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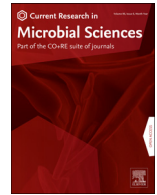
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Human macrophage polarization in the response to *Mycobacterium leprae* genomic DNA

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ABSTRACT

Infection with *Mycobacterium leprae*, the causative organism of leprosy, is still endemic in numerous parts of the world including the southwestern United States. The broad variation of symptoms in the leprosy disease spectrum range from the milder tuberculoid leprosy (paucibacillary) to the more severe and disfiguring lepromatous leprosy (multibacillary). The established thinking in the health community is that host response, rather than *M. leprae* strain variation, is the reason for the range of disease severity. More recent discoveries suggest that macrophage polarization also plays a significant role in the spectrum of leprosy disease but to what degree it contributes is not fully established. In this study, we aimed to analyze if different strains of *M. leprae* elicit different transcription responses in human macrophages, and to examine the role of macrophage polarization in these responses.

Genomic DNA from three different strains of *M. leprae* DNA (Strains NHDP, Br4923, and Thai-53) were used to stimulate human macrophages under three polarization conditions (M1, M1-activated, and M2).

Transcriptome analysis revealed a large number of differentially expressed (DE) genes upon stimulation with DNA from *M. leprae* strain Thai-53 compared to strains NHDP and Br4923, independent of the macrophage polarization condition. We also found that macrophage polarization affects the responses to *M. leprae* DNA, with up-regulation of numerous interferon stimulated genes.

These findings provide a deeper understanding of the role of macrophage polarization in the recognition of *M. leprae* DNA, with the potential to improve leprosy treatment strategies.

1. Introduction

Mycobacterium leprae is an acid-fast, obligate-intracellular bacterium and the causative organism of leprosy, a disease still endemic in numerous parts of the world (Programme, 2016, Fischer, 2017), including the southwestern United States (Sharma et al., 2015, Program, 2016). It causes various degrees of skin abnormalities, eye damage, respiratory damage, and peripheral neuropathy in humans leading to stigmatization of individuals and communities throughout human history, and still affecting millions of people worldwide to this day (Fisher 2017). The spectrum of disease ranges in severity from the milder tuberculoid leprosy (paucibacillary) to the more severe and disfiguring lepromatous leprosy (multibacillary) (Gaschignard et al., 2013) (Fig. 1).

The established thinking in the health community is that strain variations of *M. leprae* are of little consequence to disease symptoms and that differential expression of tuberculoid leprosy and lepromatous lep-

rosy are due only to variations in host response, such as TH1 or TH2 and macrophage polarization (Kibbie et al., 2016, Mi et al., 2020). Differential macrophage polarization in skin lesions of leprosy has been described, with M1 phenotype predominantly present in granulomas of TT patients, whereas macrophages in LL granulomas exhibit an M2 phenotype (Mi et al., 2020, Fachin et al., 2017).

Although the genome of *M. leprae* has been highly conserved over the past ten centuries (Schuenemann et al., 2013), comparison of variable-number tandem repeats polymorphisms has shown to be useful in effectively discriminating *M. leprae* strains (Truman et al., 2004). Further genetic differences, which led to the establishment of a new species such as *Mycobacterium lepromatosis*, are associated with a more severe presentation, known as diffuse lepromatous leprosy (Sharma et al., 2019)(Sharma CID 2020).

Genetic analysis has also shown the geographic restriction of strains in humans (Matsuoka et al., 2006) and primates (Honap et al., 2018).

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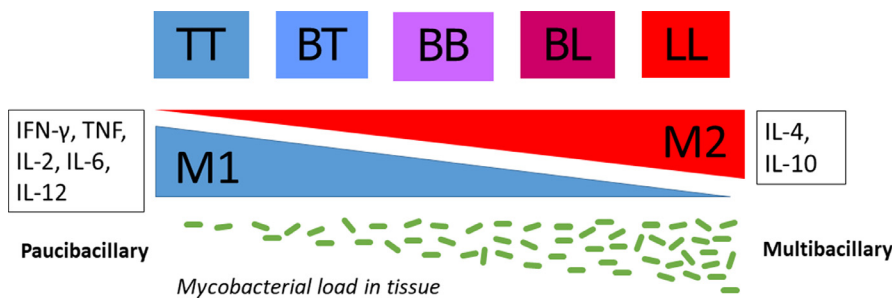


Fig. 1. Leprosy spectrum and mechanisms of pathogenesis. From least to most severe: tuberculoid (TT), intermediate borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL), lepromatous leprosy (LL). Macrophage polarization: M1 showing granulomas and M2 displaying “foamy” macrophages. Boxes depict the cytokine profile in the clinical pole of disease.

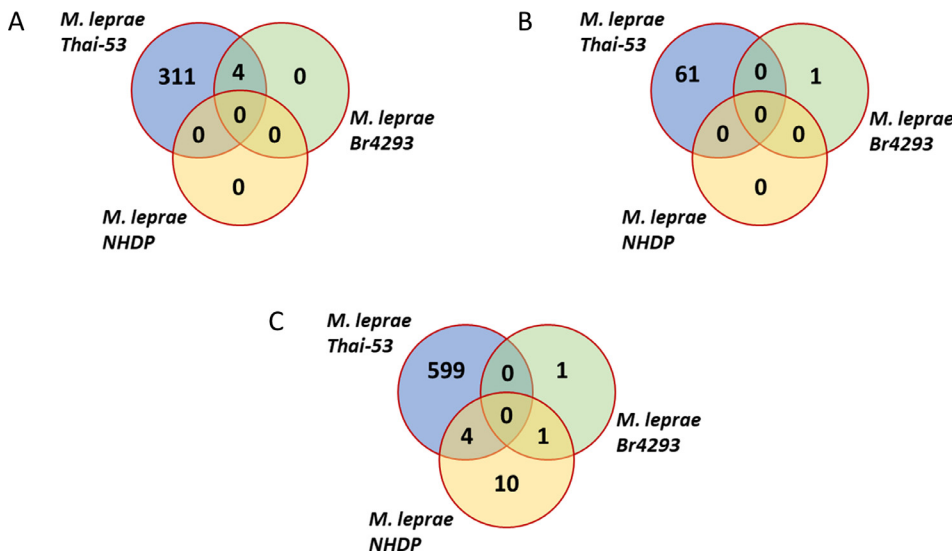


Fig. 2. (A) Venn diagram of the number of DE genes in human macrophages across the DNA from strains NHDP (Yellow), Thai-53 (Blue), and Br4923 (Green) used for stimulation. (A) M1, (B) M1activated and (C) M2 macrophages. All DE genes comprise those whose fold change is greater than 2 and adjusted p-value less than 0.05 in each NHDP, Thai-53, and Br4923.

Some evidence exists suggesting that specific *M. leprae* genotype may be associated with a particular clinical pole. Multibacillary form, LL, is more frequent in places like Saudi Arabia (Alotaibi et al., 2016). Analysis of LL skin samples from India has demonstrated that TTC repeats are abundant in these samples, and differ from other *M. leprae* strains from India (Chokkakula et al., 2014). Geographical patterning is also observed in the Pacific Islands (Blevins et al., 2020), where multibacillary disease predominates (Woodall et al., 2011). Paucibacillary disease, on the other hand, is predominant in other parts of the world like Brazil (Marciano et al., 2018).

In this study, we aim to analyze if bacterial genomic DNA from different strains of *M. leprae* (Singh and Cole, 2011, Truman et al., 2011) elicit different responses in human macrophages and the role of macrophage polarizations in these responses.

2. Material and methods

2.1. Cells and cell stimulation conditions

We utilized human monocytic cells (THP-1) under an established protocol for macrophage induction and polarization, creating M1, M1 activated (M1a), and M2 macrophages (Rey-Giraud et al., 2012). 5×10^5 cells/ml in 12-well tissue culture treated plates for 6 days in the presence of either 100 ng/ml rHuGM-CSF (M1) or 100 ng/ml rHuM-CSF (M2). For M1 activation, monocytes were first incubated with rHuM-CSF (Peprotech) for 3 days followed by stimulation with 10 ng/ml LPS (Sigma) and 50 ng/ml rHuIFN- γ (Roche) for 3 additional days (Rey-Giraud et al., 2012). Stimulation was performed with 500 ng of genomic DNA from three different strains of *M. leprae*, NHDP (NT-19350), Br4923 (NR-19351), and Thai-53 (NR-19352), acquired from BEI Re-

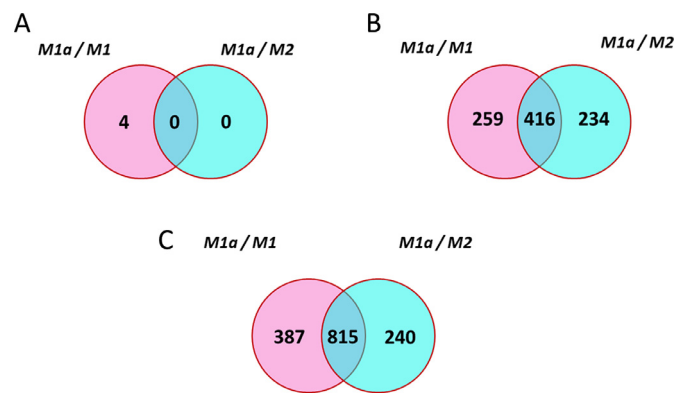


Fig. 3. Venn diagrams comparing the DE genes after *M. leprae* DNA strain stimulation for compared to M1a against M1 (magenta) and M2 (cyan) (absolute fold change value >2 and an adjusted p-value <0.05). A: *M. leprae* Br4923, B: *M. leprae* NHDP and C: *M. leprae* Thai-53 DNA stimulation.

sources (Manassa, VA). We utilized these strains as each one of them belongs to a different genetic subtype (Singh and Cole, 2011, Truman et al., 2011).

Genomic DNA was isolated from contaminating proteins and polysaccharides by organic extraction and precipitation with isopropanol respectively (Belisle and Sonnenberg, 1998). Polyethylenimine (PEI) (Polyplus) was used as an endo-lysosomal bacterial nucleic acid delivery system (Cervantes et al., 2013, Suh et al., 2012, Bieber et al., 2002), according to manufacturer’s instructions.

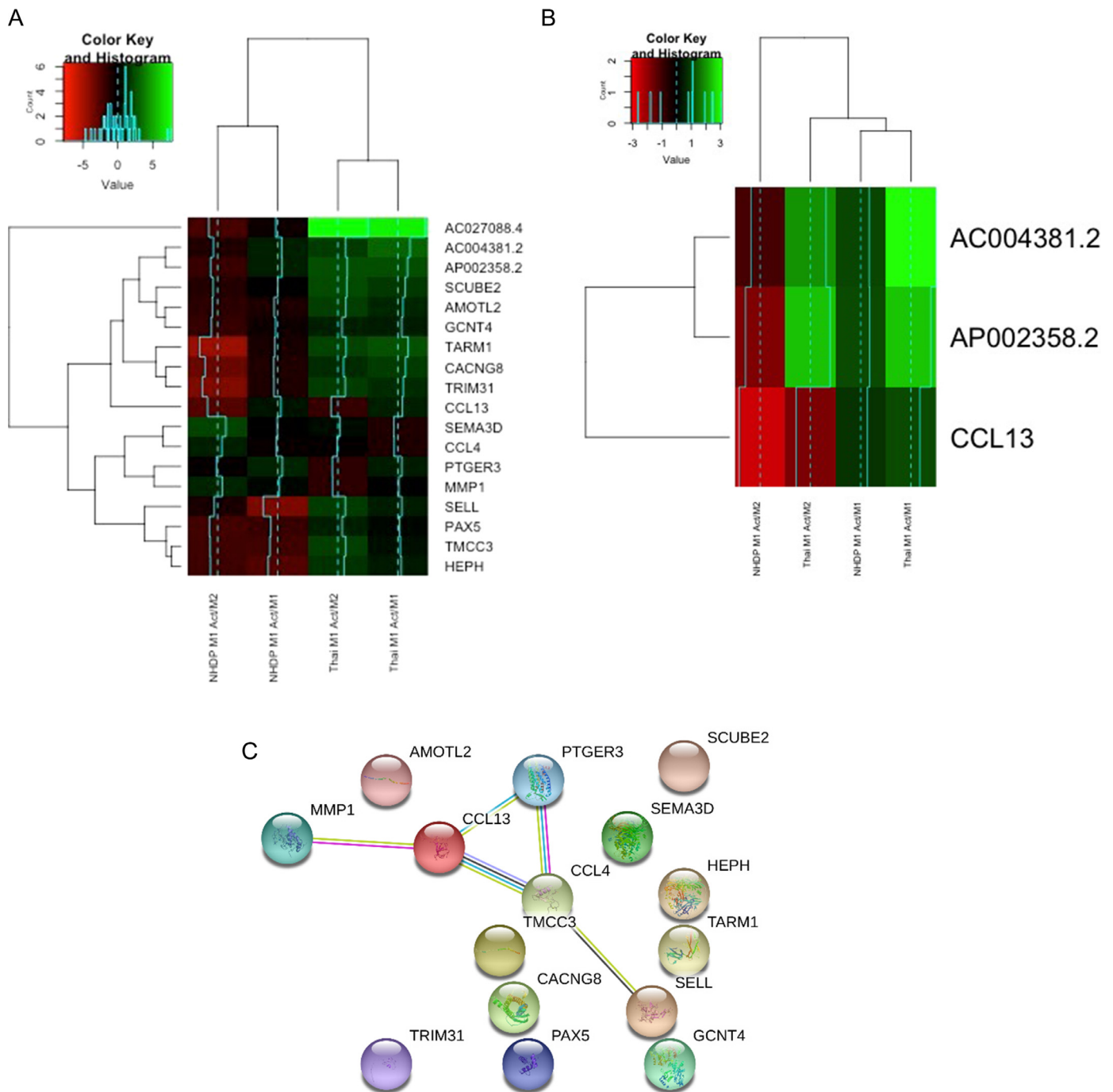


Fig. 4. Heat Map indicating DE genes in polarized macrophages. **A.** M1a compared to either M1 or M2 macrophages upon stimulation with either NHDP or Thai-53 DNA but downregulated by stimulation with the other strain. **B.** Heat map showing DE expression switches within a strain treatment. Genes are listed on the right-hand side. Green represents up-regulation and red represents down-regulation. **C.** Network analysis of genes presented in **A.**

2.2. RNA Seq and Transcriptome analysis

Total RNA was extracted after cell stimulation assays. Unstimulated cells were used as controls. A cDNA library was prepared and sequenced in an Illumina NextSeq 500 v2 High Output 150 cycle sequencing at the Center for Genomic Innovations (UConn, CT) to generate paired end (PE) 75bp reads. Sequence reads were trimmed and filtered based on read quality using Sickle (N et al., 2011) and Trimmomatic (Bolger et al., 2014). Reads were aligned with Hisat2 to the grch38 human reference genome (Pertea et al., 2016). Aligned reads were counted using ht-

seq (Anders et al., 2015). Reproducibility of the sample replicates was checked by Principle Component Analysis (PCA) in R (RC, 2017). Computation of differentially expressed (DE) genes was done using DESeq2 (Love et al., 2014) comparing the stimulated to unstimulated cells. A comparison of the significant genes with an absolute fold change of greater than 2 was done between the different *M. leprae* strains for the different cell types (M1, M1a, and M2) compared to the unstimulated control, i.e. M1 stimulated with Thai-53, Br4923, or NHDP DNA. The number of DE genes that overlap was displayed in a venn diagram using the VennDiagram (v.1.6.20) package in R (v.3.5.2) Chen (2018). Asso-

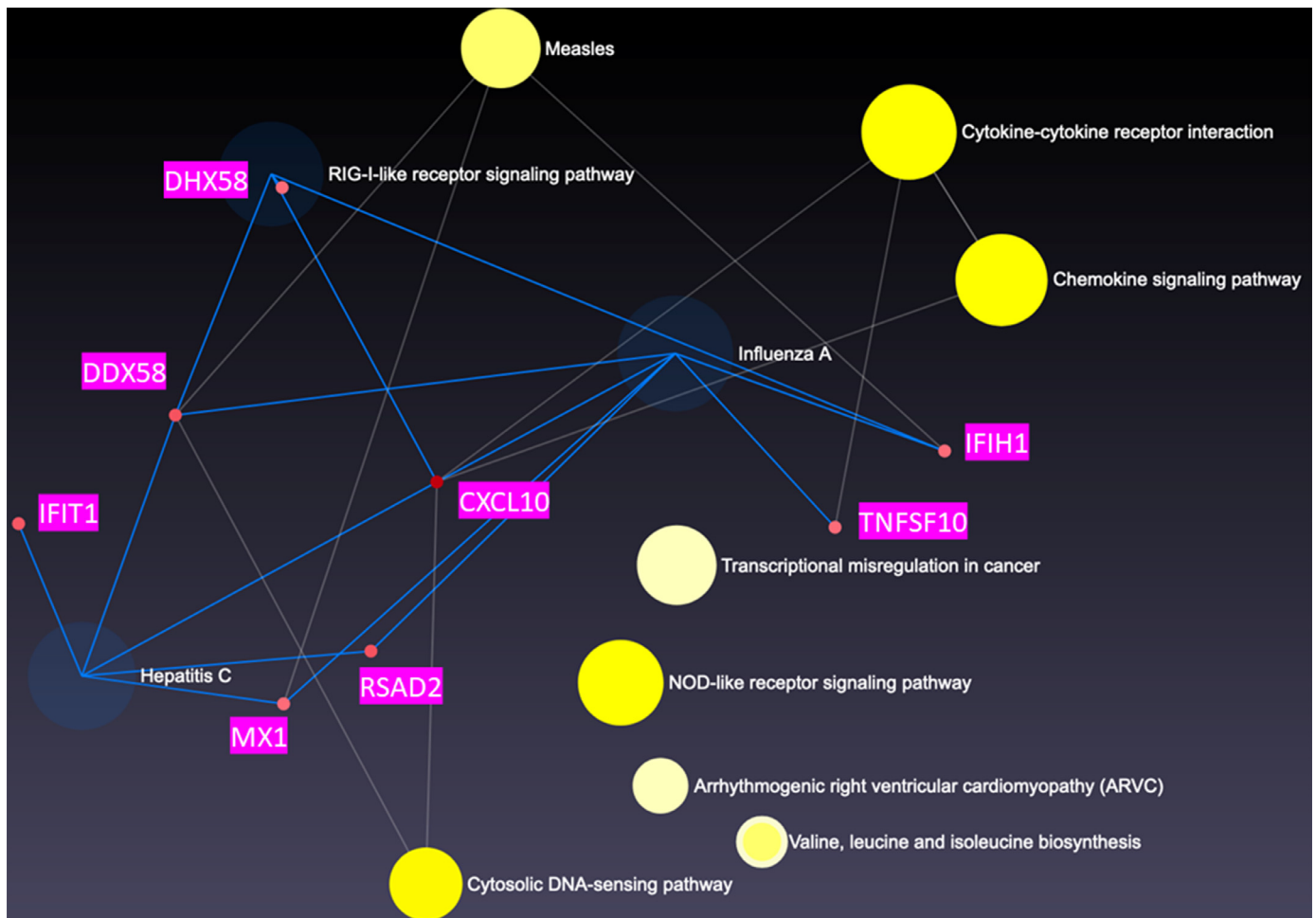


Fig. 5. Network analysis of DE genes presented in M1 macrophages upon stimulation with genomic DNA *M. leprae* strain Thai-53. A. Highly correlated genes in pairwise gene expression. B Genes that were upregulated together in a similar manner shown as connected nodes in the network. C Detail of B.

ciation networks and GO term analysis were performed using the online STRING program (v.11) (Szklarczyk et al., 2019) for the identified overlapping DE genes.

Additionally, comparisons were also made across the three types of cells (M1 and M2 expression compared to M1a) for each the *M. leprae* strain Thai-53, NHDP, Br4283, i.e. M2 versus M1a where both were stimulated with Thai-53 DNA. Heatmaps were created using the log-fold change of the genes where there was at least one value had an absolute fold change value > 2 and adjusted *p*-value < 0.05 in the various comparisons. The values were normalized across the row and displayed within the R package gplots (v3.0.4) using heatmap.2 (Warnes et al. 2020).

Network analyses were performed based on NetworkAnalyst tool (Zhou et al., 2019). The overrepresentation analysis using hypergeometric test was performed to identify significant overlap with gene-sets or pathways. The enrichment analysis was performed against KEGG. Macrophage specific gene co-expression networks were constructed based on significant genes. Highly correlated genes in pairwise gene expression profile were measured and mapped to immuno-navigator database. Genes that were up-regulated together in a similar manner were shown as connected nodes in the network.

3. Theory

Deciphering the process of macrophage polarization to enhance anti-microbial defense or to dampen detrimental inflammation is of

great importance in the pathogenesis of leprosy, as it affects clinical disease (Shapouri-Moghaddam et al., 2018). Macrophage polarization appears to play a significant role in the spectrum of leprosy disease but to what degree it contributes to the clinical presentation is not fully established (Pinheiro et al., 2018, Fallows et al., 2016). We have recently shown that different strains of *Mycobacterium tuberculosis* elicit differential NF- κ B and IRF responses in human macrophages (Cervantes et al., 2019). We aim to first determine if different strains of *M. leprae* (Singh et al., 2015) elicit different transcription responses in human macrophages and second to evaluate the role of macrophage polarization in the response to *M. leprae* DNA. Our study will provide a deeper understanding of the role of macrophage polarization and activation in the recognition of *M. leprae* which could lead to better future treatment strategies.

4. Results

4.1. DNA from different strains of *M. leprae* elicited different gene expression in human macrophages

To observe if DNA from the different strains would elicit different DE genes in stimulated macrophages, we first filtered out all expressed genes with at least one zero (missing) value sample and then used the fold change of each of NHDP, Br4923, and Thai-53 compared to unstimulated macrophages for each of the expressed genes. We observed each of the different *M. leprae* strains elicited different gene expression in

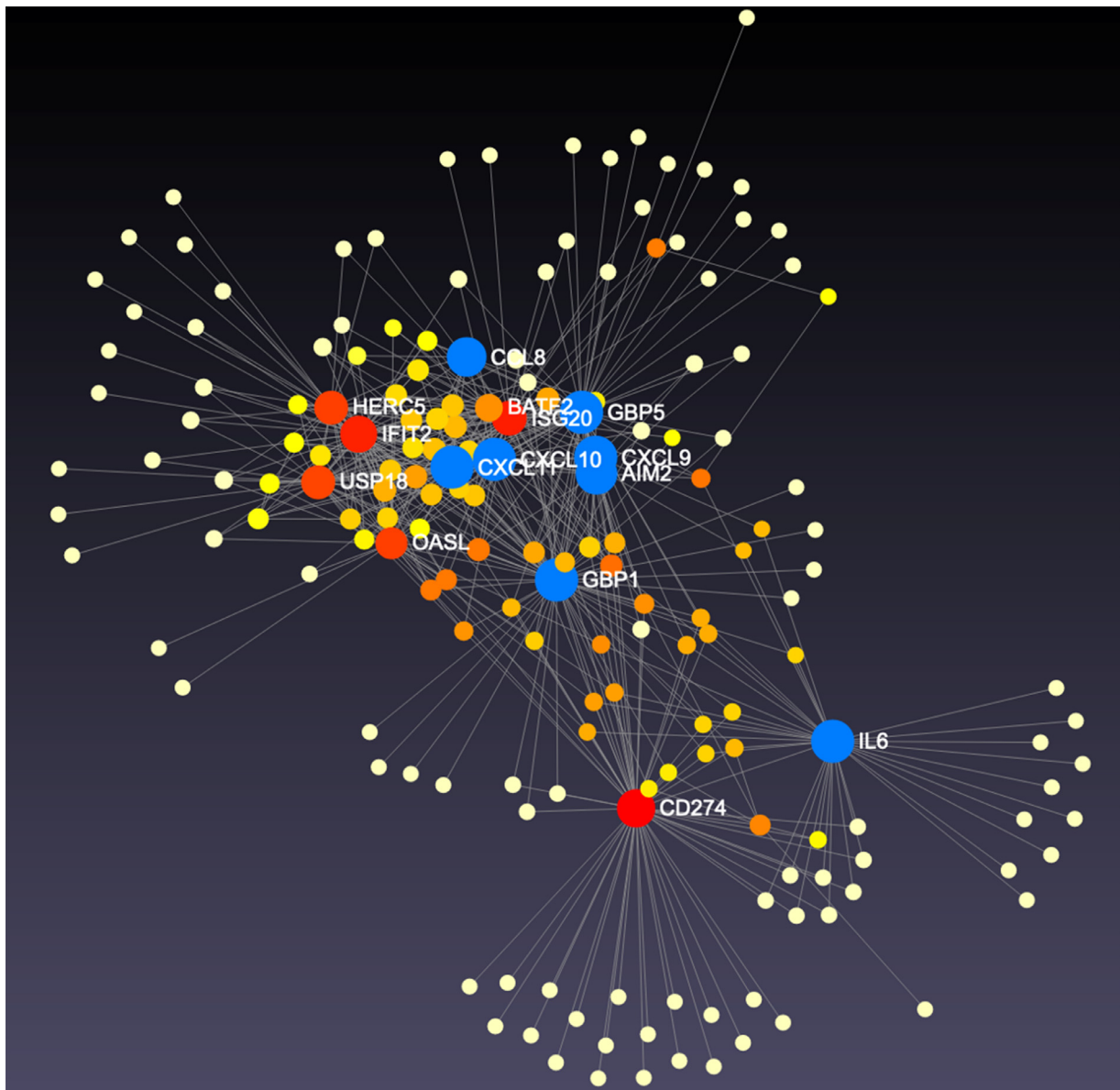


Fig. 5. Continued

M1	M2	M2
Thai-53 and Br4923	NHDP and Thai-53	Thai-53 and Br4923
OASL	MDGA1	IFIT1
IFIT1	SLAMF7	
IFIT2	IL4I1	
CXCL10	C3	

human macrophages (Fig. 2) (A complete list of genes is shown in Supplemental file 1). Common genes found between M1 stimulated with Thai-53 and Br4923 DNA included: OASL, IFIT2, IFIT1, and CXCL10 (Fig. 2A and Table 1). These four overlapped genes from the M1 stimulated with Thai53 and Br4923 DNA had eight significant GO terms and formed one significant STRING network cluster: 4Fe-4S single cluster domain, and Interferon-induced protein with tetratricopeptide. Interestingly, 311 distinct DE genes were observed in M1 uniquely stimulated by Thai-53 strain. We found 204 significant GO terms for the M1 macrophages stimulated with Thai53 DNA with top terms including “defense response to virus” (GO:0051607), “defense response to other or-

ganism” (GO:0098542), and “innate immune response” (GO:0045087). Stimulation of M1 activated (M1a) macrophages with DNA from each of three different strains identified 0, 61, and 1 DE genes for the NHDP, Thai-53, and Br4923 DNA respectively (Fig. 2B). For M1a, Thai53 DNA-stimulated cells had 157 significant GO terms and 13 network clusters. Finally, M2 showed 14, 514, and 2 DE genes for the NHDP, Thai-53, and Br4923 DNA stimulations (Fig. 2C). M2 cells stimulated with Thai-53 and Br4923 DNA both contained IFIT1, while M2-stimulated with Thai-53 and NHDP DNA contained MDGA1, SLAMF7, IL4I1, and C3. Commonly expressed DE genes upon stimulation of different *M. leprae* strains are summarized in Table 1. Thai53 DNA-stimulated M2 cells showed 410 significant GO terms and 42 network clusters. All GO terms and network cluster lists can be found in Supplemental file 2 with the associated genes for each. In this aim, we challenged macrophage with DNA from different *M. leprae* strains and observed that there were both commonly and uniquely expressed genes by each *M. leprae* strains. These genes were identified to belong to various immune cellular pathways.

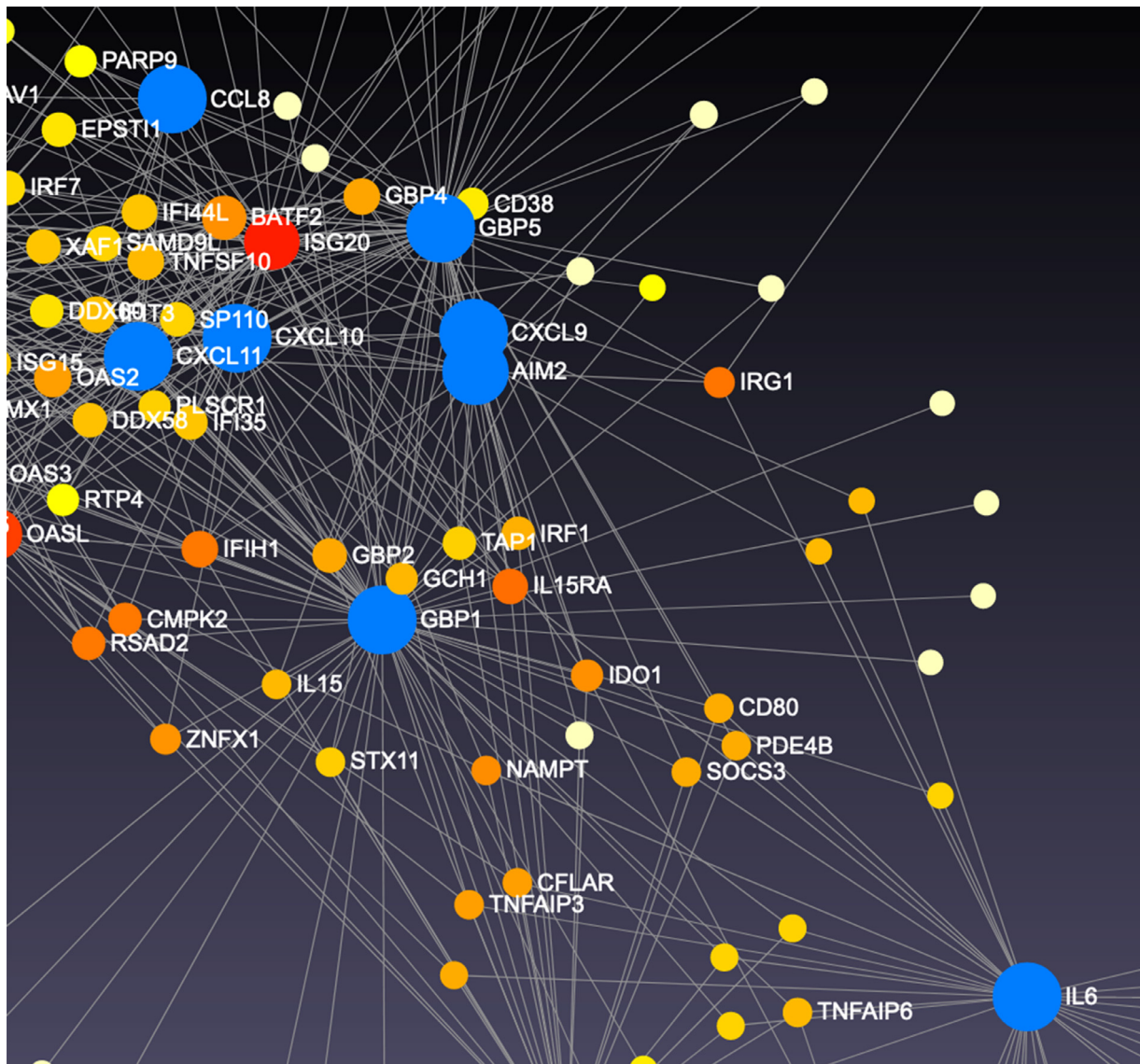


Fig. 5. Continued

4.2. Macrophage polarization in the responses to *M. leprae* DNA exposure

4.2.1. Unique gene expression profile in M1a compared to M1 and M2 polarization

We then aimed to analyze the role of macrophage polarization in the response to *M. leprae* DNA. We were particularly interested in the activated M1 phenotype (M1a) that were generated by additional LPS and IFN- γ . DE genes occurring in M1a compared to M1 and M2 were identified for Thai-53, Br4923 and NHDP DNA stimulations. M1a phenotype expressed 4 unique DE genes compared to M1 when *M. leprae* strain Br4923 was applied (Fig. 3A). Using DNA from different *M. leprae* strains for stimulation showed different profiles based on macrophage polarization status. When DNA from *M. leprae* strain NHDP was used, M1a expressed 259 and 234 unique DE compared to M1 and M2 respectively, while expressing 416 common genes (Fig. 3B). Lastly, when M1a cells were stimulated with DNA from *M. leprae* strain Thai-53, we observed 387 and 240 unique DE compared to M1 and M2 respectively, while expressing 815 common genes (Fig. 3B). (Supplemental file 3 displays the list of these significant DE genes, and supplemental file 4 lists the significant GO terms and network clusters).

4.2.2. Gene differential expression on macrophage polarization

From the list of significant genes, we selected a list of 18 genes that were up-regulated in M1a compared to either M1 or M2 upon stimulation with either NHDP or Thai-53 DNA, but down-regulated by stimulation with the other strain (Fig. 4). These included genes SCUBE2, AMOTL2, GCNT4, SELL, PAX5, TMCC3, and HEPH, which were up-regulated in M1a upon Thai-53 DNA stimulation but down regulated with NHDP DNA. It also showed that SEMA3D, CCL4, PGTGER3, and MMP1 were in turn up-regulated with NHDP DNA stimulation but some of these genes (SEMA3D and CCL4) only showed down-regulation in M1a/M1 of Thai-53 DNA stimulated, while others (PTGER3, and MMP1) showed down-regulation in M1a/M2 of Thai-53 DNA stimulated. CCL3 was unique in showing down-regulation on M1a/M2 upon either, NHDP or Thai-53 DNA stimulation (Fig. 4A). When looking at expression switches within a strain treatment, we identified CCL13 as being the sole gene that had opposite DE in M1a compared to M1 vs. M1 compared to M2 (Fig. 4B). CCL13 showed co-regulation with MMP1, PTGER3, CCL4, and TMCC3 (Fig. 4C). GO term analysis of these genes showed three significant pathways: leukocyte migration (GO:0050900), negative regulation of viral transcription (GO:0032897), and cellular response to interferon-gamma (GO:0071346).

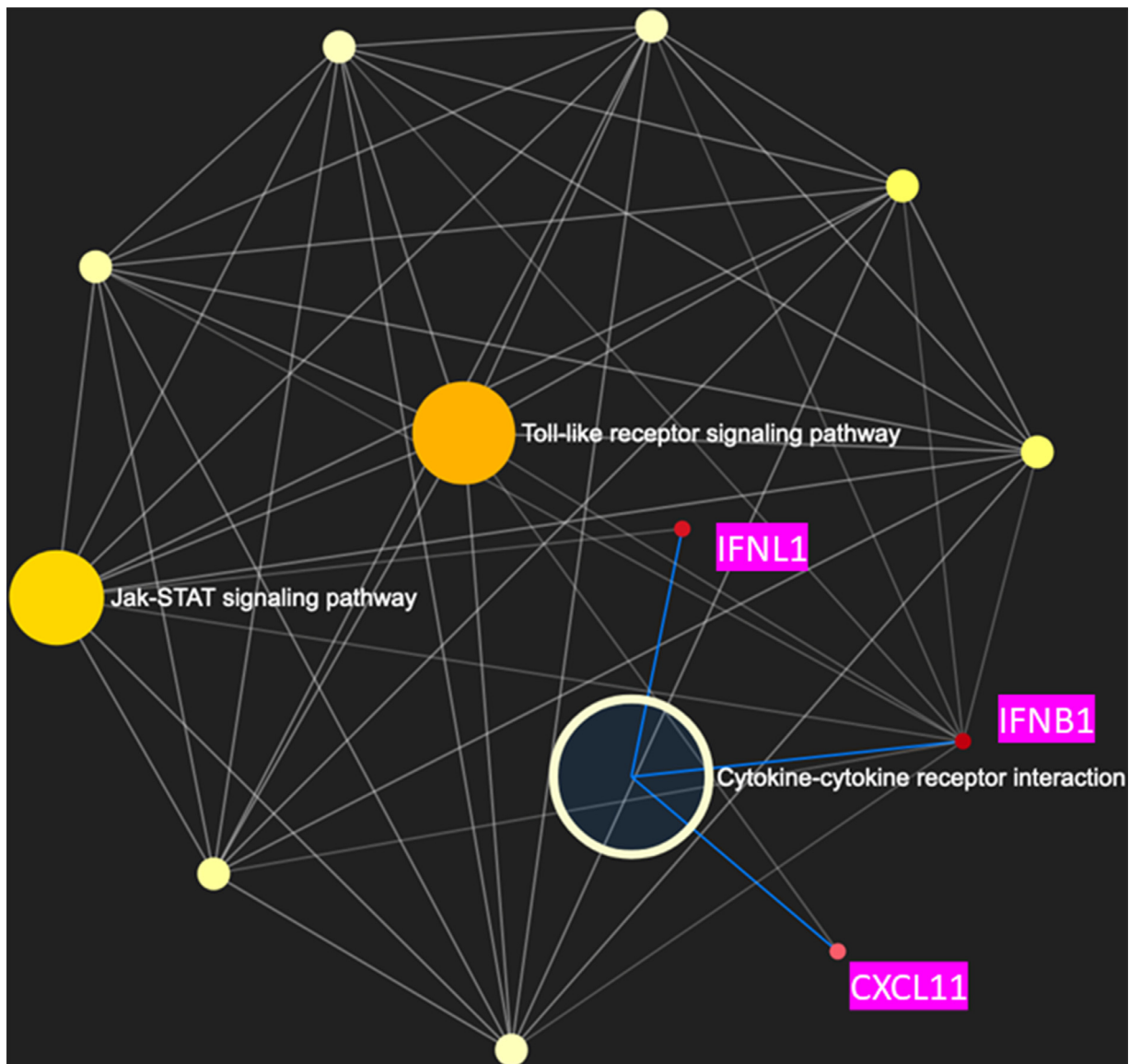


Fig. 6. Network analysis of DE genes presented in M1 activated macrophages upon stimulation with genomic DNA *M.leprae* strain Thai-53. **A.** Highly correlated genes in pairwise gene expression. **B.** Genes that were upregulated together in a similar manner shown as connected nodes in the network. **C.** Detail of **B.**

4.2.3. Network analysis for pathway identification

We then constructed a global enrichment network on cells stimulated with Thai-53 strain, given the large number of DE genes observed. We observed that DHX58, DDX58, IFIT1, CXCL10, MX1, RSAD2, IFIH1, and TNFS10 genes co-regulate influenza A ($p = 0.0114$), RIG-I-like receptor signal ($p = 0.0224$), and Hepatitis C pathways ($p = 0.0305$) (Fig. 5A) in M1 cells. When the network was constructed specifically for macrophage gene expression, CCL8, CXCL11, OASL, SP110, CXCL9, AIM2, GBP5, GBP1, and IL-6 were co-regulated in M1 cells (Fig. 5B). These genes were observed to significantly contribute to 5 macrophage gene pathways: toll-like receptor signal ($p = 0.0044$), cytokine-cytokine receptor signal ($p = 0.0077$), cytosolic DNA-sensing ($p = 0.0001$), NOD-like receptor signaling ($p = 0.0092$), and chemokine signaling ($p = 0.0095$). For M1a gene expression, the enrichment network analysis did not show any significant pathways based on our DE genes (Fig. 6A), after multiple comparison adjustments. However, the macrophage specific network analysis indicated 3 significantly distinct pathways: The toll-like receptor signal ($p = 0.0134$), chemokine signaling ($p = 0.0246$), and cytokine-cytokine receptor signaling ($p = 0.038$). CXCL11 along with

HERC5 were major hubs controlling the expression of numerous interferon stimulated genes (ISGs) (Fig. 6B), and interferon regulatory factor (IRF)-7 (Fig. 6C). M2 cells showed cytokine-cytokine receptor signal ($p = 0.0129$) pathway as a distinct profile from enrichment network analysis. M2 macrophages showed numerous co-regulations of ISGs as well, including CXCL9, CXCL10, CXCL11, CCL7, CCL8, IFNB1, IFNL1, CCR4, OSM and CCL13 (Fig. 7). The macrophage specific network analysis didn't lead to any significant pathway based on our DE genes, after multiple comparison adjustment.

5. Discussion

When investigating an infectious disease the environment, host, and pathogen all play a role in disease progression. Variation in *M. leprae* genome has been shown in findings from current molecular epidemiology studies in China (Xing et al., 2009, Weng et al., 2011) and Brazil (Fontes et al., 2017), as well as in a study comparing strains and phylogeny from ancient and modern *M. leprae* strains (Schuenemann et al., 2018). Genetic analysis has shown that Thai-53 strain displays remark-

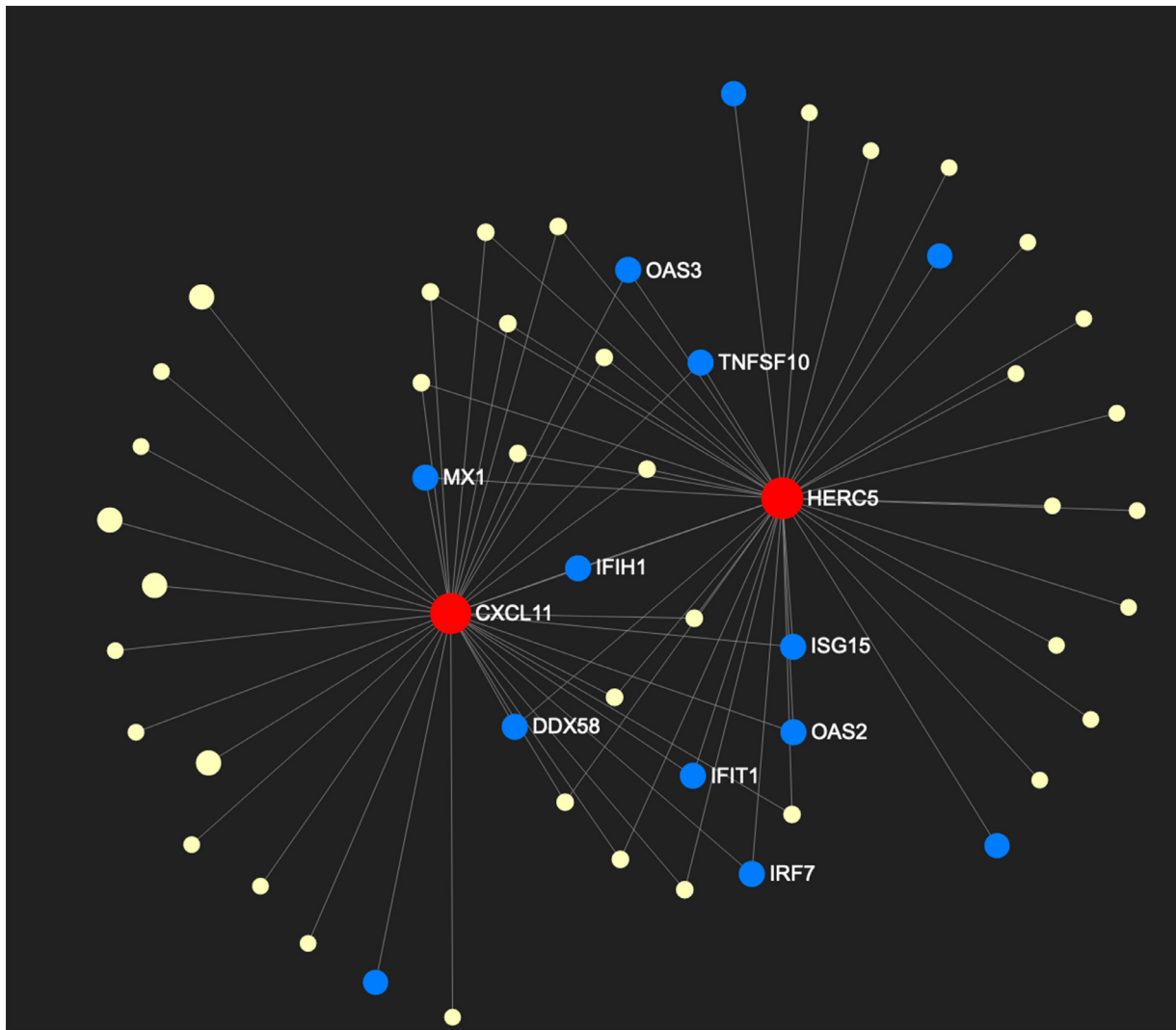


Fig. 6. Continued

able genetic variability (Truman et al., 2004). Despite the genetic variability within this pathogen (Singh et al., 2015, Benjak et al., 2018), the current consensus is that symptoms and disease course are dictated by host response (Fischer, 2017), with a focus on T-helper lymphocytes as the primary host mediators (de Sousa et al., 2017). However, recent research is now indicating that macrophage polarization also plays a central role in the clinical presentation of leprosy (Fachin et al., 2017, Pinheiro et al., 2018, Fallows et al., 2016). We herein showed that genomic DNA from various strains of *M. leprae* can induce different transcriptomes in human macrophages and that these responses are affected by macrophage polarization.

We utilized genomic DNA from three strains as each one of them belongs to a different genetic subtype (Singh and Cole, 2011, Truman et al., 2011).

We observed a large number of DE genes upon stimulation with DNA from *M. leprae* strain Thai-53 compared to strains NHDP and Br4923, independent of the macrophage polarization condition. A few genes were common in M1 macrophages after stimulation with DNA from different strains. These included OASL, which inhibits antimicrobial peptides expression and bacterial killing preventing *M. leprae* clearance (de Toledo-Pinto et al., 2016), IFIT1, and CXCL10). All these three genes have previously shown to be associated with type I IFN-activated pathway in THP1 derived macrophages (Zhang et al., 2019). Type I IFNs are associated with disseminated and progressive lepromatous lesions (Teles et al.,

2013, P et al., 2019). Shared genes observed in M2 macrophages included MDGA1, SLAMF7, IL4I1, and C3. MDGA1 has been reported in integrated stress response (Kovaleva et al., 2016). SLAMF7 is observed in all forms of leprosy (Belone Ade et al., 2015) and in M1 macrophages as well (Schulz et al., 2019). IL4I1 has been seen up-regulated in DCs after NOD2 stimulation with correlation with leprosy patients with limited disease (Schenk et al., 2012). C3 plays a role in opsonization of *M. leprae* (Schorey et al., 1997).

We also found that macrophage polarization affects the responses to *M. leprae* DNA. Genes DE in M1 macrophages upon stimulation with DNA from strain Thai-53 included pro-inflammatory receptor TARM1 (Radjabova et al., 2015), M2-associated SELL (Mould et al., 2019), and IFN- γ and IFN- β associated TMCC3 (Zhang et al., 2010). On the other hand, stimulation of NHDP DNA on M1 macrophages induced CCL4, an innate immunity cytokine associated with leprosy occurring in households (van Hooij et al., 2020), M2-associated MMP-1 (Jager et al., 2016), and PGE2 receptor PTGER3, which is associated with immune evasion of mycobacteria (Behar et al., 2010). CCL13 was clearly up-regulated in M1 activated cells compared to M1 regardless of the strain, as well in M2 macrophages upon Thai-53 DNA stimulation. This cytokine is involved in many chronic inflammatory diseases (Mendez-Enriquez and Garcia-Zepeda, 2013). No common genes were found upon stimulation with Br4923 DNA.

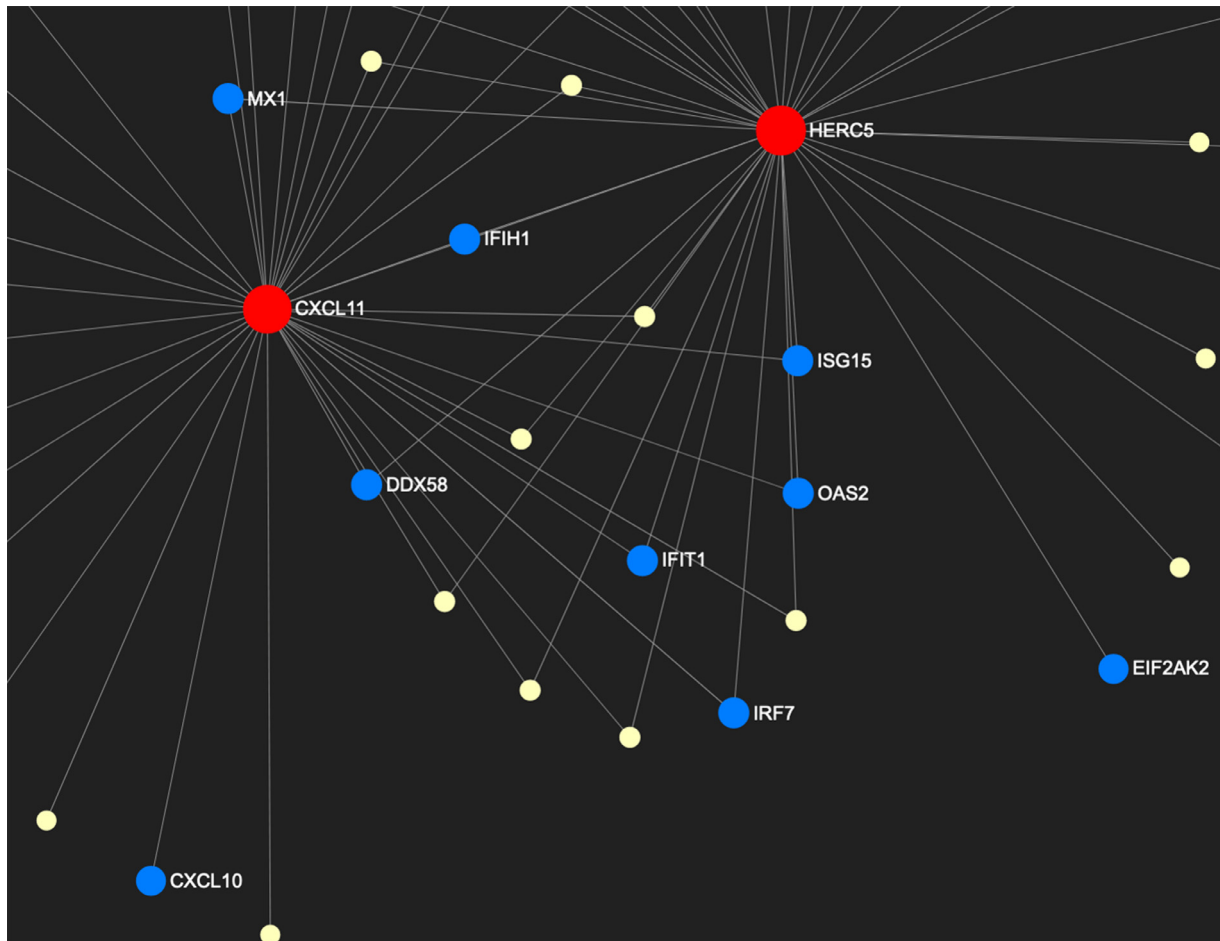


Fig. 6. Continued

As stimulation with DNA from strain Thai-53 yielded a large amount of DE genes, we were able to construct a comprehensive network analysis of co-regulated genes in each of the macrophage polarization conditions. We observed that in M1 and M1a cells there were numerous up-regulated ISGs. In M1a specifically, CXCL-11 and IFNB1 were up-regulated, underscoring Type I IFN pathway activation. CXCL10 and CXCL11 are induced by IFN- γ and by IFN- β , whereas CXCL9 induction is restricted to IFN- γ Groom and Luster (2011). The inflammatory chemokines CXCL9, CXCL10, and CXCL11 share an exclusive chemokine receptor named CXC chemokine receptor 3 (CXCR3). The CXCL11-CXCR3 axis is crucial for macrophage resistance to mycobacterial infection (Torraca et al., 2015). Increased CXCL10 without correlation with IFN- γ , is characteristic of Type 1 reaction, a systemic inflammatory syndrome seen in BL (Scollard et al., 2011). IFNL1 (IL-29), reported to be greatly down-regulated in LL and BL forms of leprosy, was also an important node in M1a (Berrington et al., 2014). CXCL11 along with HERC5 were major hubs controlling the expression of numerous (ISGs). HERC5

is up-regulated by *M. leprae*-induced Type I IFN signature, and is a major regulator of this pathway (P et al., 2019).

IRF-7 was revealed from the network in M1a cells, which is in line with knowledge of THP-1 derived macrophages utilizing this transcription factor after recognition of *Mycobacterium tuberculosis* (Mtb), and hypothesized to help patients resist Mtb infection (Zhang et al., 2019). TLR-9 has been shown to sense *M. leprae* DNA (Dias et al., 2016), and may be using IRF-7 as a transcription factor for Type I IFN induction.

In summary, despite the accepted thinking that genomic variation of *M. leprae* may not substantially contribute to the clinical manifestations of the disease, we have shown here that DNA from different *M. leprae* strains are able to induce differential gene expression in human macrophages. M1 activated macrophages presented a marked differential expression of genes involved in Type I IFN regulation, macrophage activation, pathogen DNA recognition, and recruitment of effector cells to site of inflammation upon stimulation with *M. leprae* genomic DNA.

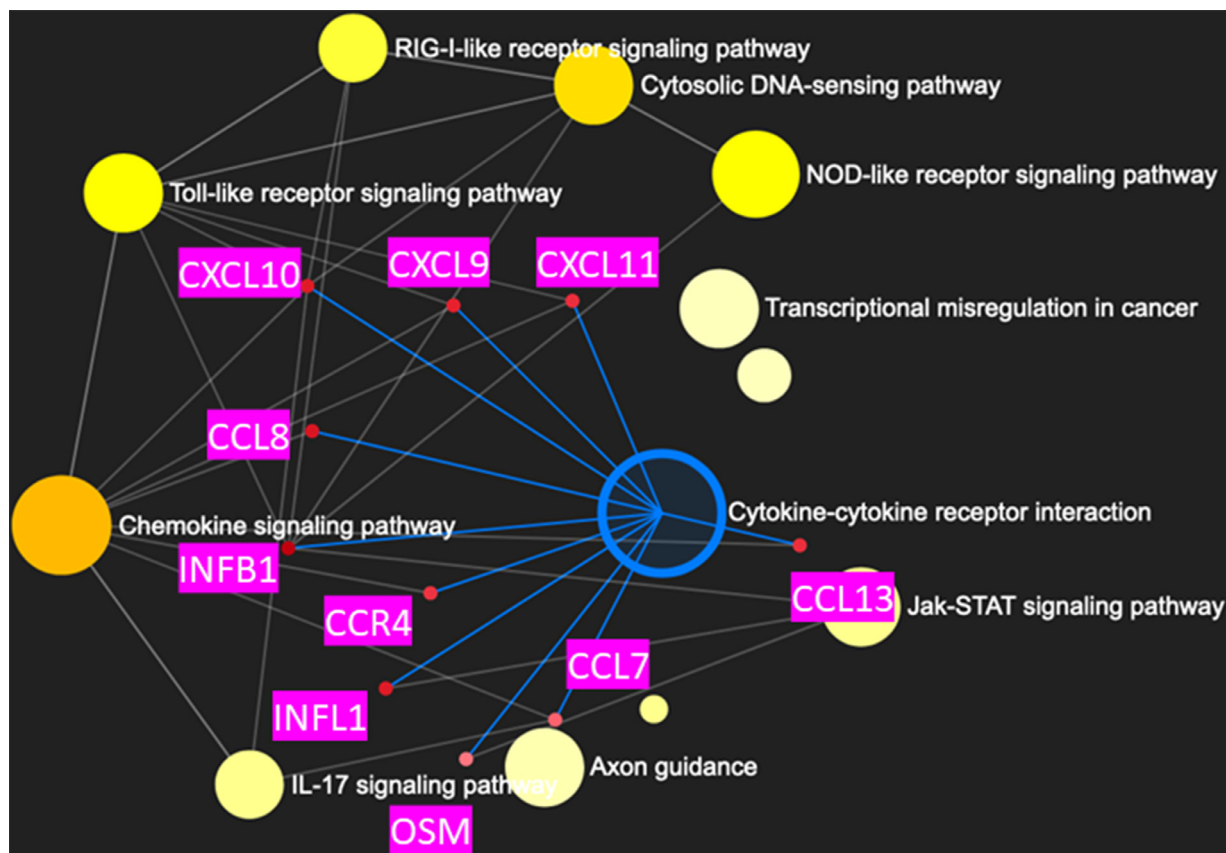


Fig. 7. Network analysis of DE genes presented in M2 macrophages upon stimulation with genomic DNA *M.leprae* strain Thai-53. A. Highly correlated genes in pairwise gene expression. B Genes that were upregulated together in a similar manner shown as connected.

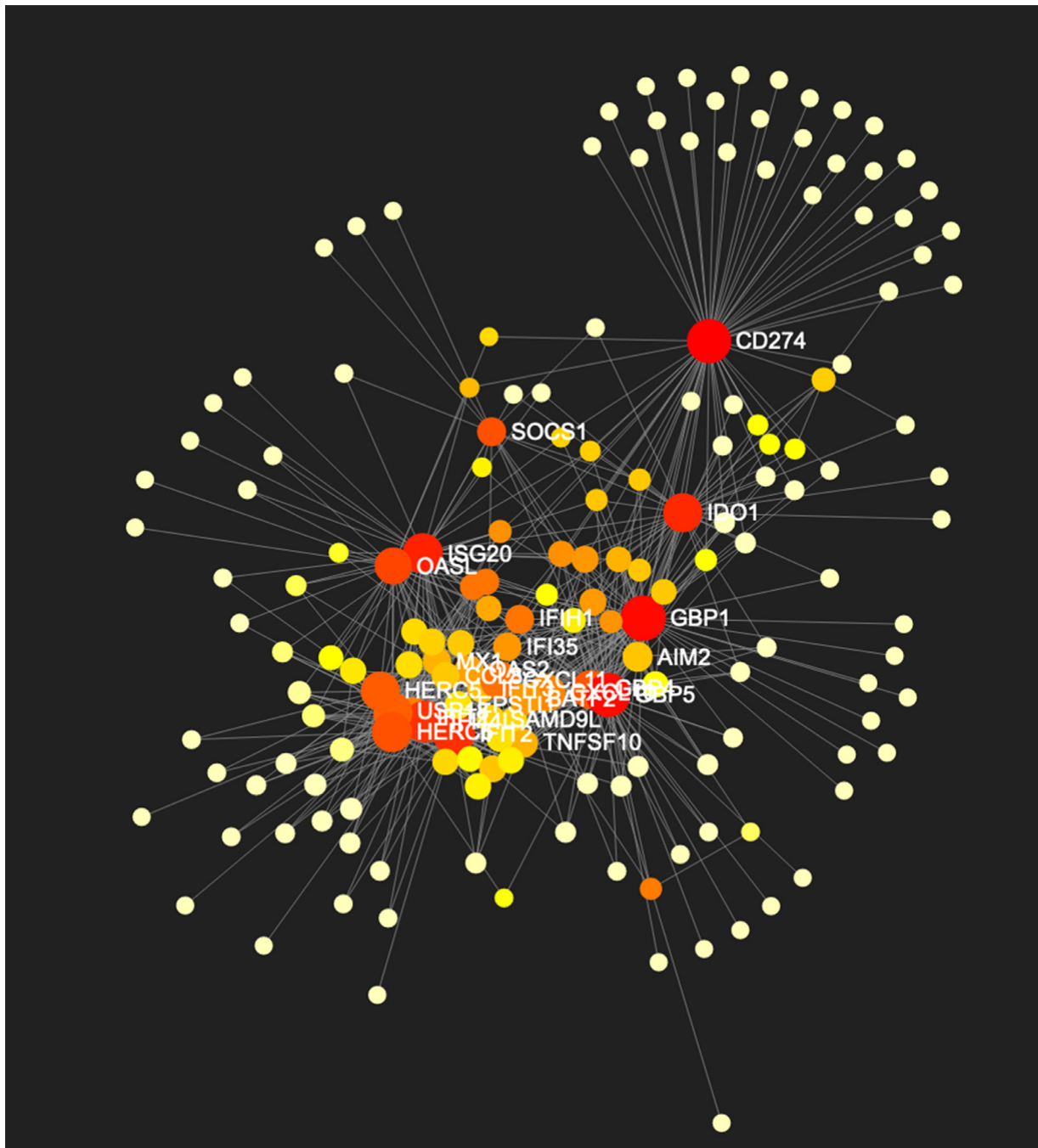


Fig. 7. Continued

6. Conclusions

While the current belief in the science and health community indicate that only host response effects gene expression and clinical exhibitions of leprosy, our data suggests *M. leprae* strain genomic variations may be able to induce differential gene expression in human macrophages. Activated M1 polarized macrophages presented a marked differential expression of genes involved in Type I IFN regulation, macrophage activation, pathogen recognition, pathogen DNA recognition, and recruitment of effector cells to site of inflammation upon stimulation with *M. leprae* genomic DNA.

Author contributions

JC conceptualized the study. AM, KVH, and JC performed the experiments. BYH, JM and SK performed the bioinformatics analysis. JC, BYH, AM, KVH, and JM prepared the manuscript.

Declaration of Competing Interest

None.

