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RESEARCH ARTICLE

# Metadichol induced expression of Toll Receptor family members in peripheral blood mononuclear cells

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## ABSTRACT

**Introduction:** Toll receptors are vital to the innate immune system. They recognize common microbial pathogen molecular patterns. The signaling pathways activated produce proinflammatory cytokines and type I interferons to start the immune response to infection. Inflammatory, autoimmune, and cancer diseases are connected to signaling abnormalities. Thus, toll receptor expression patterns and control mechanisms are essential for immune response research and treatment.

**Methods:** Metadichol, a nanoemulsion of long-chain alcohol, was applied to peripheral blood mononuclear cells to evaluate the expression of all ten toll receptor family members (TLR1-10), MYD88, and downstream genes (IRAK4, TRAF3, TRAF6 and TRIF). Quantitative real-time PCR measured gene expression.

**Results:** Toll receptors 1–10 responded as inverted U-shaped to Metadichol treatment, except Toll receptor 4. Metadichol affects TLR expression differently at low, moderate, and high dosages. Metadichol activated all 15 genes, including TLRs and downstream signalizing molecules.

Importing 15 genes into Pathway Studio produced a gene expression network analysis. Gene set enrichment analysis (GSEA) used the proprietary Elsevier pathway collection. An enriched gene list with more gene interactions than expected for a random gene collection of similar size and distribution indicated a significant biological relationship between these genes.

**Conclusions:** Metadichol expresses all the toll receptors, the MYD88 gene, and four other downfield genes. Since all immune cells express TLRs, this leads to a more robust solution for activating innate and adaptive immunity processes in humans.

**Keywords:** Toll receptors 1-10, Metadichol, Immune responses, MYD88, IRAK4, TRAF3, TRIF, PBMCs

## Abbreviations

| | |
|---|---|
| AHR | Aryl hydrocarbon receptor |
| AITD | Autoimmune thyroid disease |
| AKI | Acute kidney injury |
| FDA | Food and Drug Administration |
| GR | Glucocorticoid receptor |
| GSEA | Gene set enrichment analysis |
| IRF | Interferon regulatory factor |
| LN | Lupus nephritis |
| LRR | Leucine-rich repeat |
| LXR | Liver X receptor |
| LXRE | LXR response elements |
| MDSC | Myeloid-derived suppressor cell |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cells |
| ROR | Related orphan receptors |
| SLE | Systemic lupus erythematosus |
| TLR | Toll like receptors |

## Introduction

Ten cell surface and internal TLR receptors make up the family. They are crucial to immune system regulation. [1] TLRs 1, 2, 4, and 5 on the cell surface and TLRs 3, 7, 8, and 9 in diverse internal compartments may detect microbial molecular patterns.

TLR gene evolution has helped identify many infections. [2, 3] TLRs recognize pathogen-associated molecular patterns (PAMPs) via their transmembrane, cytoplasmic Toll/IL-1 receptor, and leucine-rich repeat (LRR) ectodomains. [4] The LRR ectodomain induces downstream signaling. TLRs recognize lipids, lipoproteins, proteins, and nucleic acids, which help detect pathogens. [5] TLRs activate transcription factors, including NF-κB and IRFs, upon ligand binding, promoting antiviral immunity and inflammatory response. [6] The pathways activate adaptor molecules with the specificity and complexity needed for a balanced immune response and to minimize tissue harm from excessive inflammation. [7]

TLR4 has an important role in cancer, infectious, and inflammatory diseases. [8,9] Gene variants impact colorectal cancer, indicating their importance to disease vulnerability. [10]

Thus, ligand-specific targeting of TLRs and their signaling pathways is a promising immune modulation therapy. [11] TLRs play an important role in innate and adaptive immunity. [12] IRAK4, TRAF3, TRAF6, and TRIF [13] are also involved in TLR signaling, leading to an effective immune response to pathogens.

IRAK4 transmits signals from all TLRs except TLR3 and leads to proinflammatory cytokine production via MAPKs and NF-κB. TRAF3 increases MyD88-dependent signaling and inhibits TRIF-dependent signaling. TRAF6, an E3 ubiquitin ligase, promotes inflammatory cytokine production via MAPKs and NF-κB. TRIF activates TLR3 and TLR4 and induces type I IFN via RIPK1 and TBK1.[14]

Monocytes, dendritic cells, B cells, and T cells in peripheral blood mononuclear cells (PBMCs) express TLRs. [15,16,17] TLR activation in PBMCs induces proinflammatory cytokines, antigen presentation, and acquired immunity. In systemic lupus erythematosus and autoimmune thyroid disease, dysregulated TLR expression and activity can cause pathogenesis. [18] TLRs are crucial to pathogen detection and PBMC immunological activity. Molecules like BCG, MPL, CBLB502, IMQ, IMO2055, and MGN1703 can induce TLR expression and activation in human cells. [19]

TLRs are versatile and clinically useful immune modulators used as vaccine adjuvants, anticancer treatments, and immunotherapies. [20] No one small molecule activates the whole TLR family, including MYD88 and other downfield transcription factors. Metadichol [21] is an exception since it effectively modulates the complete TLR family by activating all TLRs, including MYD88 transcription factors by treating PBMCs with Metadichol in concentrations of 1 picogram to 100 nanograms. This study explored Metadichol's effect on TLR expression.

## Material and Methods

All work was outsourced commercially to the service provider Skanda Life Sciences Pvt Ltd., Bangalore, India. Gene network analysis using Pathway Studio was outsourced commercially to Elsevier R&D Solutions, Inc. (Elsevier). The Primers were obtained from Saha BioSciences, Hyderabad, India, and the antibodies were from Elabscience®, Houston, Texas, USA. The raw qRT–PCR data and Western blot results are provided in the Supplemental files.

CELLS AND CELL CULTURE CONDITIONS

***Isolation of human WBCs***

*Preparation of blood samples:*

Fresh human blood was collected in EDTA-containing tubes, diluted with PBS at a 1:1 ratio, and mixed by inverting the tube. Approximately 5-8 ml of blood was collected from healthy volunteers with consent, and PBMCs were separated using the in-house SOPs and pooled. The collected PBMCs are used for research purposes. Since this was not a clinical trial, no such enrolment was done, and a detailed demographic profile was not considered in the studies. The inclusion criteria were based on the hematology and clinical parameters tested and evaluated. The Blood was collected from only those volunteers with all the tested parameters in the normal range. A No Objection Certificate for sourcing and use of PBMCs is in the Supplemental files.

***Isolation of mononuclear cells***

In a 15 ml centrifuge tube, 5 ml of Histopaque-1077 was added, and 5 ml of the prepared blood was slowly layered onto the Histopaque from the edge of the tube without disturbing the Histopaque layer. Then, the tubes were centrifuged at 400 × g for exactly 30 min at room temperature in no-brake mode. After centrifugation, the upper layer was discarded with a Pasteur pipette without disturbing the interphase layer. The interphase layer was carefully transferred to a clean centrifuge tube. The cells were washed with 1X PBS and centrifuged again at 250 × g for 10 mins; these steps were repeated two times. After centrifugation, the supernatant was discarded, and the pellet was collected in RPMI medium supplemented

with 10% fetal bovine serum (FBS). The cells were counted, and viability was evaluated with a hemocytometer.

The cells were prepared at a density of $1\times10^6$ cells/ml in medium, seeded into 6-well plates, and incubated for 24 hr at 37 °C with 5% CO2. At 24 h after seeding, the medium was carefully removed, and the cells were treated with the indicated test samples (the concentration was selected based on the MTT experiment) and incubated for 24 h at 37 °C in a CO2 incubator.

**Table 1.** Treatment concentrations

| Cell line | Sample name | Treatment details |
|---|---|---|
| Human PBMC | Metadichol | Control |
| | | 1 pg |
| | | 100 pg |
| | | 1 ng |
| | | 100ng |

SAMPLE PREPARATION AND RNA ISOLATION

The treated cells were dissociated, rinsed with sterile 1X PBS and centrifuged. The supernatant was decanted, and 0.1 ml of TRIzol was added and gently mixed by inversion for 1 min. The samples were allowed to stand for 10 min at room temperature. Then, 0.75 ml of chloroform was added to each 0.1 ml of TRIzol.

The contents were vortexed for 15 seconds. The tube was allowed to stand at room temperature for 5 mins. The resulting mixture was centrifuged at 12,000 rpm for 15 min at 4 °C. The upper aqueous phase was collected in a new sterile microcentrifuge tube, to which 0.25 ml of isopropanol was added, gently mixed by inverting the contents for 30 seconds and incubated at -20 °C for 20 minutes. The contents were centrifuged at 12,000 rpm for 10 minutes at 4 °C. The supernatant was discarded, and the RNA pellet was washed by adding 0.25 ml of 70% ethanol. The RNA mixture was centrifuged at 12,000 rpm at 4 °C. The supernatant was carefully discarded, and the pellet was air-dried. The RNA pellet was then resuspended in 20 µl of DEPC-treated water. The total RNA yield (Table 2) was quantified using a Spectradrop spectrophotometer (SpectraMax i3x, Molecular Devices, USA).

**Table 2.** RNA isolation yields

| | Test concentrations | | | | |
|---|---|---|---|---|---|
| RNA yield (ng/µl) | 0 | 1 pg/ ml | 100 pg/ ml | 1 ng/ ml | 100 ng/ ml |
| Human PBMC's | 438.00 | 304.64 | 161.92 | 376.24 | 382.88 |

Q-RT–PCR ANALYSIS

***cDNA synthesis***

cDNA was synthesized from 500 ng of RNA using the cDNA synthesis kit from the Prime Script RT Reagent Kit (TAKARA) with oligo dT primers, according to the manufacturer's instructions. The reaction volume was 20 µl, and cDNA synthesis was performed at 50 °C for 30 min, followed by RT inactivation at 85 °C for 5 min using the Applied Biosystems Veritii system. The cDNA was then used for real-time PCR analysis.

PRIMERS AND qPCR ANALYSIS

The PCR mixture (final volume of 20 µL) contained 1 µL of cDNA, 10 µL of SYBR Green Master Mix, and 1 µM complementary forward and reverse primers specific for the respective target genes. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of secondary denaturation at 95 °C for 30 s, annealing at the optimized temperature for 30 s, and extension at 72 °C for 1 min. The number of cycles that allowed amplification in the exponential range without reaching a plateau was selected as the optimal number of cycles. The obtained results were analyzed using CFX Maestro software.

- The fold change was calculated using the ΔΔCT method.
- The comparative CT method was used to determine the relative expression of target genes to that of the housekeeping gene (β-actin) in untreated control cells.

The delta CT for each treatment was calculated using the following formula:

- Delta Ct = Ct (target gene) – Ct (reference gene).
- To compare the delta Ct of individually treated samples with that of the untreated control samples, the Ct of each group was subtracted from that of the control to obtain the delta delta CT.
- Delta delta Ct = delta Ct (treatment group) – delta Ct (control group).
- The fold change in target gene expression for each treatment group was calculated using the following formula: Fold change = 2^ (−delta delta Ct).

PROTEIN ISOLATION

Total protein was isolated from $10^6$ cells using RIPA buffer supplemented with the protease inhibitor PMSF. The cells were lysed for 30 min at 4 °C with gentle inversion. The cells were centrifuged at 10,000 rpm for 15 min, and the supernatant was transferred to a fresh tube. The Bradford method was used to determine the protein concentration, and 25 µg of protein was mixed with 1× sample loading dye containing SDS and loaded

onto a gel. The proteins were separated under denaturing conditions in Tris-glycine buffer. The proteins were transferred to methanol-activated PVDF membranes (Invitrogen) using a Turbo transblot system (Bio-Rad, USA). Nonspecific binding was blocked by incubating the membranes in 5% BSA for 1 h. The membranes were incubated overnight with the corresponding primary antibodies at 4 °C, followed by incubation with a species-specific secondary antibody for 1 h at RT. The blots were washed and incubated with an enhanced chemiluminescence (ECL) substrate (Merck) for 1 min in the dark. Images were captured with appropriate exposure settings using a ChemiDoc XRS system (Bio-Rad, USA).

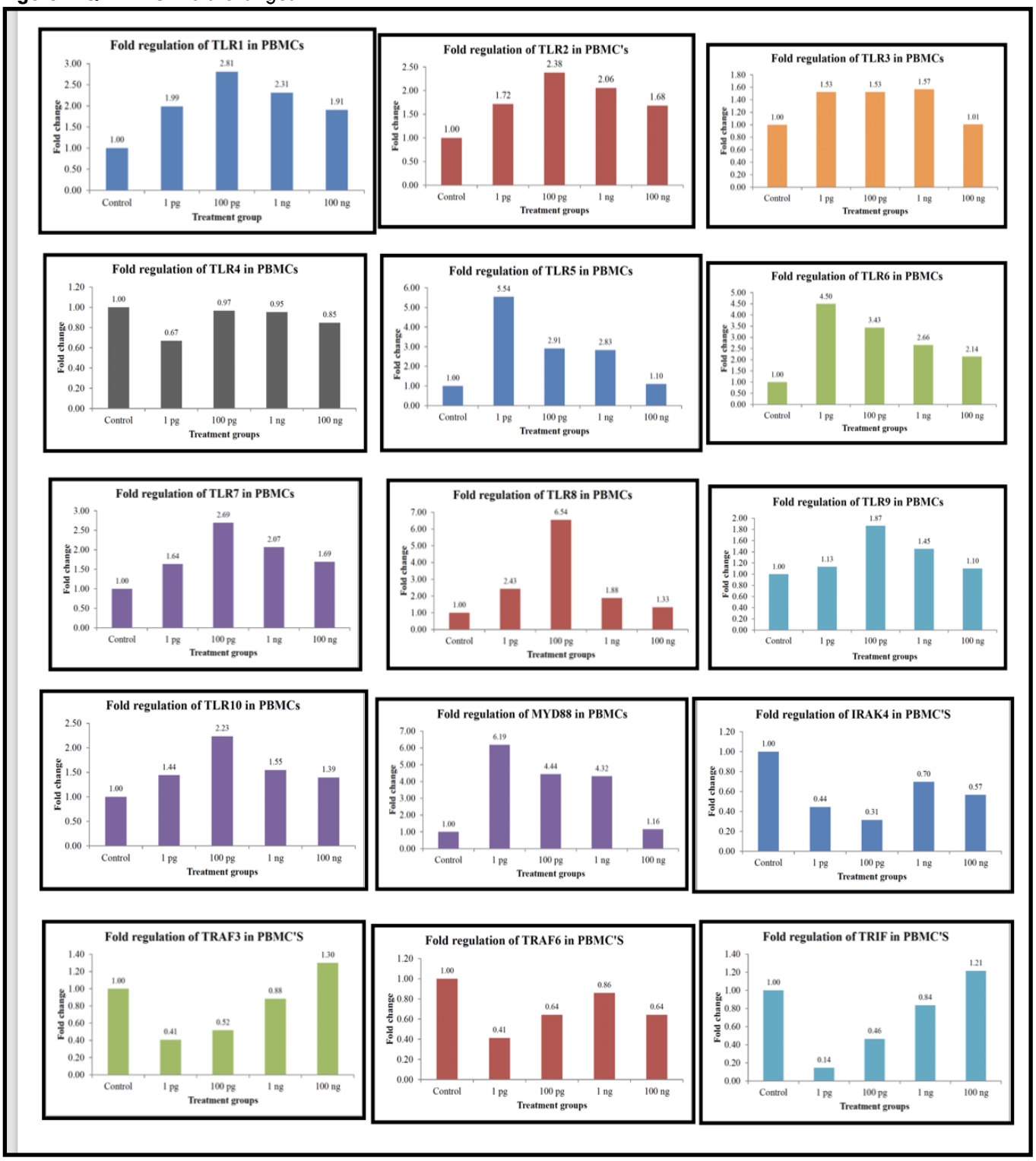
**Table 3.** Primer details

| Primer | Sequence | | Amplicon size | Annealing temperature |
|---|---|---|---|---|
| GAPDH | GTCTCCTCTGACTTCAACAGCG | Forward | 186 | 60 |
| | ACCACCCTGTTGCTGTAGCCAA | Reverse | | |
| TLR 1 | CAGCGATGTGTTCGGTTTTCCG | Forward | 145 | 67 |
| | GATGGGCAAAGCATGTGGACCA | Reverse | | |
| TLR 2 | CTTCACTCAGGAGCAGCAAGCA | Forward | 110 | 67 |
| | ACACCAGTGCTGTCCTGTGACA | Reverse | | |
| TLR 3 | CACCATTCCAGCCTCTTCGT | Forward | 157 | 65 |
| | CAGGGTTTGCGTGTTTCCAG | Reverse | | |
| TLR 4 | CCCTGAGGCATTTAGGCAGCTA | Forward | 125 | 65 |
| | AGGTAGAGAGGTGGCTTAGGCT | Reverse | | |
| TLR 5 | CCTTACAGCGAACCTCATCCAC | Forward | 129 | 65 |
| | TCCACTACAGGAGGAGAAGCGA | Reverse | | |
| TLR 6 | ACTGACCTTCCTGGATGTGGCA | Forward | 113 | 67 |
| | TGACCTCATCTTCTGGCAGCTC | Reverse | | |
| TLR 7 | CTTTGGACCTCAGCCACAACCA | Forward | 141 | 67 |
| | CGCAACTGGAAGGCATCTTGTAG | Reverse | | |
| TLR 8 | ACTCCAGCAGTTTCCTCGTCTC | Forward | 144 | 65 |
| | AAAGCCAGAGGGTAGGTGGGAA | Reverse | | |
| TLR 9 | CTGCCTTCCTACCCTGTGAG | Forward | 138 | 67 |
| | GGATGCGGTTGGAGGACAA | Reverse | | |
| TLR 10 | GGTTCTTTTGCGTGATGGAATC | Forward | 164 | 65 |
| | TGAGTGGTCTATGGCGTCCT | Reverse | | |
| MYD88 | CACCACACTTGATGACCCCC | Forward | 215 | 62 |
| | TGAGTGGTCTATGGCGTCCT | Reverse | | |
| IRAK4 | CATTCCCCGCCTTAATGCCT | Forward | 157 | 67 |
| | AGCTCTGTCAAAGGAACCCA | Reverse | | |
| TRAF3 | GCGTGTCAAGAGAGCATCGTT | Forward | 133 | 59 |
| | GCAGATGTCCCAGCATTAACT | Reverse | | |
| TRAF6 | CAATGCCAGCGTCCCTTCCAAA | Forward | 141 | 65 |
| | CCAAAGGACAGTTCTGGTCATGG | Reverse | | |
| TRIF | CCTGGAATCATCATCGGAACAG | Forward | 193 | 65 |
| | TGAGTGGTCTATGGCGTCCT | Reverse | | |

## Results

Inverted U-shaped responses were seen for toll receptors 1–10 and MYD88 but not for Toll receptor 4., which was downregulated (Fig. 1).

**Figure 1** Q-RT–PCR fold changes




## Discussion

Inversion of the U-shaped expression profile in response to small molecule concentrations reveals complex regulatory mechanisms. Low and high doses of small compounds may impact gene expression differently due to the biphasic dose-response effect. [22] Research [23] has shown an inverted U-shaped genetic response to estrogen (E2). In young women, estrogen has a monotonically increasing influence on hippocampus activity and an inverted U-shaped dose-response effect. Hippocampal genes respond to estrogen in an inverted U-shaped pattern, showing a complicated interaction between estrogen signaling and gene expression.

The antibiotic dose can also affect bacterial resistance genes inverted U-shaped response. [23] A nonlinear response pattern in antibiotic resistance genes highlights the complexity of genes with an inverted U-shaped response. This intricacy reflects the complex gene expression regulation mechanisms that respond to tiny chemical concentrations.

Few medicines with an inverted U-shaped dose-response function are licensed for therapeutic use. Fenfluramine, a 5-HT releaser used to treat seizures in Lennox–Gastaut and Dravet syndromes, has a U-shaped dose-response curve. [24] This example shows the therapeutic potential of medications with this pattern and the significance of

understanding dose-response relationships in clinical settings. Due to the structural and functional variety of toll receptors, a single tiny chemical that impacts all toll receptors is difficult to imagine. We previously showed that Metadichol-treated cells expressed all 49 nuclear receptors. [25]

Given their activities, nuclear receptor activation may indirectly regulate TLR expression rather than directly. Nuclear receptors control immunological responses, including inflammation, and interact with transcription factors that regulate toll receptor-induced gene expression [26,27,28] found that liver X receptors (LXRs), such as LXRα and LXRβ [29] regulate toll receptor signaling and inflammation through multiple methods. LXRs regulate toll receptors signaling by inhibiting toll receptors 2, 4, and 9 from signaling to downstream NF-κB and MAPK effectors via ABCA1-dependent changes in membrane lipid composition. Directly binding to LXR response sites in target promoters regulates cholesterol, fatty acid, and phospholipid metabolism genes. They activate lipid metabolism genes and inhibit toll receptor-induced inflammatory gene expression. LXR activation suppresses inflammatory gene expression in cell and mouse models. The anti-inflammatory impact is principally caused by Abca1 sterol transporter modulation, altering membrane cholesterol homeostasis and inhibiting NF-κB and MAPK signaling pathways downstream of toll receptors. LXRs, especially LXRα and LXRβ, regulate metabolism and inflammation by influencing membrane composition, toll receptor signaling, and inflammatory gene expression, highlighting their crucial role in the modulation of the immune response.

AhR regulates TLR-induced gene expression. [30] AhR activation downregulates microRNAs that target anti-inflammatory and MDSC-regulatory genes, causing chemokine and receptor synthesis. [31] This cascade upregulates inflammatory disease-related proinflammatory responses. AhR activation reduces inflammation by thymic atrophy, apoptosis, regulatory T-cell (Treg) control of MDSCs, cytokine suppression, and epigenetic alterations. [32] AhR activation can prevent inflammatory diseases such colitis, multiple sclerosis, atopic dermatitis, and psoriasis by slowing these processes. Metadichol is an AhR nuclear receptor inverse agonist.[33] AhR activation regulates inflammatory dendritic cell development, which may aid the immune response in inflammatory situations.

Other toll receptor regulators include PPAR and GR (glucocorticoid receptor). [32] GRs regulate the immune response and interact with TLR signaling pathways, affecting inflammation and immune cell activity after TLR activation.

Another nuclear receptor type that interacts with toll receptor signaling pathways is PPARs. They reduce the induced inflammation by suppressing proinflammatory cytokines and increasing anti-inflammatory responses. The orphan nuclear receptor SHP (short heterodimer partner, also known as NROB2) negatively regulates toll receptor-induced inflammatory signaling. [14]

Retinoic acid receptor-related orphan nuclear receptors regulate toll receptor signaling. They regulate immune response genes, including toll receptors. [34] The direct effect of nuclear receptor activation on toll receptor expression may include complicated regulatory processes that require additional study. Crosstalk between nuclear receptors and toll receptor signaling pathways can affect immune response genes.

Toll receptor 4 did not follow the downregulated controls inverted U-shaped response. Its inhibition may treat different disorders. [35,36] Inhibiting its signaling lowers cardiac myocyte death and inflammation. Thus, targeting toll receptor 4 may cure trauma-hemorrhage, sepsis, acute lung injury, chronic inflammatory arthritis, airway inflammation, and autoimmune disorders.

Inhibition of Toll receptor 4 has been studied in myocardial inflammation, cardiovascular illness, allergy diseases, obesity-associated metabolic diseases, and neuronal degeneration and is linked to age-related disorders, making it important for disease research. [37] Additionally, inhibitors of toll receptor 4 inhibitors are being investigated for treating severe COVID-19 and age-related illnesses. [38,17] Metadichol is a strong SARS-CoV-2 inhibitor. [39] Also Inflammatory disorders and insulin resistance have been studied with TLR4 suppression. Lipid-induced insulin resistance in skeletal muscle can be prevented by TLR4 suppression. TAK-242, a selective TLR4 inhibitor, protects muscle cells and rats from lipid- and LPS-induced insulin resistance. [40] In an animal model of autoimmune peripheral neuropathy, TLR4 suppression protects against sensory and motor impairment. [41] Inhibiting TLR4 protects neurons and regulates microglial polarization in Alzheimer's disease. [42]

TLR4 expression correlates with prostate cancer metastatic potential and promotes epithelial ovarian cancer growth and chemoresistance. TLR4 increases breast cancer cell motility, invasion, angiogenesis, and metastatic potential. TLR4 activation in immune cells may increase anticancer responses, while in tumor or stromal cells, it may promote tumor development. Eritoran, a synthetic analog of Rhodobacter sphaeroides lipid A, suppresses TLR4 by blocking its interaction with lipid A, which may prevent bacterial LPS-induced colorectal cancer. [43]

TLR agonists engage innate and adaptive immune systems. In combination therapy cohorts, TLR agonism inhibits tumor growth. [44,45] BCG stimulates TLR's 2, 4, and 9. Urothelial cell carcinomas have less cell death, proliferation, and metastasis when TLRs are activated. [46]

Breast cancer cell proliferation decreases with TLR5 expression. [47,48] Also, TLR9 activation inhibits human glioma cell lines, [49,50] making them more susceptible to radiation treatment. BCG, which activates TLR" s 2, 3, 4, and 9, and imiquimod, a TLR7 agonist, are FDA-approved cancer treatments. [51] TLRs reduce inflammation, cell proliferation, apoptosis, and chemoresistance in malignant cells. [52.53] Anti-inflammatory features distinguish TLR10 from other TLRs. The suppressive actions of TLR10 make it an

inhibitory receptor. [54] TLR10 reduces inflammatory cytokines in primary human cells by suppressing inflammatory signaling. TLR10 is a prospective target for immune response modulation in cases where excessive inflammation is harmful due to its anti-inflammatory properties. TLR10's processes and signaling pathways may provide new targets for creating therapeutics that use its anti-inflammatory effects, despite inconsistent findings. [55] TLR10 activates gene transcription via MyD88 to reduce inflammation. As a coreceptor, it shares ligands with other TLRs. [42] Additionally, a functional variant of TLR10 was linked to NF-κB activity modulation, impacting disease severity like rheumatoid arthritis. [56,57] TLR1 surface expression falls by 36% in older individuals compared to young adults, demonstrating age-related TLR1 dysfunction. [58,59] Reduced monocyte TLR1 surface expression and cytokine production in older persons indicate its role in aging. TLR1/2 function decreases with aging, and older persons have lower TLR1 surface expression than younger adults. TLR1 expression failure may decrease infection response and change immunological signaling pathways with age. Thus, increasing TLR1 expression may reverse several age-related processes.

Metadichol increased TLR8 expression by almost 6.5-fold. The immunological effects of PBMC TLR8 overexpression are considerable. [60] In the innate immune system, TLR8 and related proteins recognize pathogen-associated molecular patterns (PAMPs) and activate the immune system. PBMCs with increased TLR8 expression may promote inflammation and autoimmune disorders by identifying self-RNA. TLR8 may be used in cancer immunotherapy and vaccination adjuvants. Its expression may be a biomarker for infections and autoimmune diseases. [61,62]

Increased TLR8 expression in PBMCs may have these effects.

1. Enhanced Immune Response: TLR8 activation produces cytokines and chemokines that boost pathogen defense.
2. Abnormal activation or overexpression of TLR8 may promote inflammation and self-RNA recognition in autoimmune disorders.
3. Immunotherapy: TLR8 is being studied for cancer immunotherapy. TLR8 agonists may boost antitumor immunity by activating immune cells.
4. Vaccine adjuvants: TLR8 agonists may boost vaccine effectiveness by boosting the immune system.
5. Disease Biomarkers: TLR8 expression may indicate infections and autoimmune illnesses. Increased TLR8 expression in PBMCs indicates immunological activation and may be therapeutic.

Testicular TLR4 mRNA suggests a function in immune responses. TLR4 is also expressed in the hypothalamus, hippocampus, cortex, and cerebellum. A variety of animal species express TLR4, although it is always present. [63] Pigs and rabbits have TLR4 mRNA in their lungs. TLR2 and TLR4 are also linked to acute kidney damage and lupus nephritis. TLR4 expression levels vary among mice strains and produce pro- and anti-inflammatory cytokines.

TLRs like 2 and 4 influence the inflammatory response, which causes kidney disorders. [64] TLR activation by microbial or endogenous ligands is essential for inflammatory response. [12] Because TLRs are expressed differently throughout organs, immune response and disease consequences are affected. Variations in endothelial TLR expression can affect inflammatory diseases. [9] The testis, brain, lung, kidney, and liver have different expression patterns, suggesting they are involved in immune response and disease processes. This suggests their various roles beyond traditional immune function.

**Fig. 2** Interactions and regulatory network of TLR1-10, MYD88, IRAK4, TRAF6, TRAF3, and TICAM1 (TRIF)

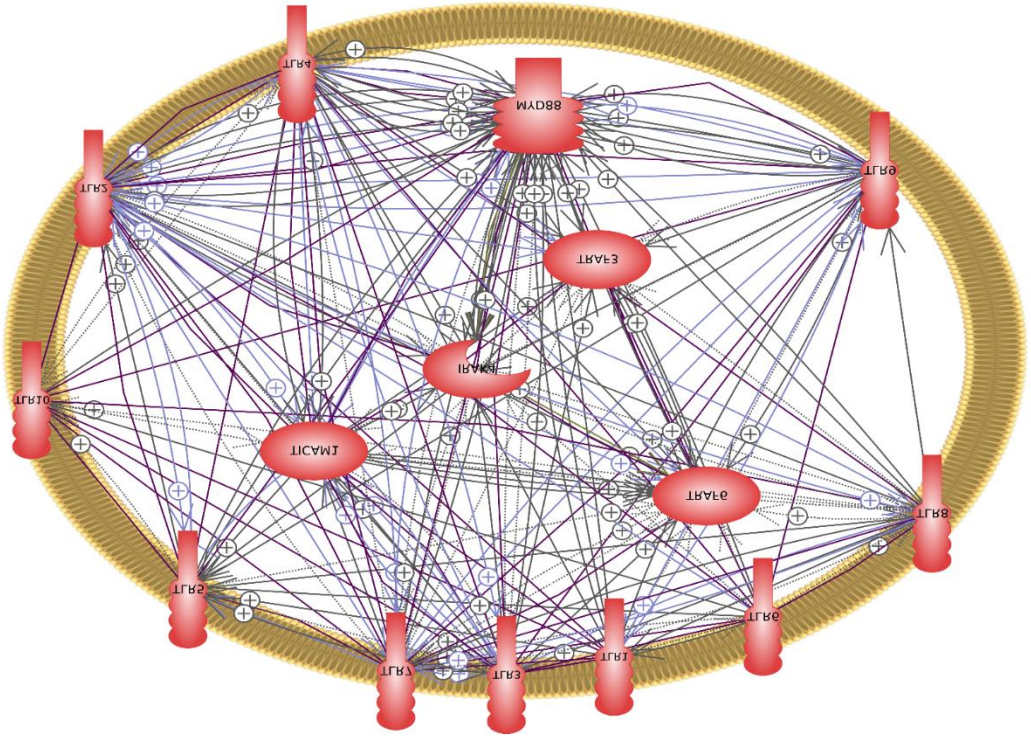


Fig. 2 displays all Elsevier Biology Knowledge Graph gene interactions for 15 genes. Gene networks analyzed using Pathway Studio and protein–protein interaction maps showed a feedback loop network. TLR genes activate and regulate each other through complex signaling pathways mediated by adaptor molecules in a closed-loop network. TLRs recruit adaptors such MyD88, TRIF, TIRAP/MAL, and TRAM to signal. Activation of transcription factors, NF-κB and IRFs, regulates immunological response. [12,5,1]

**Fig. 3** TLR gene interactions with the vitamin D receptor (VDR)

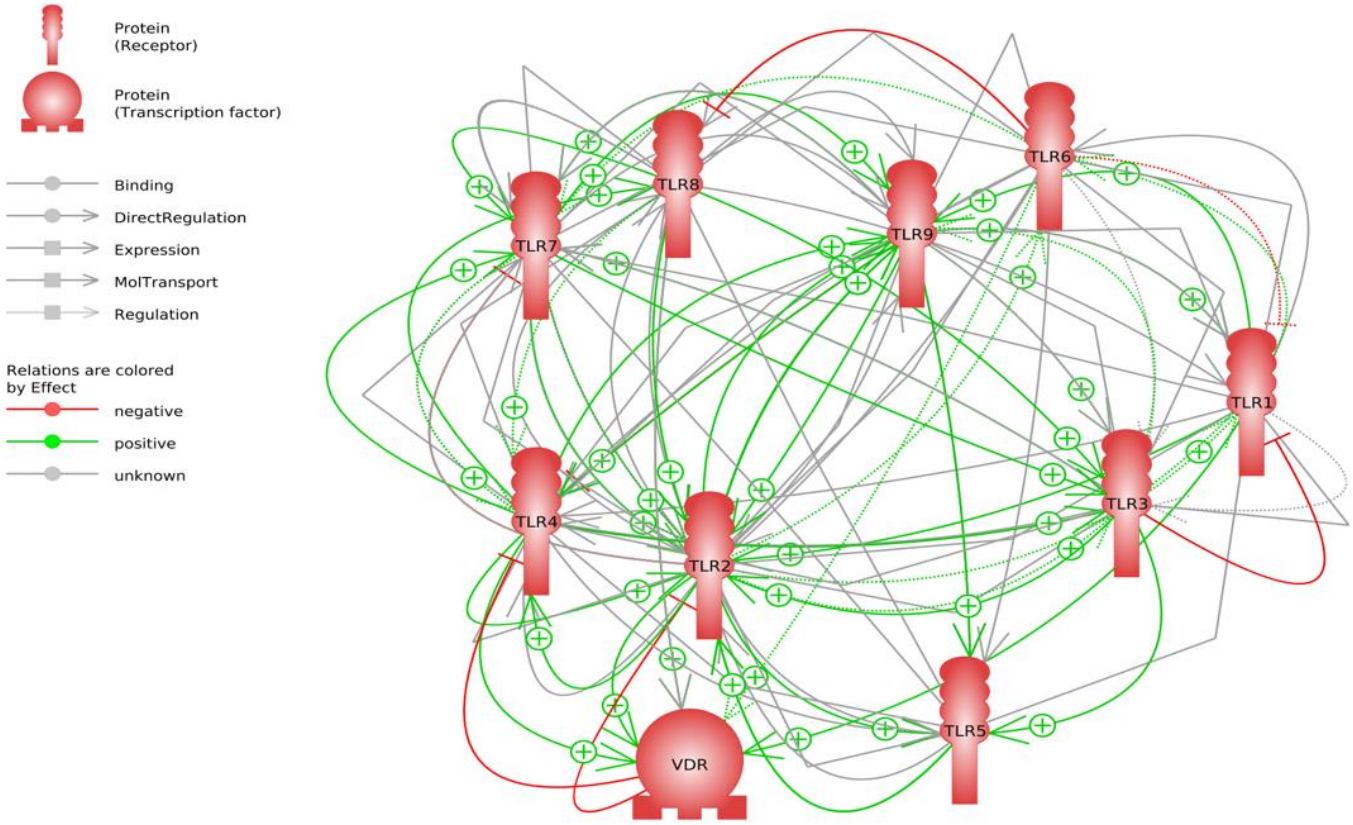



Figure 3 depicts how TLRs regulate each other and with the VDR to form closed-loop networks. VDR downregulates TLR4, conforming the result seen in Q-RT-PCR. TLR6 downregulates TLR8, TLR3 downregulates TLR1, VDR downregulates TLR 2, and TLR2 downregulates TLR7 (red lines).

Entering the gene set into the KEGG pathway database. [65,66] Fig 4 depicts metadichol-induced gene expression and its role in the TLR signaling pathway. It is unsurprising that Metadichol stimulates numerous pathways to fight germs and viruses. [39]

Importing the 15 genes into Pathway Studio software allowed one-network analysis of gene expression. [67,68] Elsevier's proprietary pathway collection was used for gene set enrichment analysis (GSEA). The gene list was enriched (table 4-6) , having more gene interactions than expected for a random gene collection of similar size and distribution. This enrichment strongly supported a biological relationship between these genes. [69]

**Fig. 4.** Input of genes expressed in TLR signaling pathway (KEGG)

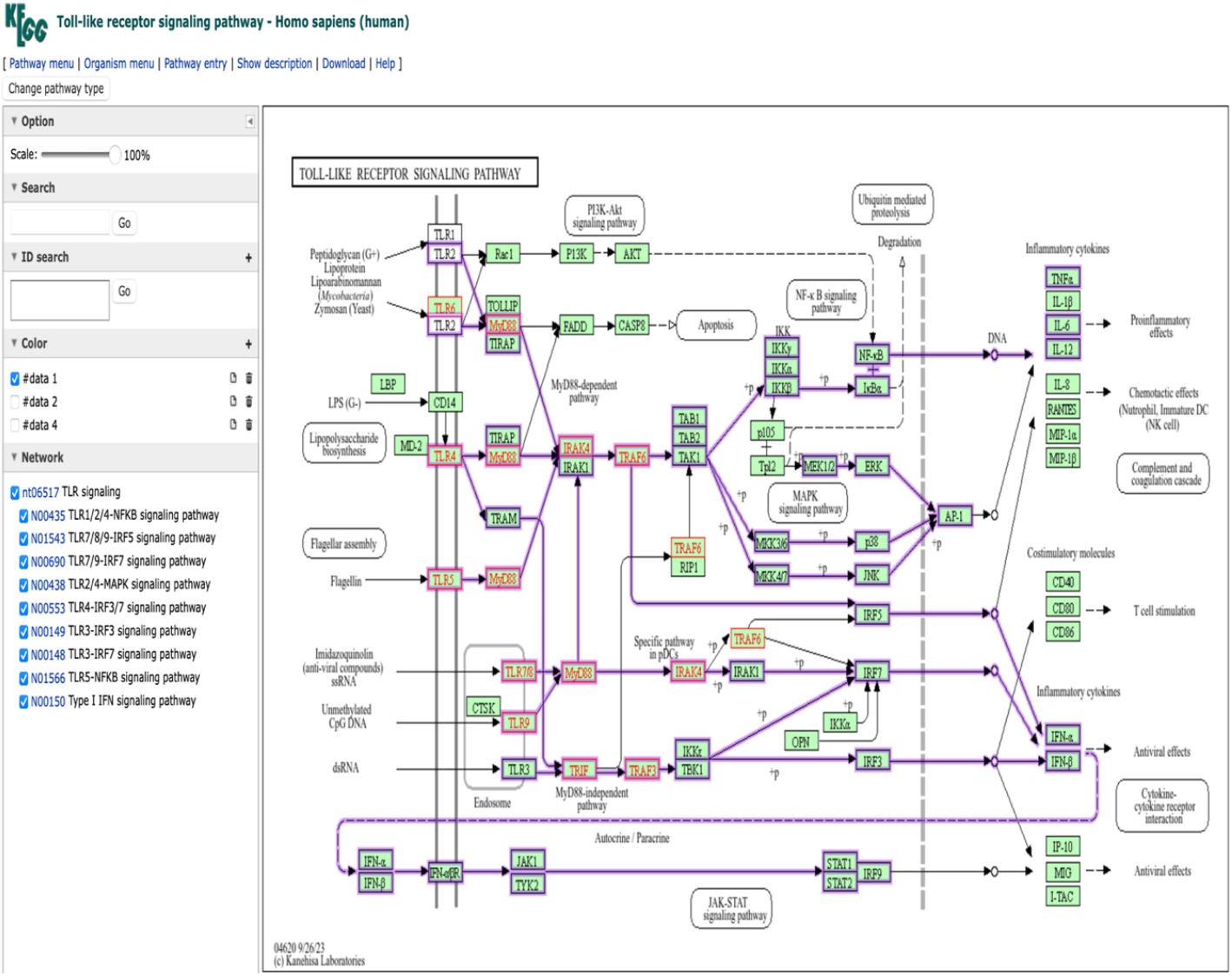



Table 4 Key biological pathways

The key biological pathways is Antiviral signaling through Pattern receptors as the top pathway in line with what we have described above. as well as the MYD88 signaling pathway

| Biological Process | Parent Folder | # of Entities | Expanded # of Entities | Overlap | Percent Overlap | Overlapping Entities | p value |
|---|---|---|---|---|---|---|---|
| Antiviral Signaling through Pattern Recognition Receptors | Antigen Recognition in the Innate Immune System; Antigen Recognition in the Innate Immune System (Immunological Pathways); COVID19-induced immune response; generic; plasma membrane; secretory vesicle | 31 | 36 | 10 | 27 | TRAF3, TRAF6, TLR2, TLR7, TICAM1, TLR3, TLR4, TLR8, MYD88, IRAK4 | 5.45964E-23 |
| Infection->TLRs in corneal ulcer | Corneal epithelium-immune system interactions | 95 | 281 | 13 | 4 | TLR7, TICAM1, TLR8, TRAF3, TRAF6, TLR6, IRAK4, TLR9, MYD88, TLR1, TLR2, TLR3, TLR4 | 6.35177E-20 |

| Biological Process | Parent Folder | # of Entities | Expanded # of Entities | Overlap | Percent Overlap | Overlapping Entities | p value |
|---|---|---|---|---|---|---|---|
| Fatty Acids Block TLR Signaling in Enteroendocrine Cells | Eating Behavior and Metabolism Regulation; Eating Behavior and Metabolism Regulation (Biological Process); digestive system; epithelium | 21 | 69 | 9 | 13 | TLR7, TLR9, TLR8, TLR10, MYD88, TRAF6, TLR3, TLR5, IRAK4 | 2.34166E-17 |
| Toll-like Receptors Act through MYD88 Signaling | Antigen Recognition in the Innate Immune System; Antigen Recognition in the Innate Immune System (Immunological Pathways); generic; generic; plasma membrane; secretory vesicle | 28 | 39 | 8 | 20 | TRAF6, TLR7, TLR5, TLR9, TLR8, TLR10, MYD88, IRAK4 | 4.31449E-17 |
| Toll-like Receptors in Sterile Inflammation | Inflammation Initiation; Inflammation Initiation (Inflammation Pathways); generic; generic | 75 | 103 | 9 | 8 | TICAM1, TRAF3, TRAF6, IRAK4, TLR9, MYD88, TLR2, TLR3, TLR4 | 1.02082E-15 |
| Mast-Cell Activation without Degranulation through IL1R1 and TLR Signaling | Mast Cell Activation; Mast Cell Activation (Myeloid Cell Activation in Inflammation); generic; hematological system | 27 | 155 | 9 | 5 | TLR7, TLR8, TRAF6, IRAK4, TLR9, TLR10, MYD88, TLR3, TLR5 | 4.45974E-14 |
| AXL Receptor Inhibits Macrophage and Dendritic Cell Function | Monocyte Activation; Monocyte Activation (Myeloid Cells Activation in Inflammation); generic; hematological system | 46 | 62 | 7 | 11 | TICAM1, TLR9, MYD88, TRAF6, TLR3, TLR4, IRAK4 | 4.98267E-13 |
| Mast-Cell Activation without Degranulation | Mast Cell Activation; Mast Cell Activation (Myeloid Cell Activation in Inflammation); generic; hematological system | 63 | 214 | 9 | 4 | TLR7, TLR8, TRAF6, IRAK4, TLR9, TLR10, MYD88, TLR3, TLR5 | 8.44955E-13 |
| Macrophage M1 Lineage | Monocyte Activation; Monocyte Activation (Myeloid Cell Activation in Inflammation); generic; hematological system | 66 | 345 | 10 | 2 | TLR7, TICAM1, TLR8, TRAF6, IRAK4, TLR9, TLR10, MYD88, TLR3, TLR5 | 1.09802E-12 |
| I-cell: CCK-8 Secretion and Regulation of Eating Behavior | Activation of Cells of the Gastrointestinal Tract; Activation of Cells of the Gastrointestinal Tract (Eating Behavior and Metabolism Regulation); digestive system; epithelium | 54 | 231 | 7 | 3 | TRAF6, IRAK4, TLR9, MYD88, TLR2, TLR4, TLR5 | 5.81468E-09 |

**Table 5.** Key signaling pathways

It shows key signaling cell processes pathways with Antiviral Signaling through Pattern Recognition Receptors as the key result

| Pathway | # of Entities | Expanded # of Entities | Overlap | Percent Overlap | Overlapping Entities | p value |
|---|---|---|---|---|---|---|
| Antiviral Signaling through Pattern Recognition Receptors | 31 | 36 | 10 | 27 | TRAF3, TRAF6, TLR2, TLR7, TICAM1, TLR3, TLR4, TLR8, MYD88, IRAK4 | 5.20380E-25 |

| Pathway | # of Entities | Expanded # of Entities | Overlap | Percent Overlap | Overlapping Entities | p value |
|---|---|---|---|---|---|---|
| Toll-like Receptors (TLR) in Antiviral Innate Immune Response | 30 | 48 | 10 | 20 | TLR7, TICAM1, TLR9, TLR8, MYD88, TRAF6, TLR2, TLR3, TLR4, IRAK4 | 1.33514E-23 |
| COVID19+>AGER feed forward loop in innate immunity | 30 | 38 | 9 | 23 | TRAF6, TLR6, TLR2, TLR7, TLR4, TLR9, TLR8, MYD88, IRAK4 | 1.03891E-21 |
| Dendritic Cell Activation in Systemic Lupus Erythematosis | 73 | 242 | 12 | 4 | TLR7, TICAM1, TLR8, TRAF3, TRAF6, TLR6, IRAK4, TLR9, MYD88, TLR2, TLR3, TLR4 | 7.25697E-21 |
| Dendritic Cell Activation in COVID19 | 76 | 247 | 12 | 4 | TLR7, TICAM1, TLR8, TRAF3, TRAF6, TLR6, IRAK4, TLR9, MYD88, TLR2, TLR3, TLR4 | 9.32148E-21 |
| Plasmacytoid Dendritic Cell in Diabetes Mellitus Type 1 | 53 | 91 | 9 | 9 | TLR7, TICAM1, TRAF6, IRAK4, TLR9, MYD88, TLR2, TLR3, TLR4 | 4.91915E-18 |
| Apoptotic Keratinocyte Clearance Recession in Systemic Lupus Erythematosus | 79 | 379 | 11 | 2 | TICAM1, TRAF6, TLR6, IRAK4, TLR9, TLR10, MYD88, TLR1, TLR2, TLR4, TLR5 | 2.56647E-16 |
| Toll-like Receptors (TLR) in Periodontitis | 39 | 78 | 8 | 10 | TLR6, TICAM1, MYD88, TRAF6, TLR1, TLR2, TLR4, IRAK4 | 3.93659E-16 |
| Dendritic Cells Function in Atherosclerosis | 53 | 80 | 8 | 10 | TLR6, TLR7, TICAM1, TLR9, MYD88, TRAF6, TLR4, IRAK4 | 4.86359E-16 |
| IL36 Receptor Antagonist (IL1F5) Deficiency (DITRA) | 37 | 165 | 9 | 5 | TLR7, TLR8, TRAF6, IRAK4, TLR9, TLR10, MYD88, TLR3, TLR5 | 1.22898E-15 |

**Table 6.** Presence of genes in key organs.
The table shows the presence of genes in key organs like the testis, brain, liver, and other key organs with a p-value of zero. This shows the need for expression of TLR's in these key organs to maintain their normal functioning and has diverse implications for immune responses and disease development

| Protein entities related to | Total # of Neighbors | Overlap | Percent Overlap | Overlapping Entities | p value |
|---|---|---|---|---|---|
| Testis | 10388 | 13 | 0 | TLR3, TLR4, TLR10, TLR5, MYD88, IRAK4, TLR9, TLR1, TLR8, TLR6, TLR2, TLR7, TICAM1 | 0.00000E+00 |
| Cerebral cortex | 8885 | 12 | 0 | TLR3, TLR4, TLR5, MYD88, IRAK4, TLR9, TRAF3, TRAF6, TLR8, TLR6, TLR2, TLR7 | 0.00000E+00 |
| Brain | 11791 | 15 | 0 | TLR3, TLR4, TLR10, TLR5, MYD88, IRAK4, TLR9, TRAF3, TRAF6, TLR1, TLR8, TLR6, TLR2, TLR7, TICAM1 | 0.00000E+00 |
| Neuron | 9349 | 14 | 0 | TLR3, TLR4, TLR5, MYD88, IRAK4, TLR9, TRAF3, TRAF6, TLR1, TLR8, TLR6, TLR2, TLR7, TICAM1 | 0.00000E+00 |
| Lung | 10058 | 15 | 0 | TLR3, TLR4, TLR10, TLR5, MYD88, IRAK4, TLR9, TRAF3, TRAF6, TLR1, TLR8, TLR6, TLR2, TLR7, TICAM1 | 0.00000E+00 |
| Kidney | 10916 | 14 | 0 | TLR3, TLR4, TLR5, MYD88, IRAK4, TLR9, TRAF3, | 0.00000E+00 |

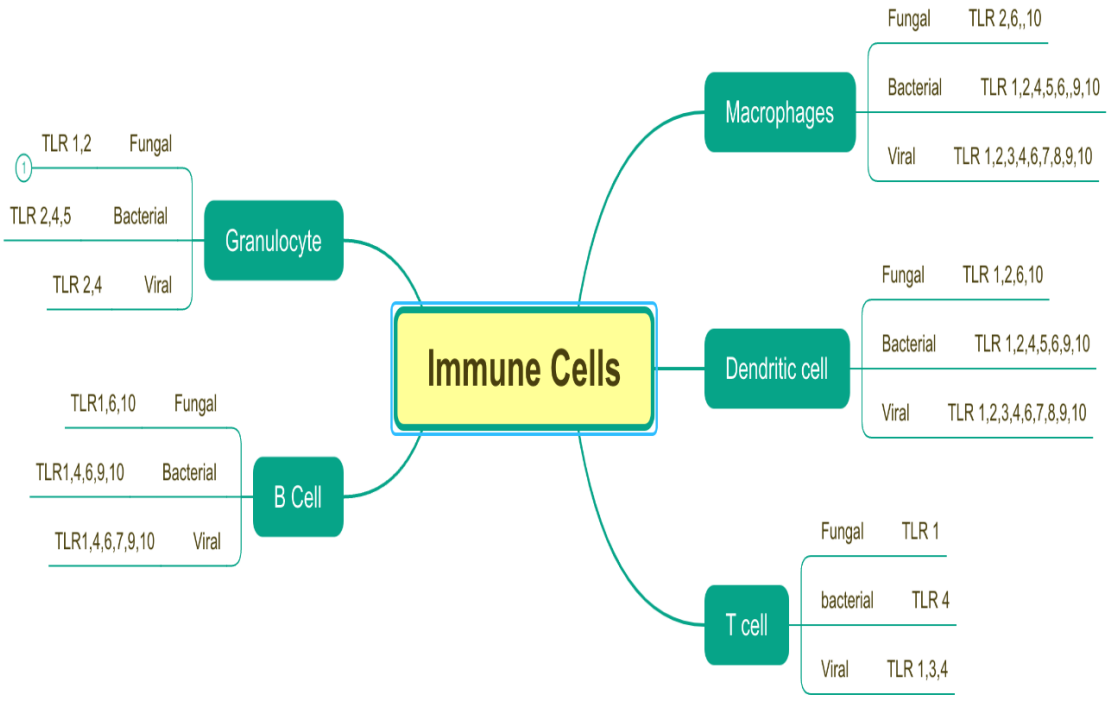
| Protein entities related to | Total # of Neighbors | Overlap | Percent Overlap | Overlapping Entities | p value |
|---|---|---|---|---|---|
| | | | | TRAF6, TLR1, TLR8, TLR6, TLR2, TLR7, TICAM1 | |
| Liver | 10017 | 15 | 0 | TLR3, TLR4, TLR10, TLR5, MYD88, IRAK4, TLR9, TRAF3, TRAF6, TLR1, TLR8, TLR6, TLR2, TLR7, TICAM1 | 0.00000E+00 |
| EPC | 84 | 11 | 12 | TLR3, TLR4, TLR9, TLR10, TLR5, MYD88, TLR1, TLR8, TLR6, TLR2, TLR7 | 5.27370E-20 |
| OKF6 | 46 | 9 | 19 | TLR3, TLR4, TLR9, TLR5, TLR1, TLR8, TLR6, TLR2, TLR7 | 7.73300E-18 |
| IPEC-J2 | 234 | 11 | 4 | TLR3, TLR4, TLR9, TLR10, TLR5, MYD88, TLR1, TLR8, TLR6, TLR2, TLR7 | 6.04630E-15 |
| | | | | | |

## Conclusion

Our work shows that Metadichol activates all immune cell Toll-like receptors (TLRs) (Figure 5), to generate an immunological response. Activation allows immune cells to recognize and respond to pathogen-associated molecular patterns (PAMPs) like flagellin, peptidoglycans, bacterial lipopolysaccharides, lipoproteins, and bacterial and viral nucleic acids.

Understanding the immune response in many species requires activating the Toll-like receptor (TLR) network, which governs many biological processes. Recent genome scans have shown that TLRs are evolutionarily

**Figure 5;** TLR's expressed by various Immune cells.




conserved and ecologically important outside mammals. [70] Metadichol activates, all TLR family members, including MYD88, and selectively suppresses TLR4, which may reduce numerous illnesses. Metadichol is the sole substance that activates all TLRs. It also induces neuronal,[71]Yamanaka,[72] nuclear receptors, [25] cardiovascular, [73], and Sirtuins 1-7 in fibroblasts [74] at a low dose of one picogram per milliliter to 100 nanograms per milliliter. Control of transcription factors could provide a way to mitigate many diseases.[75] Conventional research has generated extremely specific compounds that target a single biomolecule involved in disease development.[76] This method works for diseases with clear pathways. Single-target, single-molecule treatments for thousands of human diseases have failed [77] To treat many disorders, drugs must target several genes [78,79,] or gene network-based approaches to numerous diseases are thus important. [80] As we have shown, metadichol can target telomerase, sirtuins, TLRs, nuclear receptors, and other transcription factors to promote cell reprogramming

and act on multiple diseases. Metadichol could revolutionize our approach to diseases. It has no known toxicity of [81,82,83] and could treat many human disorders without viable treatments.

## Declarations

ACCESS TO DATA AND MATERIALS

The manuscript and supplementary resources contain all raw data. The author states that the paper is also been published as a preprint and available at https://www.biorxiv.org/content/10.1101/2024.04.04.588068v1

CONFLICTING GOALS

Author is founder and main shareholder of Nanorx, Inc., NY, USA.

FUNDING

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## Supplementary files

Name of File. Raw Data:

This file contains Raw Data of Fold changes by Q-Rt-PCR and of the evaluation of effect of Metadichol on gene expression of Toll like receptor using Human peripheral mononuclear blood cells (PBMCs). The western blot results of PBMCS treated with test sample suggests the upregulation of MYD88, TRL3, and TRAF3 of 2.32, 1.87, and 2.77 fold, respectively, in the case of the 1 ng treated group. While, 1 ng treated group showed down-regulation of 0.13, 0.53 and 0.25 fold of IRAK4, TLR4 and TRAF6 respectively compared to control cells.

Name of File .Western Blot .

Contains Data and pictures of western Blot studies of TLR gene proteins expressed. The western blot results of PBMCS treated with test sample suggest the upregulation of MYD88, TRL3, and TRAF3 of 2.32, 1.87, and 2.77 fold, respectively, in the 1 ng treated group. Meanwhile, the 1 ng treated group showed downregulation of 0.13, 0.53, and 0.25 fold of IRAK4, TLR4, and TRAF6, respectively, compared to control cells.

File name: PBMC

Letter of No Objection Certificate for use of PBMC blood samples used in study

File name; Kegg

Permission letter for use of copyrighted figure 4 from Kegg Consortium

Declaration of writing-related generative AI and AI-assisted technologies.

No AI tolls were used to prepare this manuscript.

All supplementary files are found here.

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