of inflammatory responses in human macrophages, as demonstrated by previous studies upon Mtb infection(Volpe et al., 2006). Hierarchical clustering analysis further elucidated gene modules associated with the host immune response to Mtb, with most immune regulatory genes showing upregulation in infected samples. Furthermore, the incorporation of a pregnancy timeline as an integral design factor in the analysis unveiled discernible monocyte transcriptomic signatures during the latter stages of gestation. Notably, comparisons between third trimesters and before pregnancy illuminated nuanced differential expression patterns, evident in genes associated with pivotal pathways in controlling Mtb infection including calcium signaling, MAPK signaling, TNF signaling, MTORC signaling pathways, and hypoxia(Flynn et al., 1995; Kalia et al., 2023; Liu et al., 2018; Pagan et al., 2022; Pasquinelli et al., 2013). Downregulated genes like GBP5 which is a candidate gene in RSK11 signature(Kim et al., 2023) and one of the 3 genes TB-score targeted as a benchmark for non-sputum method for progression and diagnosis of active TB(Warsinske et al., 2018), TNFRSF4 and NIBAN1are likely to be linked to altered inflammatory and stress response observed in late pregnancy. The congruence observed in gene expression profiles before and after pregnancy underscores the robustness and resilience of the maternal immune system. Moreover, the minimal observed changes during the second trimester of pregnancy align seamlessly with prior investigations into similar T-cell immune response profile within the same cohort, thereby providing further validation and coherence to the established findings(Saha et al., 2022).

The distinct findings in the third trimester compared to the second trimester highlight the temporal dynamics of immune modulation during pregnancy. Another aspect of the study is the potential implications of altered immune responses during pregnancy on TB outcomes. Pregnancy-induced immunological changes, particularly in the third trimester may lead to a compromised ability to control TB infection. The downregulation of key immune genes observed during late pregnancy, such as NIBAN1 and TNFRSF4, could contribute to reduced immune surveillance response efficacy against Mtb by altering MTORC and IL-2 STAT5 signaling pathway respectively. Moreover, it has been found recently that TNFRSF4, encoding costimulatory receptor OX40 shifts activated parenchymal effector CD4 T-cell towards more protective phenotype in controlling active Mtb infection and downregulation of this gene would likely contribute to impaired host ability to control and clear the infection(Gress et al., 2023). Together these observations lead to an idea of a critical window of vulnerability during pregnancy where TB infection may progress more aggressively, or reactivation of latent TB could occur.

I would like to acknowledge notable limitations of the study. The limited number of samples used may affect the statistical power of the findings, potentially leading to less robust conclusions and limiting the generalizability of the results. Future studies with larger cohorts are necessary to validate these findings. Another limitation pertains to the inadequate peripheral blood mononuclear cell (PBMC) count. The insufficient PBMC counts resulted in low RNA quality in several samples which were discarded. This limitation restricted the depth of the analysis, reducing the ability to detect subtle but biologically significant changes in gene expression. Future analyses will include examining the directionality of pathways associated with the differentially expressed genes (DEGs) using Gene Set Enrichment Analysis (GSEA) software. Additionally, protein validation of the DEGs identified through mRNA sequencing was beyond the scope of this study, which could be considered as an important avenue for future research.

Conclusion

We found that monocyte responses to Mtb changed substantially during the third trimester of pregnancy. Employing transcriptomic analyses, we identified critical host defense genes and pathways against Mtb that are altered during the third trimester of pregnancy. These findings provide evidence for possible mechanisms leading to altered T cell responses and TB susceptibility leading to increased risk of Mtb progression postpartum. These may define targets for therapeutic interventions as well. In its entirety, this work enriches our comprehension of TB pathogenesis and its complex interplay with immune changes during pregnancy which can be used in strengthening public health strategies to improve TB prevention, diagnosis, and treatment during pregnancy, reducing the global burden of the disease.

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