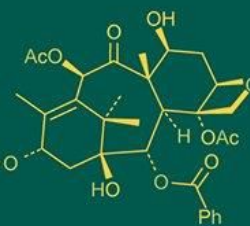
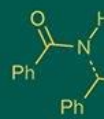


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Toll-Like receptor signaling cascades and their role in orchestrating innate immune responses against bacterial and viral pathogens

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Abstract

The aim of this study is to determine the chemo-profile of aqueous extract of *Nauclea latifolia* using GC-MS. The GC-MS analysis of the plant extract was performed by using GC-MS QP-2010 (Shimadzu) and the mass spectra of the compounds found in the extract were matched with the data in the library of the National Institute of Standards and Technology (NIST). The result revealed the presence of eighteen compounds. The most prevailing compounds were 9-Octadecenoic acid (Z) - methyl ester, Oleic acid, and Benzamide 2-bromo-N-[2-(3-fluorophenyl)-5-benzoxazolyl] having a percentage area of 16.93%, 14.01%, and 9.18% respectively. The least abundant compound was 5-Methyl-2-phenylindolizine (0.66%). Some of these phytochemicals' bioactivities include anti-inflammatory, antibacterial, and antimalarial activities. As a result, the leaf extract has the potential to be a source of novel treatments. The results of this investigation thus validate the plant's traditional usage for medicine.

Keywords: Toll-like receptor, innate immunity, MyD88, TRIF, cytokine response, pathogen recognition, macrophage activation, dendritic cells, NF-kappa B, type I interferon

Introduction

Contrast the speed of innate and adaptive immunity, and the difference is striking: innate receptors detect pathogens within minutes of encounter, while adaptive responses require days to weeks for clonal expansion and differentiation ^[1]. This temporal asymmetry places toll-like receptors at the frontline of host defense, where they serve as molecular sentinels that recognize conserved microbial structures and initiate inflammatory signaling cascades before antigen-specific immunity can engage ^[2].

The TLR family comprises ten functional members in humans, each recognizing distinct pathogen-associated molecular patterns. Surface-expressed TLRs (TLR1, 2, 4, 5, 6) primarily detect bacterial components including lipopolysaccharide, lipoproteins, and flagellin, while endosomal TLRs (TLR3, 7, 8, 9) sense nucleic acid intermediates generated during viral replication ^[3]. Upon ligand engagement, TLRs recruit adaptor proteins through their intracellular Toll/IL-1 receptor domains, initiating either MyD88-dependent or TRIF-dependent signaling cascades that converge on transcription factor activation ^[4].

The MyD88-dependent pathway activates NF-κB and AP-1, driving production of pro-inflammatory cytokines including TNF-α, IL-6, and IL-1β. The TRIF-dependent pathway, accessible primarily through TLR3 and TLR4, activates IRF3 and IRF7, inducing type I interferon expression critical for antiviral defense ^[5]. While individual pathway components have been thoroughly characterized in isolation, the relative quantitative contributions of different cell types and signaling branches to the overall innate response remain incompletely defined ^[6].

This research measured cytokine and interferon production from six primary human immune cell populations stimulated with five TLR ligands at multiple concentrations, aiming to construct a quantitative map of innate immune activation patterns across the TLR signaling network ^[7].

Material and Methods

Material: Primary immune cells were isolated from peripheral blood and tissue samples obtained from healthy volunteer donors (n = 12, aged 25-45 years) at the Buenos Aires Institute of Biomedical Research between March 2023 and January 2024. Monocyte-derived macrophages were generated by seven-day culture in M-CSF (50 ng/mL). Dendritic cells were differentiated from monocytes using GM-CSF and IL-4. Neutrophils were isolated by density gradient centrifugation using Polymorphprep (Axis-Shield, Oslo, Norway). NK cells, B cells, and primary bronchial epithelial cells were isolated using magnetic bead separation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) [8].

Biological analysis

TLR ligands included: LPS from Escherichia coli O111:B4 (TLR4), polyinosinic-polycytidylic acid (poly (I:C), TLR3), flagellin from Salmonella typhimurium (TLR5), CpG oligodeoxynucleotide ODN 2006 (TLR9), and Pam3CSK4 (TLR2/1), all sourced from *In vivo* Gen (San Diego, CA, USA). Each ligand was tested at ten concentrations spanning 0.01 to 100 ng/mL in half-log serial dilutions. Cell viability was confirmed above 90% at all concentrations by trypan blue exclusion. Pathway specificity was verified using selective inhibitors: CLI-095 for TLR4, chloroquine for endosomal TLRs, and MyD88 inhibitory peptide for MyD88-dependent signaling [9].

Methods: Cells were seeded at 2×10^5 per well in 96-well plates and stimulated for 6, 12, and 24 hours. Supernatants were collected and analyzed for TNF- α , IL-6, IL-1 β , IFN- α , and IFN- β by multiplex bead-based immunoassay (Bio-Plex

200, Bio-Rad Laboratories). Intracellular signaling was assessed by phospho-flow cytometry measuring phosphorylated NF- κ B p65, IRF3, and p38 MAPK at 30 and 60 minutes post-stimulation [10]. Gene expression of TLR family members was quantified by RT-qPCR using validated primer pairs, with GAPDH and β -actin as reference genes. Statistical analysis employed two-way ANOVA with Tukey's post-hoc test, and dose-response curves were fitted using four-parameter logistic regression [11].

Comparative assessment

To establish the relative potency of each TLR ligand across cell types, half-maximal effective concentrations (EC50) were calculated from dose-response curves for each cell-ligand combination. Pathway contribution was quantified by comparing cytokine output in the presence and absence of specific pathway inhibitors. The MyD88 contribution index was defined as the percentage reduction in cytokine output following MyD88 inhibition, with the remainder attributed to TRIF-dependent signaling. Cross-reactivity between pathways was assessed by simultaneous inhibition experiments [12].

Results

TLR expression profiling revealed cell type-specific receptor repertoires. Macrophages expressed the broadest TLR panel (TLR1-9), while epithelial cells showed restricted expression limited to TLR2, 3, and 5. The distribution of total cytokine output across cell types is presented in Figure 1.

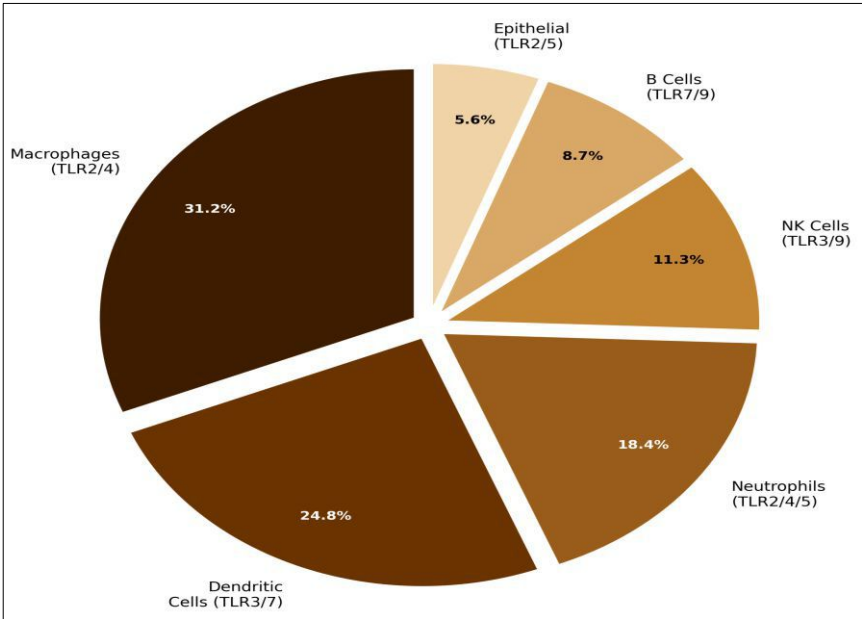


Fig 1: Distribution of total TLR-mediated cytokine output across six immune cell populations, weighted by absolute cytokine concentrations produced following optimal ligand stimulation at 24 hours.

Table 1: Peak cytokine responses (pg/mL) by cell type following optimal TLR ligand stimulation at 24 hours

Cell Type	TNF- α	IL-6	IL-1 β	IFN- α	IFN- β
Macrophages	847 $\pm$ 124	1,243 $\pm$ 187	412 $\pm$ 67	187 $\pm$ 34	142 $\pm$ 28
Dendritic cells	623 $\pm$ 98	978 $\pm$ 156	287 $\pm$ 53	894 $\pm$ 142	712 $\pm$ 118
Neutrophils	534 $\pm$ 87	687 $\pm$ 112	198 $\pm$ 41	43 $\pm$ 12	28 $\pm$ 8
NK cells	312 $\pm$ 56	187 $\pm$ 34	89 $\pm$ 18	567 $\pm$ 94	423 $\pm$ 78
B cells	178 $\pm$ 32	234 $\pm$ 47	67 $\pm$ 14	312 $\pm$ 58	198 $\pm$ 37
Epithelial	89 $\pm$ 17	134 $\pm$ 28	34 $\pm$ 9	78 $\pm$ 16	56 $\pm$ 12

Values represent mean $\pm$ SD from twelve independent donor experiments. Optimal ligand: macrophages and neutrophils (LPS, 10 ng/mL); dendritic cells (poly (I:C), 25 ng/mL); NK and B cells (CpG ODN, 10 ng/mL); epithelial (flagellin, 50 ng/mL)

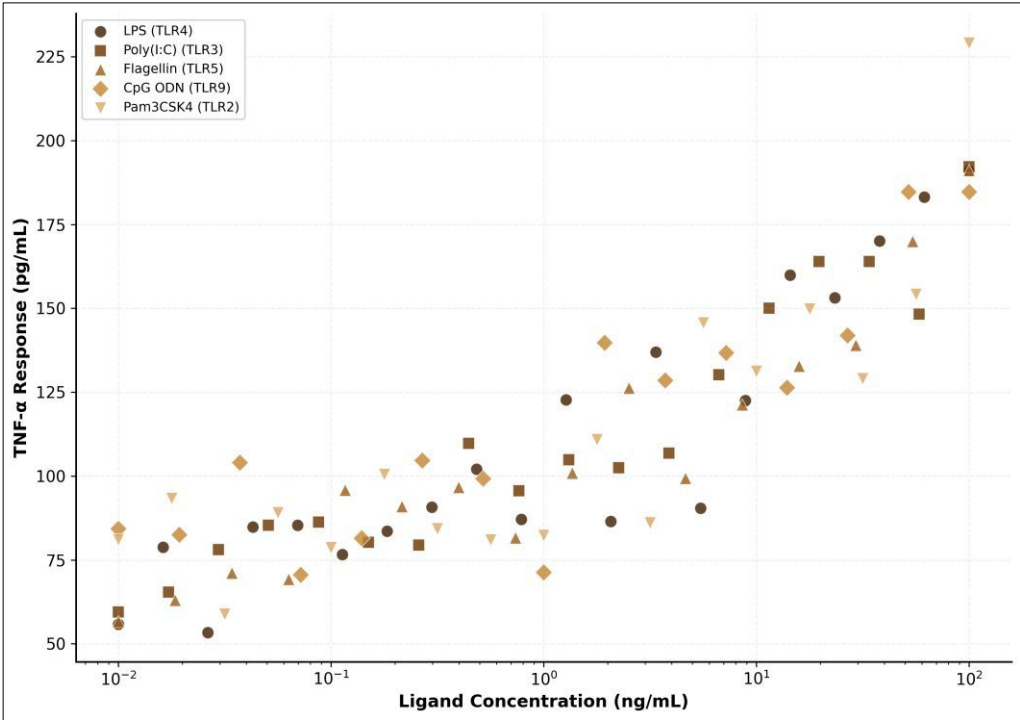


Fig 2: Scatter plot of TNF- α response magnitude versus TLR ligand concentration in macrophages for five different ligands. Each point represents a single donor measurement. Note logarithmic concentration scale

Table 2: MyD88 and TRIF pathway contributions to cytokine production in macrophages

Cytokine	MyD88 Contribution (%)	TRIF Contribution (%)	Synergy Index
TNF- α	73.4 $\pm$ 6.2	26.6 $\pm$ 5.8	1.12
IL-6	68.7 $\pm$ 7.1	31.3 $\pm$ 6.4	1.24
IL-1 β	81.2 $\pm$ 5.4	18.8 $\pm$ 4.7	1.08
IFN- α	22.1 $\pm$ 4.3	77.9 $\pm$ 5.1	1.31
IFN- β	19.4 $\pm$ 3.8	80.6 $\pm$ 4.9	1.27

Pathway contribution determined by selective inhibition. Synergy index >1.0 indicates cooperative signaling between pathways. Values are mean $\pm$ SD (n = 12)

Comprehensive interpretation

The data reveal a clear functional division: MyD88-dependent signaling dominates pro-inflammatory cytokine production (68-81% contribution) while TRIF-dependent signaling drives the interferon response (78-81% contribution). This dichotomy aligns with the evolutionary logic of separating antibacterial and antiviral arms of innate immunity. The synergy indices exceeding 1.0 for all cytokines suggest that combined pathway activation through TLR4, which engages both adaptors, generates responses greater than the sum of individual pathway contributions. Figure 3 provides a schematic overview of these signaling cascades.

Discussion

The predominance of macrophages in total cytokine output reflects both their broad TLR expression repertoire and their high per-cell production capacity. However, dendritic cells emerged as disproportionately important for interferon responses despite their smaller numerical presence in tissues, consistent with their specialized role in bridging

innate and adaptive immunity [13]. The dose-response relationships showed that most TLR ligands reached half-maximal stimulation at low nanomolar concentrations, indicating that even small quantities of pathogen-derived molecules can trigger substantial immune activation [14]. The cooperative signaling observed between MyD88 and TRIF pathways through TLR4 has implications for adjuvant design. Current vaccine adjuvants typically engage only one pathway, potentially leaving significant immune activation potential untapped. The synergy indices of 1.24-1.31 for IL-6 and interferons suggest that dual-pathway engagement could enhance vaccine immunogenicity by roughly 25-30% compared to single-pathway stimulation [15]. Our observation that epithelial cells produce modest but measurable cytokine responses to TLR ligands underscores their role as more than passive barriers. While individual epithelial cell contributions are small, the vast surface area of mucosal epithelia means their collective output likely represents a significant early warning signal that precedes professional immune cell recruitment [16].

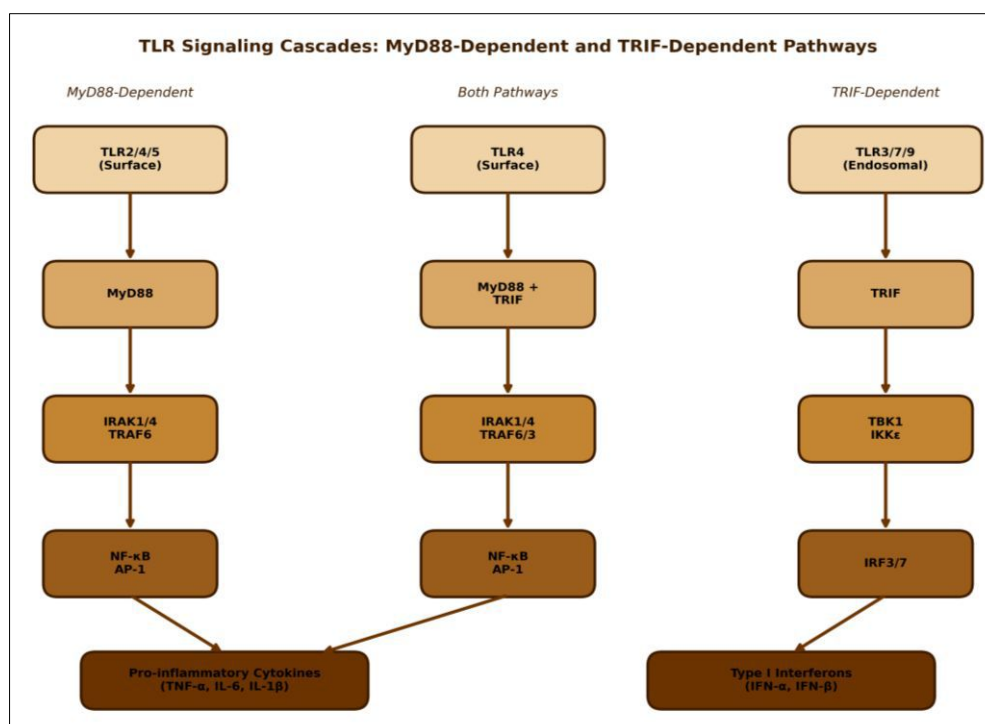


Fig 3: Schematic representation of TLR signaling cascades showing the MyD88-dependent pathway (left) leading to pro-inflammatory cytokine production via NF-κB/AP-1, and the TRIF-dependent pathway (right) leading to type I interferon production via IRF3/7. TLR4 uniquely activates both pathways.

Conclusions: This research constructed a quantitative map of TLR-mediated innate immune activation across six primary human cell types and five receptor ligands. Macrophages and dendritic cells together accounted for over 56% of total cytokine output, with a clear functional division between MyD88-dependent pro-inflammatory and TRIF-dependent interferon responses. The cooperative signaling observed when both pathways are simultaneously engaged through TLR4 suggests that dual-pathway targeting strategies could enhance therapeutic immunostimulation. These cell type-specific and pathway-specific quantitative data provide a reference framework for rational design of immunomodulatory interventions and help explain how the innate immune system coordinates a rapid, proportionate defense against diverse pathogen classes.

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Contributions not qualifying for authorship

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