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REVIEW Article

Theranostic significance of Toll Like Receptors through an in silico approach: Research and pre-clinical Perspectives

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ABSTRACT

Toll Like Receptors are transmembrane receptors that belong to the pattern recognition receptor family of proteins. They are specifically involved in the activation of innate and/or adaptive immunity against pathogens and biological molecules as well as eliciting inflammatory reactions by recognizing various pathogen associated and damage associated molecular patterns. This paper aims to coalesce the available information about TLR1 through 10 in case of humans, compiling information regarding their structures, locations, functions and signaling pathways. The focus on understanding the variations in TLRs across various species is also considered for clinical importance and in silico screening of various agonists. The selection of a few clinically significant species for this purpose ranged from Zebrafish to non-human primates due to their importance in various pharmacological and theranostic approaches during the screening and drug development processes. The TLR genes of these species were compared against the corresponding human homologs. In the review we tried to compare and analyze the ligand binding region of TLRs across the species to understand the potential variations in response. Our studies show that across all TLRs, a similar trend in terms of homology is observed between the species, indicating the availability of conserved sequences in the ligand binding domain throughout evolution.

Keywords
Toll Like Receptors, Toll Like Receptor Agonists, Pathogen Associated Molecular Patterns, Homology, Theranostic Application.

Abbreviations
Pattern Recognition Receptor (PRRs); Toll Like Receptors (TLRs); Damage Associated Molecular Patterns (DAMPs); Pathogen Associated Molecular Patterns (PAMPs); Toll-Interleukin 1 Receptor (TIR); TIR Domain Containing Adapter Protein (TIRAP); Myeloid Differentiating Factor 88 (MyD88); TRIF Related Adapter Molecule (TRAM); TIR Domain Containing Adapter Inducing Interferon B (TRIF); IL-1-Receptor Associated Kinase (IRAK); Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6); Nuclear Factor κB (NFκB); Interferon (IFN); Leucine Rich Repeats (LRRs); Macrophage Activating Lipopeptide (MALP); Single Strand RNA (ssRNA); Bacterial Lipopolysaccharide (LPS[1.1]); Inhibitor of Nuclear Factor-κB (IκB) Kinase (IKK); NF-kappa-B Essential Modulator (NEMO); Dendritic Cells (DCs); Macrophage Activating Lipopeptide (MALP).

1. Introduction

Pattern Recognition Receptors (PRRs) are a family of membrane bound proteins that are involved in eliciting immune responses through the recognition of specific biological pattern(s). Their signaling also triggers various cellular processes such as, boosting cellular immunity, autophagy, apoptosis, cytokine processing, phagocytosis, etc. Toll Like Receptors (TLRs) are specifically involved in the activation of innate and/or adaptive immunity against pathogens and cancer, as well as eliciting inflammatory reactions in cells. Therefore, these receptors, are key players in the cellular defense against pathogens, as they have the ability to recognize specific molecular patterns called pathogen associated and damage associated molecular patterns (PAMPs and DAMPs)[1].

TLRs, much like the other members of the PRR family, are germline encoded proteins and are able to recognize a plethora of self- and non-self-molecules. Named because of their similarity to Toll Receptor genes that were originally discovered in Drosophila in the early 1980s, TLRs are amongst the most studied PRR subfamily[2]. These proteins were first identified in humans by Nomura et al. in 1994[3]and they are a subfamily of type-1 transmembrane receptors and are evolutionarily conserved between vertebrates and invertebrates. TLR4, due to its high clinical significance, is one of the most well-studied members of this receptor family[4].

2. TLR Family of Proteins

TLRs are involved in the immune system of organisms and can be found either on the cell membrane or on the membranes of other cell organelles. Cell membrane TLRs include TLR1, 2, 4, 5, 6, 7, 8 and 10 while organelle bound TLRs includes TLR3 and 9. Some TLRs are associated with both, cell membrane and organelles (TLR4, 7, 8 and 10)[4] (**Figure 1**).

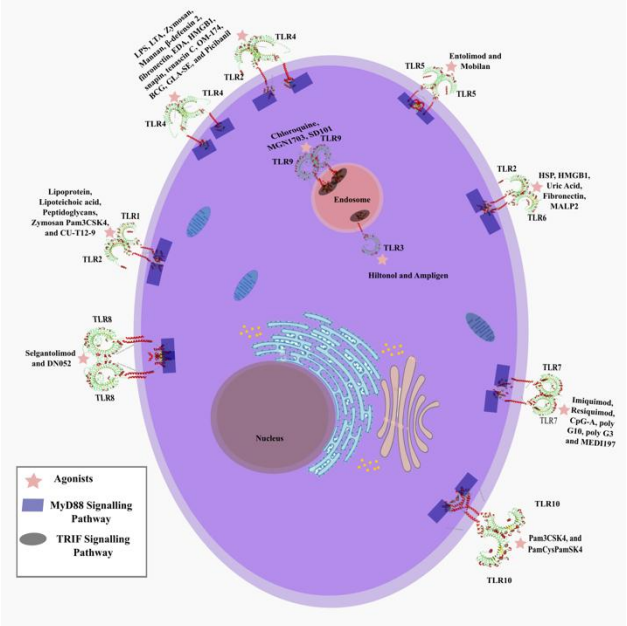


Figure 1. A diagram showing the various TLRs as they are found on human cells

Table 1 describes all the known human TLRs, their general subcellular locations and origins, the co-receptors for each TLR, and their active forms. Aside from this, it also describes the chromosomal location of the TLR gene, the loci on which these genes are present, and their Protein IDs, as shown in the UniProt database.

3. Classification of TLR and Structure

TLRs belong to the category of Type 1 Integral Transmembrane proteins and are known to possess an N-terminal domain, a Single Helix Transmembrane domain, and a C-terminal domain [2].

The N-Terminal domain of most TLRs is located outside the membrane in the extracellular space and is responsible for interacting with various ligands present outside the membrane. However, in the case of TLR3, the N-terminal domain is located inside the cytoplasm[5] whereas in TLR9, this domain is found inside organelles such as the endoplasmic reticulum and endosomes[6]. The C-Terminal Domain for most TLRs is located inside the cytoplasm. For TLR3, this domain lies inside the organelle to which it is bound[5,6]. The interaction of agonists with

Table 1. An Overview of the TLR Family of Proteins

Name	Location in the Cell and Origin	Co-receptor	Active Form	Chromosome Number and Gene location (For Humans)	Protein ID (For Humans)	Reference
TLR1	Cell Membrane; with Non-Viral Origin	Found in conjunction with TLR2	TLR1-TLR2	4p14; NC_000004.12 (38787569..38805644, complement)	Q15399	63,64
TLR2	Cell Membrane; with Non-Viral Origin	Found bound toTLR1, TLR2, TLR6, TLR10, CD14, CD36, Integrin, RP105, MBI, LBP	TLR2-TLR1; TLR2-TLR6	4q31.3; NC_000004.12 (153684280..153710637)	O60603	34,38,63,65–68
TLR3	ER, Lysosomal Membrane; with Viral Origin	Found bound to CD14 or Mex3B	TLR3-TLR3	4q35.1; NC_000004.12 (186069156..186088073)	O15455	65,69
TLR4	Cell Membrane, ER, Lysosomal Membrane; with Non-Viral Origin	Found Bound to MD2, LY96, CD14, LBP, CD36 and RP105	TLR4/MD2-TLR4/MD2, TLR4-TLR6, TLR4-TLR2	9q33.1; NC_000009.12 (117704403..117724735)	O00206	68,70
TLR5	Cell Membrane; with Non-Viral Origin	-	TLR5-TLR5	1q41; NC_000001.11 (223109404..223143248, complement)	Q15399	50,71–73
TLR6	Cell Membrane; with Non-Viral Origin	TLR2, CD36, LBP	TLR2-TLR6, TLR4-TLR6	4p14; NC_000004.12 (38822897..38868390, complement)	Q9Y2C9	30
TLR7	Cell Membrane, ER, Lysosomal Membrane; with Viral Origin	CD14	TLR7-TLR7	Xp22.2; NC_000023.11 (12867072..12890361)	Q9NYK1	74,75
TLR8	Cell Membrane, ER, Lysosomal Membrane; with Viral Origin	-	TLR8-TLR8	Xp22.2; NC_000023.11 (12906620..12923169)	Q9NR97	74,75
TLR9	ER, Lysosomal Membrane; With Viral Origin	CD14	TLR9-TLR9	3p21.2; NC_000003.12 (52221080..52225645, complement)	Q9NR96	74,75
TLR10	Cell Membrane, ER, Lysosomal Membrane; With Non-Viral Origin	-	TLR1-TLR10, TLR2-TLR10, TLR10-TLR10	4p14; NC_000004.12 (38772238..38782990, complement)	Q9BXR5	76,77

corresponding TLRs activates associated signaling pathways. This signaling within the cell is made possible due to the Toll-Interleukin 1 Receptor (TIR) homologous domain. This domain shows the degree of evolutionary conservation across prokaryotic and eukaryotic species[7,8]. The N-Terminal domain also has a glycan moiety that acts as the binding site for ligands[9–11]. The N-Terminal domain contains multiple short tandem Leucine Rich Repeats (LRR) that vary in number from 19 to 25 and are responsible for the ligand specificity. The LRR may contain 24 to 29 amino acids arranged in a xLxxLxLxx pattern.[3]

4. TLR Signaling

TLR signaling overall has been extensively explored, and two major signaling pathways have been identified for TLR i.e. MyD88 Dependent and MyD88 Independent (**Figure 2**). In most cases, the dimerization of TLR occurs following interaction with ligand(s) through the TIR portion. This leads to conformational changes in the receptors and initiation of intracellular signaling through the recruitment of TIR domain containing adapter molecules[12,13].

There are four known TIR domains containing adapters that belong to one of the two aforementioned

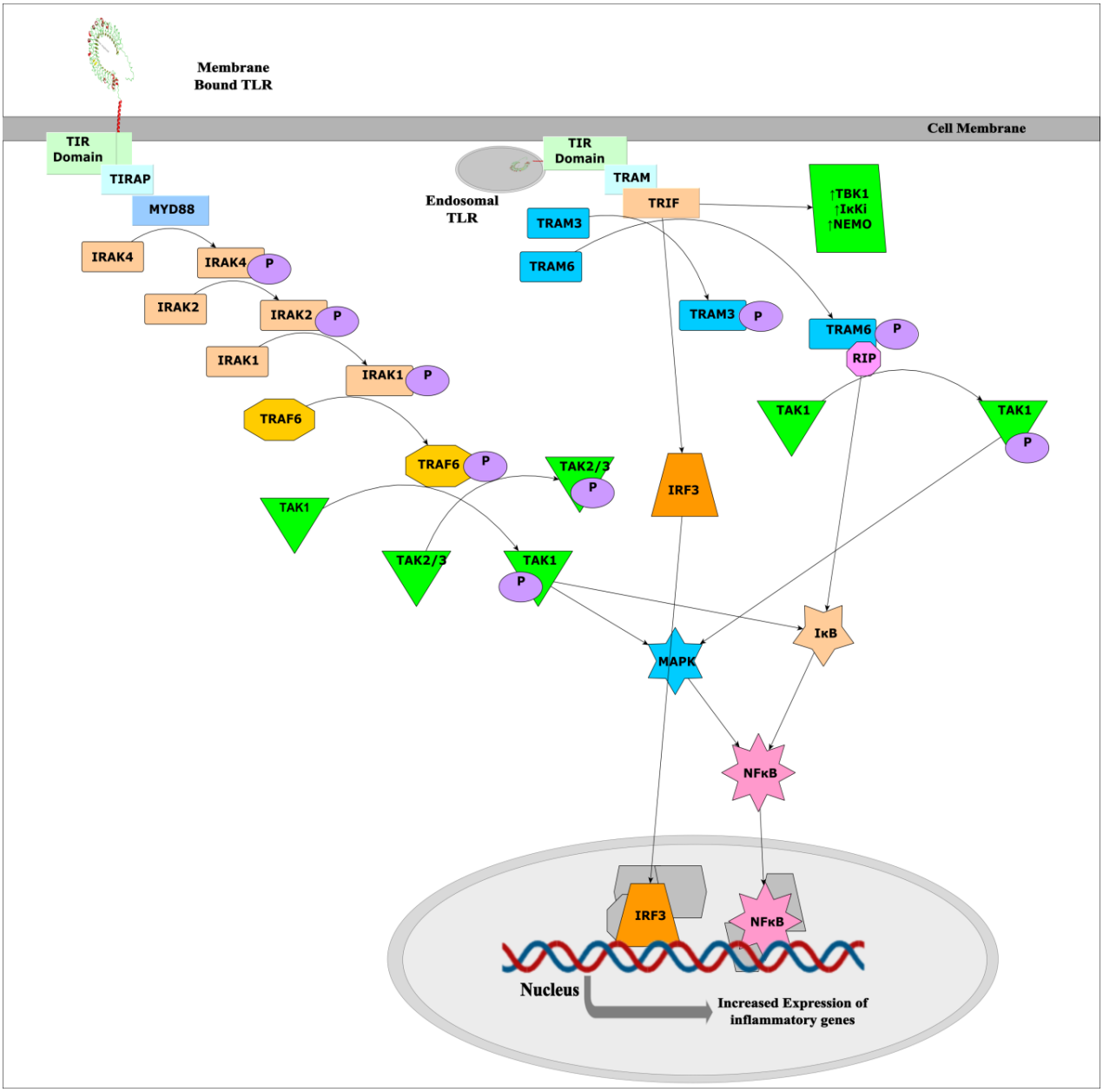


Figure 2. TLR Signaling
TLRs perform signaling through either the Myd88 dependent or the Myd88 independent pathway through the TIR domain on the C-terminus of the TLR proteins

pathways. These four proteins are: Myeloid differentiating factor 88 (MyD88) adapter like protein (also known as TIR domain containing adapter protein or TIRAP), TIR domain containing adapter inducing interferon B (TRIF; also known as TIR domain containing adapter molecule, TICAM-1) and TRIF related adapter molecule (TRAM; also known as TIR containing protein, TIRP). TLRs interact with MyD88 or TRIF molecules through TIRAP and TRAM, respectively, leading to either the MyD88 signaling cascade through the formation of a Myddosome complex or the TRIF signaling cascade through the Triffosome complex[12,13].

4.1. MyD88 Pathway of TLR Signaling

The signaling cascade related to myddosome begins with the binding of TIRAP and MyD88 to the TIR domain of the TLR. The myddosome includes proteins such as IL-1-Receptor Associated Kinase

(IRAK) 1, 2, 4 and M. IRAK4 is the first protein to bind to MyD88 and is phosphorylated at Threonine-345 (Thr345). Together this complex of MyD88 and IRAK4 functions to recruit IRAK1 and IRAK2 leading to the phosphorylation of IRAKs. IRAK4-MyD88 complex first phosphorylates IRAK1 at the Threonine-209 (Thr209) and the Thr987 position, leading to autophosphorylation of IRAK1 and activation of IRAK2 *via* phosphorylation. Phosphoryalted-IRAK2 then can also undergo autophosphorylation, activating other IRAK2 molecules in the cell[13,14].

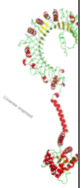
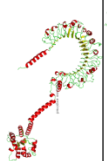
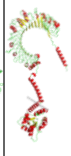
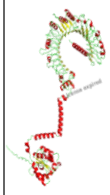
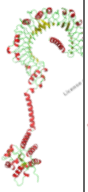
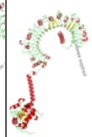
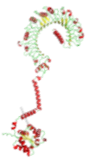
Phosphorylation of IRAK1 activates Tumor Necrosis Factor Receptor Associated Factor 6 (TRAF6) by phosphorylation leading to the activation of TAK1 through phosphorylation at Thr187 and Serine-192 (Ser192) and TAK2/3. This cascade leads to the activation of IκB by phosphorylation at Ser32 and Ser36 and Mitogen Activated Protein Kinase (MAPK) resulting in the translocation of Nuclear Factor κB (NFκB) and AP-1 to the nucleus. In case of TLRs expressed on endosomes, the MyD88 cascade leads to the translocation of Interferon Regulatory Factor-7 (IRF7) which eventually leads to the activation of interferons[4,13,14].

4.2. MyD88 independent pathway or TRIF dependent signaling cascade

This pathway is observed in macrophages in conventional dendritic cells and is more commonly observed in endosomal TLR4 where it functions to induce interferon (IFN) expression by increasing IRF3 in the cells. This pathway is known to involve the formation of a Triffosome which comprises of TRAM that is bound to the TIR domain of the TLR.

Table 2. TLR1

A comparison of TLR1 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 1											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	14	4	5 C3.1; 5 33.53 cM	14p11	3	6	8	5	3	3	4p14
Gene Location	NC_007125.7 (736575-732606)	NC_052535.1 (69035725..69041254)	NC_000071.7 (65082023..65090945, complement)	NC_086032.1 (43737761..43750389)	NC_051807.1 (74285167..74325228)	NC_091765.1 (58751900..58754779, complement)	NC_010450.4 (30147600..30155454, complement)	NC_041758.1 (37935710..37945703, complement)	NC_072401.2 (39122226..39171850, complement)	NC_073227.2 (46829489..46839015, complement	NC_000004.12 (38787569..38805644, complement)
Number of Exons	2	4	5	4	8	1	4	4	8	5	7
Overall similarity at the protein level	35.41	50.64	74.01	72.87	76.79 – 84.69	78.48	78.88	95.29	98.47	97.84	100
Similarity between First Ligand Binding (LRR) Region	37.9	50.41	75.83	75.47	88.68	87.06	82.79	Graphics were not available for the chosen gene for *M. fascicularis*	99.19	97.56	100
Similarity between Secon Ligand Binding (LRR) Region	31.75	46.07	67.23	68.54	74.84	73.1	75.43	Graphics were not available for the chosen gene for *M. fascicularis*	99.04	97.25	100
Protein Structure											

TRAM is bound to TRIF which in turn activates and binds to TRAF3 and 6. It has been found that activated TRAF6; together with RIP1 can leads to the formation of a complex that involves TAK1 and IκB, causing an increase in inflammatory cytokines in the body using the NFκB and MAPK pathways. TRIF is also known to increase activation of TBK1, IκBi and NEMO. It also causes IRF3 activation, which homodimerizes and translocates to the nucleus, where it increases Type 1 IFN genes and IFN stimulated genes. However, it has also been shown that TRAM is unable to interact with the TIR domain of TLR3 suggesting that a different mechanism of interaction occurs between TLR3 and TRIF, however, this mechanism is still not well understood.[12–14].

5. Sequence Alignment and Phylogenetic Analysis

Information from NCBI was used to gather the protein sequences of TLRs. The following organisms: Humans *(Homo sapiens)*, Zebrafish (*Danio rerio)*, Hen*(Gallus gallus)*, Mouse (*Mus musculus)*, Rat (*Rattus norvegicus)*, Dog (*Canis lupus* and *Canis lupus familiaris)*, Cattle (*Bos taurus* and *Bos indicus)*, Pig (*Sus scrofa)*, Monkey (*Macaca mulatta* and *Macaca facicularis)*, Chimp (*Pan troglodyte)* and Gorilla (*Gorilla gorilla gorilla)* were considered for the studies. Comparisons were made between TLR sequences found in each of the chosen species with respect to the humans.

These organisms were chosen due to their clinical significance as well as the presence of completely sequenced genomes for all the species. Zebrafishes have been a cornerstone in drug development due to the ease with which their development can be observed[15]. Since their embryos are transparent and they show ex-utero development, they can be observed for changes in response to drugs right from the fertilization stage, going all the way to their adult form. Hens have been employed in epigenetic studies to improve our understanding of human disease, and in fields such as developmental biology, oncology, and virology amongst others[16]. Rats and mice have long been used as model organisms due to their short life cycles, relatively low breeding costs, and widespread availability as well as their ability to survive in most environments[17,18]. Dogs have also been a very common model organism to study genetic diseases, congenital malformations, disorders of sexual development, and various forms of cancer, helping in not just improving our understanding of these disorders but also the development of drugs against them[19]. Pigs have been used as an alternative model to study anatomy and physiology due to their similarities to humans, allowing for the successful study of metabolic, cardiovascular, infectious diseases, xenotransplantation, and neurological disorders[20]. Bovines display physiological parallels to humans with their ovarian functions, folliculogenesis, and pre-implantation embryo developments; they also share more gene expression profiles with humans than mice. Additionally, their larger sizes also allow for higher amounts of samples to be collected and possess a longer digestive tract with diverse microbiomes that allow for the study of gut microbiomes, fetal programming, and maternal microbiome studies as well[21]. Finally, the last three species chosen for this study, monkeys, chimpanzees and apes, are classified broadly as non-human primates and are closer to humans on the evolutionary tree than all the other species. They possess a genome size comparable to humans and possess other similarities such as a similar number of chromosomes, roughly the same gestation period, and the same number of embryos produced per cycle. Non-human primates have been used for various studies, including not just behavioral studies, but are also used as models for infectious diseases, viral infections, vaccine development, and drug safety studies. Additionally, they are also used as models to understand higher brain dysfunction, immunology, neurophysiology, and stem cell development[22].

Gene information and details about chromosomal location of the respective TLR genes were retrieved from NCBI's Gene database while protein sequences were obtained from NCBI's Protein Database. The protein sequences were then aligned and compared using EMBL's online Clustal W webserver[23] as well as MEGA X[24]. For the settings, we used Clustal W with character counts, while all the advanced settings for the alignment were kept the same. Default parameters were used while performing multiple sequence alignment in MEGA X as well. Details regarding percentage identity were retrieved from the results obtained in Clustal W alignment while phylogenetic trees were generated using MEGA X alignments after cross checking the similarity of the data obtained through both methods. Maximum likelihood algorithm with the default parameters was used to generate the phylogenetic trees in MEGA X.

The results for the overall similarity, the leucine rich repeats (LRRs) of all the proteins were selected. This was done using the graphics for protein which were also available on NCBI's Protein Database. Protein sequences that lacked the graphics were excluded from this part of the study. Based on observations made in all chosen TLR sequences among the species of interest, the LRRs were divided into two subsections, LRR1 and LRR2. This was performed because of the lack of LRRs in specific regions of the overall ligand binding domains. These regions were then aligned and compared among the various species with respect to humans. To obtain the 3D structures for the TLRs, UniProt database[25] and AlphaFold[26] were accessed. AlphaFold predicted structures were selected to ensure uniformity across the protein structures. This overcomes the limitations related to the lack of experimentally proven protein structures. For some TLRs, protein structures had no AlphaFold predicted structures, and SWISS Modeller[27] was used to model and predict the 3D structure using templates (**Table 2-11**). Finally, PyMol[28] was used to generate images of these proteins from the retrieved, predicted, or modelled structures.

Toll-like Receptor 1

TLR1 is known to participate in innate immunity following recognition of various agents *viz.* lipoprotein, lipoteichoic acid, peptidoglycans, zymosan and certain molecules such as Pam3CSK4, CU-T12-9 etc.[29] (Table 12). It is known to form heterodimer with TLR2 to produce an immune response[30–33] by forming an activation cluster with CD14 upon binding to an agonist. This leads to signaling from the cell surface to the golgi apparatus through either lipid raft dependent or independent pathways[34]. TLR1 and 2 are available in close proximity on cellular membrane and are expressed macrophages, dendritic cells (DCs), lymphoid, myeloid, epithelial tissues, etc. TLR1 is also expressed on CD11 positive DCs whereas plasmacytoid DCs show low levels of TLR1. The prominence of TLR1 on plasmacytoid DCs has also been reported to be much lower than that on B Cells[35]. Alignment results of TLR1 among various species with respect to humans showed an expected increase in similarity from *D. rerio* to *G. gorilla* to humans. However, the LRR pattern that interacted with the ligands showed a distribution similar to that of the other proteins. Moreover, the overall similarity of LRR is over 90% conserved in case of the primates with respect to humans. However, it varied from 35-75% among the other species studied. In these proteins, the first LRR showed more similarity to humans across all species than the second LRR, suggesting conservation during the evolutionary process. (**Table 2**, **Figure 3A**)

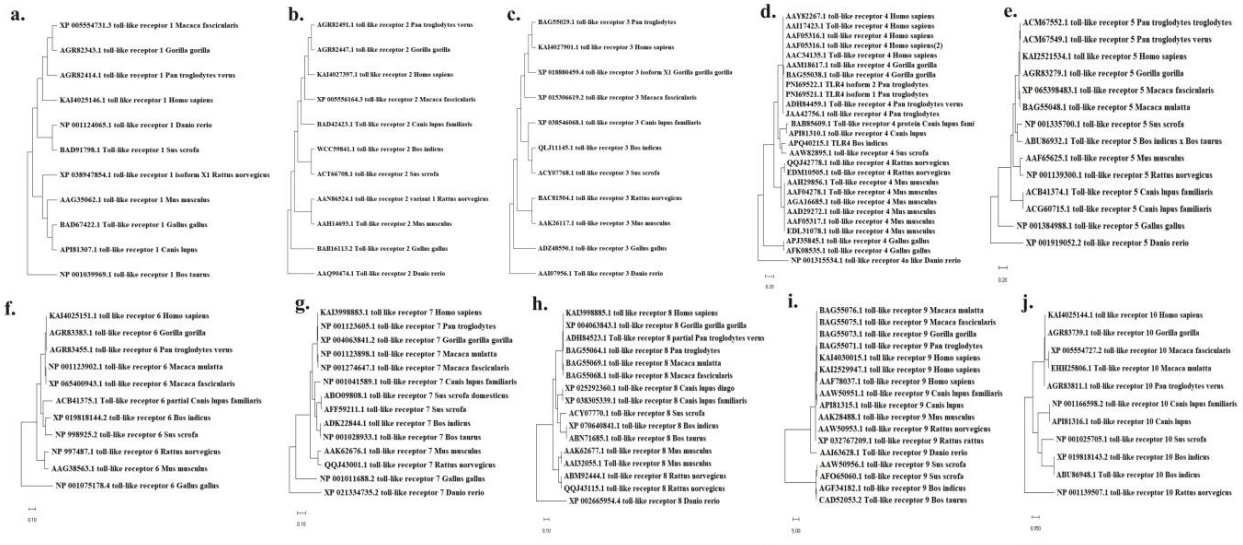
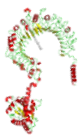
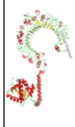
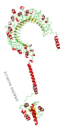
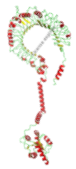
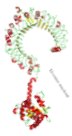


Figure 3. A compilation of the Phylogenetic Trees based on the Sequence Alignment of each TLR. a.) Phylogenetic Tree of TLR1. b.) Phylogenetic Tree of TLR2. c.) Phylogenetic Tree of TLR3. d.) Phylogenetic Tree of TLR4. e.) Phylogenetic Tree of TLR5. f.) Phylogenetic Tree of TLR6. g.) Phylogenetic Tree of TLR7. h.) Phylogenetic Tree of TLR8. i.) Phylogenetic Tree of TLR9. j.) Phylogenetic Tree of TLR10.

Table 3. TLR2

A comparison of TLR2 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 2											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	1	4	3 E3; 37.4 cM	2q34	15	17	8	5	3	3	4q31.3
Gene Location	NC_007112.7 (8983965..8990541)	NC_052535.1 (19496158..19502594)	NC_000069.7 (83743579..83749045, complement)	NC_086020.1 (171499189..171504831, complement)	NC_051819.1 (52140775..52151566)	NC_091776.1 (39643591..3977469, complement)	NC_010450.4 (75416257..75428370, complement)	NC_041758.1 (151322435..151347090)	NC_072401.2 (155466219..155492875)	NC_073227.2 (174717649..174739230)	NC_000004.12 (153684280..153710637)
Number of Exons	2	3	3	3	5	4	5	3	4	5	4
Overall similarity (average)	41.49	52.3	70.79	71.68	84.69	77.14	78.06	95.79	99.49	99.74	100
Similarity between First Ligand Binding (LRR) Region	41.91	50.63	61.33	63.24	79.88	68.48	69.02	Graphics were not available for the chosen gene for *M.fascicularis*	99.61	99.63	100
Similarity between Second Ligand Binding (LRR) Region	43.81	45.96	74.11	73.18	86.71	80.17	81.15	Graphics were not available for the chosen gene for *M.fascicularis*	99.7	99.11	100
tein ucture											

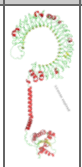
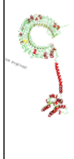
Toll-like Receptor 2

TLR2 is known to initiate signaling in response to agonists such as bacterial lipoproteins, lipopeptides, microbial cell wall components, peptidoglycan, lipoarabinomannan, fungal mannan, HSP, HMGB1, uric Acid, fibronectin, ECM proteins, and ligands of synthetic nature, etc. as well as single strand RNA (ssRNA) virus and Macrophage Activating Lipopeptide (MALP)[29,31,36]. The protein initiates immune responses by associating with LY96, TLR1 and TLR6 to mediate innate immune response and forms activation clusters, which initiate intracellular signalling cascade[30,31,37,38]. Studies have also revealed that certain TLR2 agonists, such as CBLB613 and heat killed *Mycobaterium tuberculosis* can protect cells and tissues from damage due to ionizing radiation[39] (**Table 12**).

A study done by Ochoa et al.[40] suggested that TLR2 is found to be expressed in the medullary cords of the lymph node, the marginal zone and splenic cords of the red pulp of the spleen where one would find the phagocytic macrophages, in the corticomedullary junction of the thymus and the medulla of the tonsils. Amongst these locations, the highest levels of expression, which also was

Table 4. TLR3

A comparison of TLR3 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 3											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	1	4	8 B1.1; 8 25.31 cM	16q11	16	27	15	5	3	3	4q35.1
Gene Location	NC_007112.7 (17892944..17898227)	NC_052535.1 (60833481..60843093)	NC_000074.7 (45848702..45864112, complement)	NC_086034.1 (53554916..53572321)	NC_051820.1 (46830974..46859705, complement)	NC_091786.1 (15862058..16235096)	NC_010457.5 (46966262..46977774)	NC_041758.1 (183349248..183372582)	NC_072401.2 (187711535..187729590)	NC_073227.2 (207053023..207071025)	NC_000004.12 (186069156..186088073)
Number of Exons	5	5	9	9	9	19	6	6	5	5	5
Overall Similarity at Protein level	48.48	57.11	79.42	80.31	83.96	82.61	83.41	95.69	98.89	99.34	100
Similarity between First Ligand Binding (LRR) Region	44.02	51.7	77.9	77.33	82.25	80.77	82.97	Graphics were not available for the chosen gene for *M. fascicularis*	98.91	98.18	100
Similarity between Second Ligand Binding (LRR) Region	53.61	61.52	78.51	79.94	86.28	85.71	83.76	Graphics were not available for the chosen gene for *M. fascicularis*	99.1	99.64	100
Protein Structure											

correlated to a higher frequency of cells was the spleen. Cells that were found to be TLR positive, were also found to be correlated to the distribution of CD14 positive monocytes and CD1a positive DCs in the same study. TLR2 was found to be absent in plasmacytoid DCs[40]. They are also found on tonsil epithelial cells indicating the ability of these cells to recognize microbial patterns and respond to them. These receptors are also found on the epithelial cells of the GI tract, urinary and respiratory system, as well as endothelial cells, fibroblasts, and neural cells such as microglia.

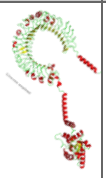
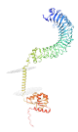
Interspecies alignment results showed that *D. rerio* had the lowest similarity percentage (just over 41%) in comparison to humans while the non-human primates showed a similarity of 95-99%. *C. lupus* showed a much higher similarity to humans when compared to cattle and pig, showing close to an 85% overall similarity. Interestingly, two key takeaways were noted from the analysis. First, with the exception of *G. gallus*, LRR2 had higher conservation across all of the chosen organisms in comparison to LRR1. Second, the two LRRs showed a similar trend in conservation pattern was observed together. (**Table 3**, **Figure 3B**).

Toll-like Receptor 3

TLR3 is known to recognize double stranded RNA (dsRNA) and a few synthetic molecules. Upon binding to the ligand, it initiates downstream signaling that upregulates antiviral proteins and interferons. TLR3 distinguishes itself from the other TLRs because it lacks a conserved proline residue between human TLR3 and mouse TLR4, leading to a variation in the signaling[41]. It is the only known TLR that depends on the TRIF pathway for signal transduction[5,42]. Some of the PAMPs recognized by TLR3 are currently in clinical trials for use in various forms of cancer such as Hiltonol (poly-ICLC) against

Table 5. TLR4

A comparison of TLR4 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 4											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Gorilla**	**Chimp**	**Human**
Chromosome	13	17	4 C1; 4 34.66 cM	5q24	11	8	1	15	13	11	9q33.1
Gene Location	NC_007124.7 (18514663..18517495)	NC_052548.1 (4245618..4251245)	NC_000070.7 (66745788..66765338)3	NC_086023.1 (85161247..85174882)	NC_051815.1 (72483514..72494289)	NC_091767.1 (106965156..106976019)	NC_010443.5 (258044610..258054641)	NC_088389.1 (40351418..40364531, complement)	NC_073237.2 (114064035..114077038)	NC_072409.2 (20728788..20742096, complement)	NC_000009.12 (117704403..117724735)
Number of Exons	3	3	3	3	3	3	3	4	4	4	4
Overall similarity (average)	38.51	45.36 - 45.48	67%	66.50%	73-74% in Canis lupus familiaris 94% in Canis lupus	75-76%	72-73%	94-99%	99-100%	99%	100
Similarity between Ligand Binding (LRR) Region	35.39	42.40 - 42.51	62-63%	65-66%	62	66.30%	62-69%	91-92%	99.66%	99.30%	100
Protein Structure											

melanomas, breast cancer, prostate cancer, mesotheliomas etc. Apart from this another agonist Ampligen (poly-IC12U; Rintatolimod) is currently in phase II clinical trials against colorectal cancer, melanomas and prostate cancer[29]. TLR3 expression levels have been found to be moderately high in T cells, NK cells, and in low levels in B cells and dendritic cells; however, this has been suggested to be due to their intracellular location rather than being bound to the cell membrane like the other TLRs[5]. It is also expressed across endothelial cells, epithelial cells, and endosomes of microglial under certain conditions (**Table 12**).

TLR3 maintains the expected trend of increasing similarity in the genes as we get closer to humans on the evolutionary tree. Interestingly, while TLR3 also showed a similar pattern of conservation of each LRR between the species to TLR1 and 2, unlike them, the conservation between LRR1 and LRR2 showed a difference of almost 10% in *D. rerio* and *G. gallus* while a difference of <5% in the other organisms (**Table 4**, **Figure 3C**).

Toll-like Receptor 4

TLR4 is a transmembrane receptor that induces the innate immune response in response to molecules such as bacterial lipopolysaccharide (LPS) and is also known to have LPS independent inflammatory responses that are triggered by free fatty acids and certain metals such as Ni(2+)[43–45]. TLR4 also recognizes molecules from various organisms *viz.* bacteria (LPS and LTA), fungi (mannan oligosaccharides, zymosan), plants (mannan), endogenous molecules (β-defensin 2, fibronectin, EDA, HMGB1, snapin, tenascin C) and synthetic molecules (OM-174, a synthetic derivative of Lipid A) [52]. TLR4 achieves this through homodimerization or heterodimerization with other receptors and/or molecules, such as LY96, TLR2, etc.[4]. Characterized for the first time in Toll5, these receptors are

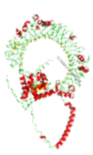
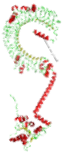
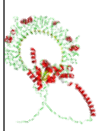
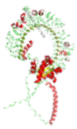
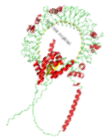
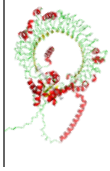
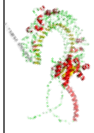
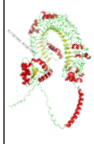
expressed in various cell types, being predominantly present on immune cells such as antigen presenting cells (APC) and macrophages. They function as signal transducers, binding with molecules such as LPS or other similar oligosaccharide molecules like zymosan or mannan(s). It also recognizes LTA53, a type of lipoteichoic acid, and heat sensitive cell associated factor (derived from *Mycobacterium tuberculosis*). For the activation of TLR4 and its subsequent binding to ligands such as LPS, TLR4s require multiple accessory molecules and proteins such as LPS Binding Protein (LBP), CD14, and Myeloid Differentiation factor 2 (MD-2) which is a protein expressed on the cell surface in association with the TLR4 ectodomain.

TLR4 expression on B and T cells, and NK cells found to be lower with respect to monocytes. Moreover, its presence is almost negligible in dendritic cells. Studies suggest that TLR4 is involved in the recognition of heat shock protein HSP60, thereby initiating inflammatory responses in necrotic cells. This cascade of responses plays a potential role in the remodeling and healing of tissues[29].

Some TLR4 agonists are also being used as part of vaccines or as treatments/potential treatments for diseases such as cancer; these BCG (bacille Calmette-Guerin), an attenuated variant of *Mycobacterium bovis which* is a vaccine for tuberculosis and for the treatment of bladder cancer[46], and Picibanil which is used to treat forms of head and neck cancer. Moreover, MPLA is currently under clinical trials for the treatment of various forms of cancer *viz.* melanoma, ovarian, lung and soft tissue sarcoma. GLA-SE is yet another TLR4 agonist that is currently in clinical trials for tuberculosis and leishmaniasis[29]. Mannan Oligosaccharides, extracted from yeast is

Table 6. TLR5

A comparison of TLR5 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 5											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	20	3	1 H5; 1 86.12 cM	13q26	38	8	10	1	1	1	1q41
Gene Location	NC_007131.7 (51471979..51476180)	NC_052534.1 (17528808..17565492)	NC_000067.7 (182782317..182804010)	NC_086031.1 (97166430..97190642)	NC_051842.1 (23873004..23878847)	NC_037335.1 (107057826..107068837)	NC_010452.4 (19387666..19410024, complement)	NC_041754.1 (80784492..80817882, complement)	NC_072398.2 (25935256..25969322)	NC_073224.2 (36674350..36706195, complement)	NC_000001.11 (223109404..223143248, complement)
Number of Exons	2	6	9	6	3	3	2	5	5	8	7
Overall similarity (average)	35.28	52.04	72.7	70.28	64.92	77080	78.55	95.22 - 95.35	98.37 - 98.60	98.83	100
Similarity between First Ligand Binding (LRR) Region	-	52.53	68.8	68.25	69.92	78.33	79.3	*Macaca mulatta*- 96.23	99.37 - 99.38	99.38	100
Similarity between Second Ligand Binding (LRR) Region	32.48	51.37	75.55	73.55	64.02 - 64.15	80.71	79.93	*Macaca mulatta*- 93.93	97.6	98.15	100
Protein Structure											

also used as a TLR4 agonist due to its ability to protect cells from radiation[4,47–49] (**Table 12)**.

While TLR4 also maintains the overall trend of similarity seen across the species, it interestingly shows a close similarity (~94%) between the sequences of humans and wolves (*C. lupus*) and a lower similarity between humans and *C. lupus familiaris* (74%). The greater TLR4 similarity between humans and wolves is probably due to the fact that, as untamed animals, wolves were subject to intense natural selection to preserve the ancestral receptor sequence and maintain high levels of pathogen detection. On the other hand, throughout breed development, domestic dogs experienced severe artificial selection and population bottlenecks, which may have loosened selected constraints on TLR4 and permitted more sequence divergence from the human ortholog. (**Table 5**, **Figure 3D**)

Toll-like Receptor 5

TLR5 recognizes ligands such as bacterial flagellins and induces inflammatory responses through both the MyD88 and TRIF pathways. It plays a major role in the relationship between the intestinal epithelium and enteric microbes, contributing to the composition of the gut microbiota throughout life[50,51]. Naturally, TLR5 levels are higher in immune cells such as T cells, moderate in NK cells, monocytes, and low in B cells, and plasmacytoid DCs. Entolimod and Mobilan are truncated forms of bacterial flagellin that are currently in clinical trials against solid tumor cancer and prostate cancer etc.[29] and as a radiation countermeasure agent (**Table 12**).

While aligning and analyzing the sequences for TLR5, it was found that the sequence domains for *M. fascicularis* were not available; as such, sequence

Table 7. TLR6

A comparison of TLR6 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 6											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	No reported protein sequences in NCBI's database for *Danio rerio*	4	5 C3.1; 5 33.54 cM	14p11	3	6	8	5	3	3	4p14
Gene Location		NC_052535.1 (69023529..69029651)	NC_000071.7 (65109373..65128387, complement)	NC_086032.1 (43715809..43727019)	NC_006585.4 (76051457..76071053)	NC_037333.1 (58050661..58091640, complement)	NC_010450.4 (30159744..30180650, complement)	NC_041758.1 (37964499..37994060, complement)	NC_072401.2 (39148782..39182830, complement)	NC_073227.2 (46857640..46901157, complement)	NC_000004.12 (38822897..38868390, complement)
Number of Exons		4	5	3	3	8	2	7	3	4	7
Overall similarity (average)		48.83	73.84	74.21	77.46	80.38	79.52	95.60 - 95.98	98.12	98.87	100
Similarity between First Ligand Binding (LRR) Region		Reported Protein sequence only has a single LRR Domain	72.65	72.65	73.83	No reported gene graphics to depict the domains of the chosen protein	80.34	95.73 (*Macaca mulatta*)	98.11	99.14	100
Similarity between Second Ligand Binding (LRR) Region		44.03	74.65	76.71	76.41	No reported gene graphics to depict the domains of the chosen protein	81.25	97.54 *(Macaca mulatta)*	100	97.89	100
Protein Structure											

comparisons for the LRR were done using only the data for *M. mulatta*. Unlike TLR4, in TLR5 it was observed that LRR2 had lower conservation than LRR1, however, second LRR for *M. musculus* and *R. norvegicus* also showed a higher similarity to humans, both being over 70% while the first LRR regions of the TLR5 genes in these organisms had less than 70% conservation (**Table 6**, **Figure 3E**).

Toll-like Receptor 6

TLR6 can recognize PAMPs of bacterial and endogenous origin, such as HSP, HMGB1, uric Acid and fibronectin, as well as ECM proteins and synthetic PAMPs such as the previously mentioned Pam3CSK4[29] (**Table 12**). TLR6 participates in the innate immune response of the body in response to gram positive bacteria and fungi, diacylated lipopeptides, through the formation of a TLR2-TLR6-CD14-CD16 activation cluster that leads to the initiation of an intracellular signaling cascade[34]. It also recognizes mycoplasmal macrophage-activating lipopeptide-2kD (MALP-2) and other bacterial factors alongside TLR2 and promotes inflammation in immune cells, such as monocytes and macrophages in response to molecules, such as Amyloid-Beta 42[52]. TLR6 expression has been found to be the highest in B cells amongst the immune cells.

As shown in **Table 7**, TLR6 was the first among the TLRs focused in this study to not have a homologous sequence in *D. rerio*. Additionally, the LRR sequences for *G. gallus, B. taurus,* and *M. fascicularis* were unavailable, resulting in their exclusion from the alignments performed. It was also noted that of the species that were compared, there was a lower level of conservation in the second LRR when compared to the first LRR between *M.*

Table 8. TLR7

A comparison of TLR7 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 7											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	9	1	X F5; X 78.31 cM	Xq13	X	X	X	X	X	X	Xp22.2
Gene Location	NC_007120.7 (54114154..54147139)	NC_052532.1 (123561394..123568153, complement)	NC_000086.8 (166086376..166113570, complement)	NC_086039.1 (30644324..30670796)	NC_051843.1 (9283446..9312699)	NC_037357.1 (130768546..130786344, complement)	NC_010461.5 (9573178..9576987)	NC_041774.1 (12511357..12534481)	NC_072421.2 (13444223..13467441)	NC_073247.2 (23336150..23359900)	NC_000023.11 (12867072..12890361)
Number of Exons	2	4	5	5	7	4	1	3	3	3	3
Overall similarity (average)	55.99	64.05	80.84	80.65	85.61	86.18-86.46	85.13 - 85.94	97.9	99.52	99.52	100
Similarity between First Ligand Binding (LRR) Region	No reported gene graphics to depict the domains of the chosen protein	66.12	76.83	77.74	83.02	86.54	82.87-83.18	98.37	99.39	99.08	100
Similarity between Second Ligand Binding (LRR) Region	No reported gene graphics to depict the domains of the chosen protein	64.19	80	79.64	86.99	86.64-87.33	86.35	97.61-97.63	99.35	99.35	100
Protein Structure											

musculus, R. norvegicus and *C. lupus*. (**Table7**, **Figure 3F**)

Toll-like Receptor 7

TLR7 is an endosomal receptor that is known to control host immune responses against pathogens that possess uridine containing ssRNA as well as guanosine analogs and is involved in both adaptive and innate immune responses [53–55]. Upon binding to its agonist, TLR7 homodimerizes and involves the TIR domains of dimerized molecules, leading to signaling through the MyD88 Dependent pathway[56]. This TLR shows increased levels of expression plasmacytoid DCs and B cells. Studies have also suggested that this receptor is absent or shows low expression levels in other types of immune cells. TLR7, in conjugation with TLR8, recognizes synthetic compounds such as CpG-A, poly G10, and poly G3. Imiquimod, a TLR7 agonist that, has shown potential in cancer therapy, is currently undergoing phase III trials for superficial basal cell carcinoma, phase II trials for high-risk melanoma, and phase I trials for malignant melanoma[57]. At the same time, Resiquimod, which is a TLR7/TLR8 agonist, is currently undergoing phase I clinical trials for stage 2, stage 3 and stage 4 melanomas[58]. MEDI9197, a TLR7/TLR8 agonist can be used against certain forms of skin cancer such as melanomas[29]. (Table 12) Unlike the previously discussed members of the TLR family, it was observed that the genes for TLR7 were found on the X chromosome of all the chosen species with the exception of *D. rerio* and *G. gallus*. This protein also shows a much higher similarity at the protein level across species, with almost 56% similarity between humans and *D. rerio,* and only increasing, as we got to the non-human primates in the same manner as the other TLRs while also maintaining the trend of LRR2 having more conservation than LRR1 across species. (**Table 8**, **Figure 3G**)

Table 9. TLR8

A comparison of TLR8 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 8											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	Unplaced	No reported gene or protein sequences in NCBI's database for *Gallus gallus*	X F5; X 78.29 cM	Xq13	X	X	X	X	X	X	Xp22.2
Gene Location	NW_003336636.1 (6147..13421, complement)		NC_000086.8 (166024119..166047325, complement)	NC_086039.1 (30708714..30733104)	NC_051843.1 (9333237..9351758)	NC_037357.1 (130727684..130745770, complement)	NC_010461.5 (9551284..9616762)	NC_041774.1 (12563581..12566701)	NC_072421.2 (13496425..13499548)	NC_073247.2 (23375972..23392539)	NC_000023.11 (12906620..12923169)
Number of Exons	2		6	3	3	6	8	1	1	3	3
Overall similarity (average)	42.72		71.53	71.73-77.83	77.51	74.49	73.39	96.63-99.73	99.52	99.72	100
Similarity between First Ligand Binding (LRR) Region	42.9		66.85	66.67-67.12	73.78-74.11	72.85-72.91	66.44	97.21-97.24	99.74	99.4	100
Similarity between Second Ligand Binding (LRR) Region	47.43		67.52-67.93	69.52	73.26	69.18	71.58	96.14	100	100	100
Protein Structure											

Toll-like Receptor 8

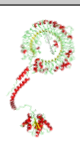
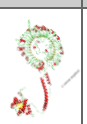
TLR8 controls the immune response of the host against pathogens by recognizing products of RNA degradation that are specific to microorganisms and recognizing GU rich ssRNA that originates or is derived from various viruses such as, SARS-CoV-2, SARS-CoV-1, and HIV-1[59,60]. It produces this immune response in conjugation with TLR7. TLR8 has been reported to have moderate expression in different immune cells; however, it has almost negligible expression in DCs[61]. TLR8 agonists include molecules such as Selgantolimod, used for treatment of chronic Hepatitis B and DN052 which are in clinical trial as a tumor growth inhibitor. TLR8, in conjugation with TLR7, also recognizes synthetic compounds such as CpG-A, poly G10 and poly G3[29]. (**Table 12**)

Gene information for this gene in *D. rerio* also mentioned that the gene is currently unplaced in the chromosomes of the species even though the loci of the gene is known. Because TLR8 has no reported gene or protein sequence for *G. gallus,* an inference can be made that the species used a different TLR to compensate for the lack of this specific receptor. The remaining species displayed the same pattern of sequence conservation as the other TLRs (**Table 9**, **Figure 3H**).

Toll-like Receptor 9

TLR9 is localized in endosomal and vacuole compartments of neuronal cells such as microglia and astrocytes. It is known to recognize unmethylated CpG DNA motifs found in bacterial DNA in humans and mice through heterodimerization with TLR9. It is reported to induce type 1 IFNs in plasmacytoid DCs through the PI3K-mTOR-p70S6K pathway and is also reported to be involved in influencing hematopoiesis for the production of plasmacytoid and

Table 10. TLR9 - A comparison of TLR9 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 9											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	8	No reported gene or protein sequences in NCBI's database for *Gallus gallus*	9 F1; 9 57.46 cM	8q32	20	22	13	2	1	2	3p21.2
Gene Location	NC_007119.7 (53730368..53733659, complement)		NC_000075.7 (106099797..106104075)	NC_086026.1 (115743407..115747523)	NC_051824.1 (37879856..37897682)	NC_037349.1 (48676670..48680933)	NC_010455.5 (34353444..34370990, complement)	NC_041755.1 (108361487..108379379, complement)	NC_086015.1 (56266220..56284354, complement)	NC_086017.1 (62100631..62118759, complement)	NC_000003.12 (52221080..52225645, complement)
Number of Exons	1		2	2	12	2	10	11	10	11	2
Overall similarity (average)	39.6		74.98	72.84 - 73.42	81.55 - 81.75	79.57 - 79.77	81.44 - 81.73	95.83 - 96.03	99.32	99.42	100
Similarity between First Ligand Binding (LRR) Region	38.11		71.72	73.48	81.49	76.11 - 80.43	80.36 - 81.36	95.45 - 95.96	99.49	99.49	100
Similarity between Second Ligand Binding (LRR) Region	43.75		72.78	68.92 - 71.30	76.07 - 76.27	75.89 - 76.28	77.69 - 78.40	94.12 - 94.62	98.22	99.2	100
Protein Structure											

killer DCs by utilizing B lymphocytes as precursors[62]. It has been reported that B cells show moderate expression of this receptor while, other immune cells show low levels of the receptor[40]. TLR9/TLR7 heterodimers are also known to recognize chloroquine. MGN1703 is a TLR9 agonist that is currently undergoing phase III clinical trials for use against metastatic colorectal carcinoma while SD101 is currently undergoing phase I clinical trials for non-Hodgkin lymphoma[29]. (**Table 12**)

It was found that *G. gallus* possessed no reported proteins for TLR9, suggesting that the species uses a different TLR in place of TLR9 to compensate for the lack of this specific receptor. It is in TLR9 where the pattern of conservation between LRR1 and 2 between the species is finally broken. Here, instead of LRR1 being less conserved, the opposite is seen as LRR2 shows lesser conservation across species; however, the exceptions to this is *D. rerio* where LRR2 is more conserved (**Table 10**, **Figure 3I**).

Toll-like Receptor 10

TLR10 is a plasma membrane bound receptor that belongs to the same gene cluster as TLR1 and TLR6 and has been reported to recognize synthetic agonists such as Pam_3CSK_4, and $PamCysPamSK_4$ while naturally occuring agonists for this TLR are unknown [29]. As a pseudogene in mice, this receptor has been poorly studied compared to other members of the family. It was also thought to be an orphan receptor with almost no ligands; however, recent studies have identified some ligands for this receptor. It is also the only known TLR that possesses anti-inflammatory properties. Low levels of TLR10 have been observed in Plasmacytoid DCs. Levels of TLR10 on B cells have been reported to be quite high[40] (**Table 12**).

Figure 3A-J shows the phylogenetic trees for each TLR, shedding light on how these sequences show evolutionary conservation and variation between *D. rerio* to humans. **Figure 4** shows a complete phylogenetic tree made using sequences for all the TLRs across all chosen species, giving us a comprehensive overview of the evolutionary relation of each TLR, not just across species, but also with respect to other TLRs for any chosen species. Considering all of these differences and variations across the species for the TLRs, two key facts emerge: first, TLR agonist interactions vary across the species

Table 11. TLR10
A comparison of TLR10 across the chosen species, with their Chromosome numbers, Gene locations, Number of Exons in their Gene, similarities w.r.t humans at the protein level and their 3D structures as seen on AlphaFold

Toll Like Receptor 10											
	Zebrafish	**Hen**	**Mouse**	**Rat**	**Dog**	**Cattle**	**Pig**	**Monkey**	**Chimp**	**Gorilla**	**Human**
Chromosome	No reported gene or protein sequences in NCBI's database for *Danio rerio*	No reported gene or protein sequences in NCBI's database for *Gallus gallus*	No reported gene or protein sequences in NCBI's database for *Mus musculus*	14p11	3	6	8	5	3	3	4p14
Gene Location				NC_086032.1 (43758940..43768352)	NC_051807.1 (74341903..74394720)	NC_037333.1 (58034097..58054261, complement)	NC_010450.4 (30124895..30134854, complement)	NC_041758.1 (37910552..37921489, complement)	NC_072401.2 (39098172..39108973, complement)	NC_073227.2 (46805450..46816191, complement)	NC_000004.12 (38772238..38782990, complement)
Number of Exons				2	6	3	2	2	3	2	4
Overall similarity (average)				71.02	81.91 - 82.03	80.15 - 80.52	80.27	96.67	99.12	99.25	100
Similarity between First Ligand Binding (LRR) Region				76.27	87.65	81.01 - 81.86	84.94	97.03 - 97.05	99.15	99.58	100
Similarity between Second Ligand Binding (LRR) Region				75.98	80.68 - 81.71	79.66	78.86	96.63 - 96.65	98.88	98.88	100
Protein Structure											

due to this variation, and second, appropriate species selection is crucial for drug screening from a theranostic standpoint. These expression differences and similarities in the protein across species lead to changes in the effects seen in response to a given agonist, leading to a variation in the response of the body and its immune system. These variations and conservations in the structure and sequence of TLRs play an important role not only in drug screening studies but also in *in vitro, in vivo,* and clinical studies.

6. Conclusion

The human TLR family contains 13 members, with TLR11, 12, and 13, being the understudied members of this family. These proteins show evolutionary conservation. This conservation extends to the ligand

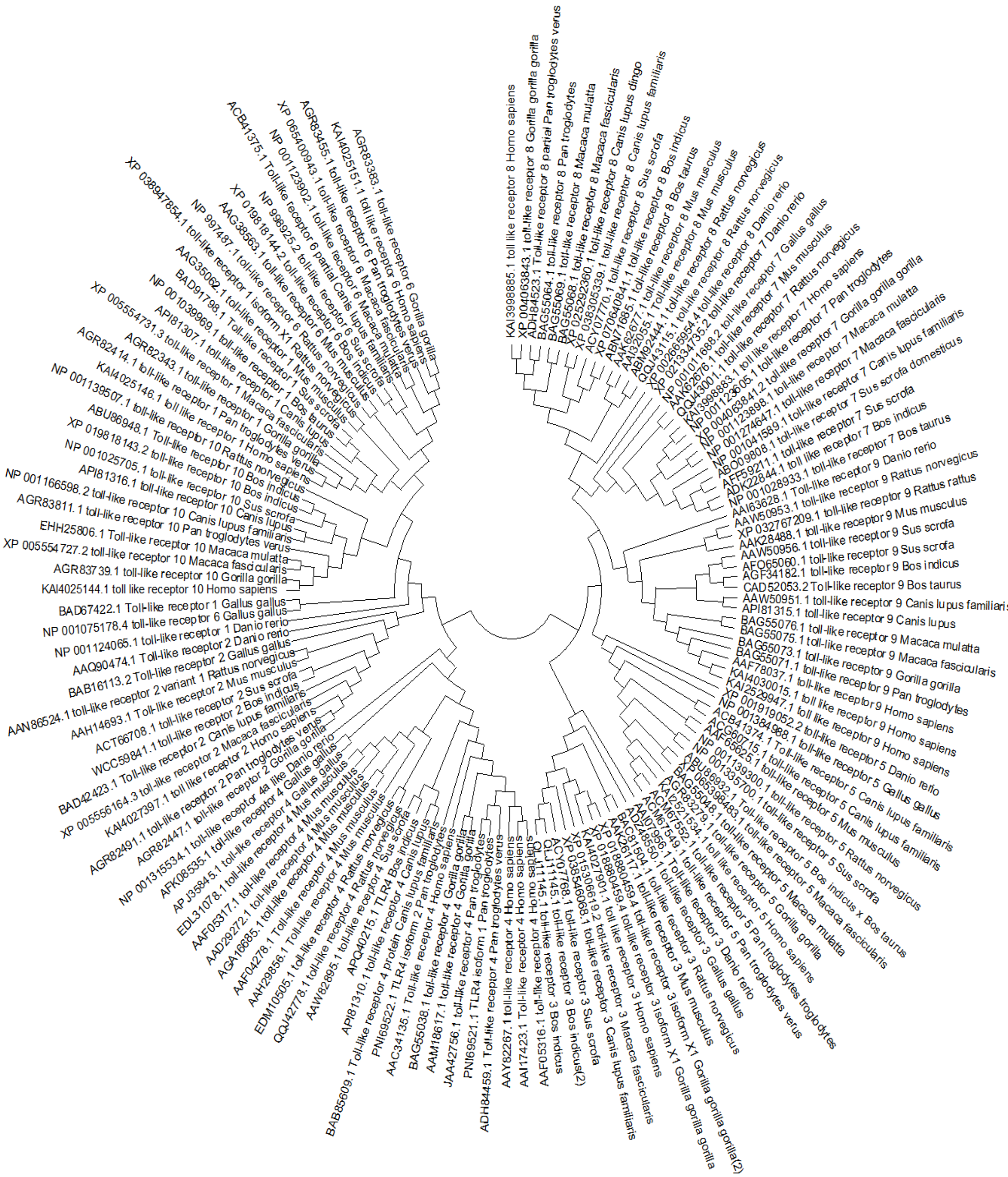


Figure 4. Phylogenetic Tree for All TLRs across all chosen species

binding regions of the proteins; however, the degree of conservation is less than what is observed in the TIR domains. This paper was written to coalesce the available information for all TLRs found in humans while also comparing and contrasting their sequences across various species. The same information may be utilized for clinical and pharmacological significance at various levels. The similarities found in the TLRs and the degree of conservation between the chosen species when compared to humans supports the fact that these organisms are good candidates for various theranostic applications. The higher degree of conservation also benefits this as it enables researchers and clinicians to safely extrapolate their data to humans based on pre-clinical studies conducted in these models. While further studies are needed to understand the reason for the differences that exist between the organisms, the insights gained through our analysis could also potentially aid in bridging the gaps between *in vitro* studies, pre-clinical findings and clinical studies for the development of theranostics by potentially providing more details on how these model organisms would respond differently to a given molecule and what changes to the administration can be done to avoid major changes in response due to said differences Moreover, this could potentially help researchers work towards furthering our understanding of various TLR associated pathological and oncological changes, immunological profiles, responses towards

Table 12. **TLR Agonists**
TLRs and some of their known agonists along with their applications in various fields of research or study

		Application	
TLR1	Lipoprotein	Adjuvant for cancer therapy	[10,29,31,36]
	Lipotechoic Acid	Cancer immunotherapy and as a vaccine adjuvant.	
	Peptidoglycan	Key component of Bacillus Calmette-Guerin (BCG)	
	Zymosan	Inhibition of melanoma progression.	
	Pam3CSK4	Improves antibacterial response of immune cells and has potential role in cancer therapy	
	CU-T12-9	To understand the immune responses induced by TLR1/2	
TLR2	Peptidoglycan	Adjuvant in vaccines	[10,29,31,33,36,44]
	Lipoarabinomannan	Adjuvant in cancer vaccines and	
	Fungal Mannan	Used as a prebiotic and as a potential radioprotectant	
TLR3	Hiltonol	Treatment of Breast, lung, and gynecological cancers, solid tumors, Gliomas, multiple myeloma,	[41,43]
	Ampligen	Treatment of colorectal cancer	
TLR4	BCG	Used to treat melanomas	[4,29,45–50,79]
	Picibanil	To treat lymphangiomas, and treatment of gastrointestinal, head and neck, and lung cancers	
	MPLA	Part of certain vaccine formulations	
	Mannan	Used as a prebiotic and as a potential radioprotectant	
	GLA-SE	Stimulates Th1 Cell–Promoting Cytokines and can potentially be a vaccine adjuvant	
TLR5	Entolimod	Used as a radiation counter measure and to treat cancers that express TLR5 by suppressing their growth	[29,50,71–73]
	Mobilan	As a potential cancer immunotherapy	
	MALP-2	As a vaccine adjuvant, for wound healing,	
TLR6	Pam3CSK4	Improves antibacterial response of immune cells and has potential role in cancer therapy	[29,30]
70TLR7	Imiquimod	Cervical intraepithelial lesions, genital warts, chronic lymphocytic lymphoma,	[10,29,55]
	Resquimod	Melanoma treatment	
	MEDI9197	Monotherapy and in combination with other immunotherapies, for the treatment of various cancers.	
TLR8	CpG-A	A vaccine adjuvant, for cancer immunotherapy, and treatment of autoimmune disorders	[2029,59–61,75]
	poly G10	Cancer immunotherapy	
	poly G3	Cancer immunotherapy and anti-cancer immunotherapy	
	Selgantolimod	To treat chronic hepatitis B	
TLR9	Chloroquine	Treatment of malaria	[29,80]
	MGN1703	Has anti-tumor activity	
	SD101	As an *in situ* vaccine for lymphoma	
TLR10	Pam_3CSK_4	Improves antibacterial response of immune cells and has potential role in cancer therapy	[29,76,77]
	$PamCysPamSK_4$		

the screening, discovery and development of new agonists (**Figure 5**).

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Autor contribution

Viraaj K. Kulshreshtha contributed to the drafting of the sections TLR Family of Proteins, Classification of TLR and Structure, Conclusion, and Sequence Alignment and Phylogenetic Analysis. Pritam Samal contributed to writing the abstract, introduction, TLR Signaling and assisted in preparing the figures & tables. Dr. Sweta Sanguri contributed to conceptualizing the TLR signaling pathways to the in silico approach and various TLR agonist identifications in the tables. Dr. Damodar Gupta conceptualized and supervised the progress of work and provided critical concepts as per guidelines and revisions. Also, defended the final version of the manuscript for submission during the institutional research and ethics committee meeting. All authors have read and agreed to the final version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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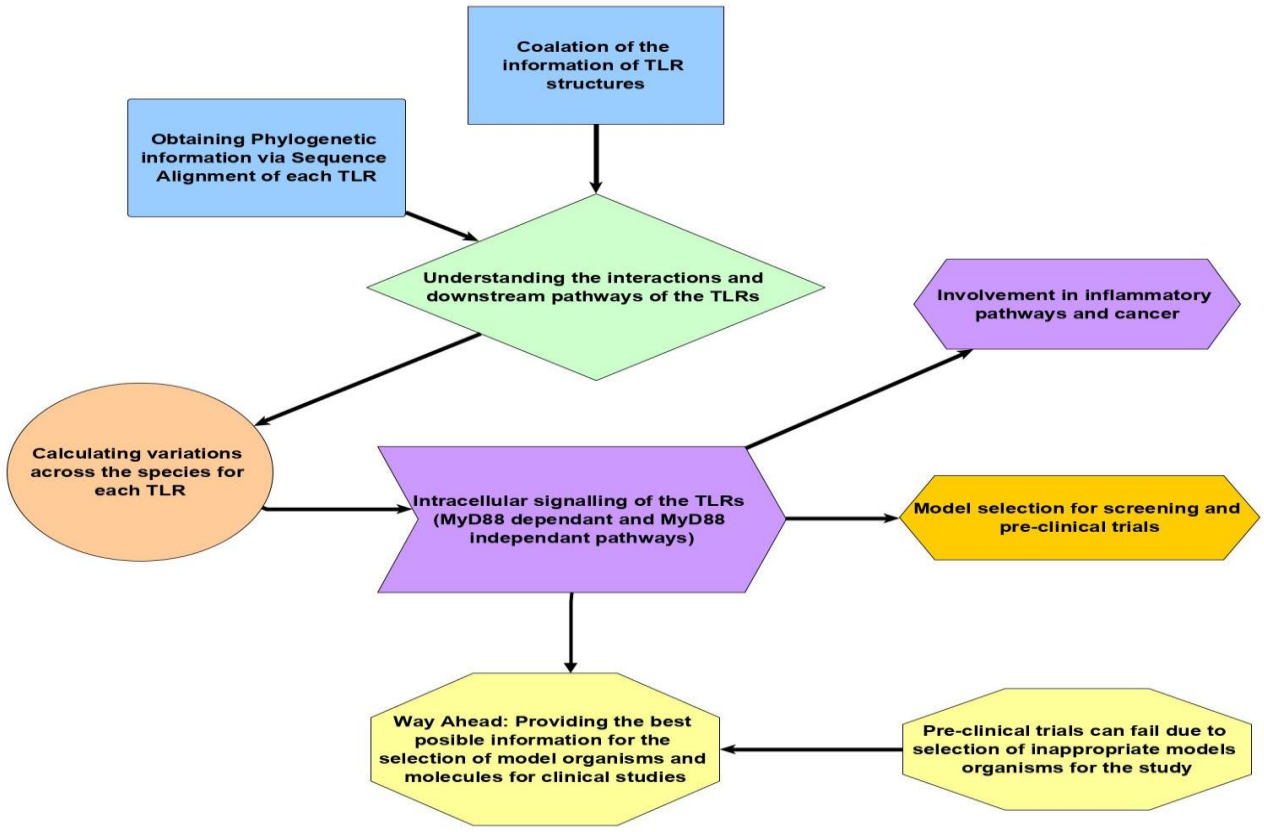

Figure 5. Perspectives in therapeutical targeting the TLR associated inflammatory and oncological pathologies

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