Recent advances in studying Toll-like receptors with the use of computational methods

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Abstract:

Toll-like receptors (TLRs) are transmembrane proteins which recognise various molecular patterns and activate signalling that triggers the immune response. In this review, our goal was to summarise how, in recent years, various computational solutions contributed to a better understanding of TLRs, regarding both their function and mechanism of action. We updated the recent information about small-molecule modulators and expanded the topic towards next-generation vaccine design, as well as studies of the dynamic nature of TLRs. Also, we underlined problems which remain unsolved.

Introduction

Toll-like receptors (TLRs) represent one of the families of pattern recognition receptors (PRRs) and are an important part of the innate immune system[1,2]. They are able to recognise various molecular patterns (MPs) in the host organism - damage/danger-, microbial/microbe-, pathogen- or xenobiotic-associated (DAMPs, MAMPs, PAMPs, or XAMPs, respectively)[3–5]. Recognition of those MPs activates downstream signalling cascades that lead to the induction of the innate immune system[6–8]. In humans, TLRs comprise ten functional members (TLR1-10) which share similar domain organisation: an N-terminal domain containing the leucine-rich repeats (LRR), a single transmembrane helix (TM), and a C-terminal cytoplasmic Toll-interleukin-1 receptor (TIR) domain **(Figure 1A)**. TLR7-9 possess an additional long-inserted loop region (so-called Z-loop) in their LRR domain **(Figure 1B)** which needs to be cleaved proteolytically. The LRR domain is responsible for ligand recognition, while the TIR domain interacts with adaptor proteins and is responsible for initiating signal transduction. A characteristic feature of the TIR domain in all TLRs is the conserved and functionally important BB-loop **(Figure 1C)**. TLRs are expressed either on the cell surface (TLR1, 2, 4, 5, 6, 10; occasionally TLR7) or in the various intracellular compartments (TLR3, 7, 8, 9; occasionally TLR4). The location of TLRs determines the spectrum of ligands they are able to recognise. For instance, TLRs expressed on the cell surface primarily recognise microbial membranes and/or components of the cell wall, while intracellular TLRs principally recognise nucleic acids[9–11]. The full list of the recognised ligands is much larger and has been discussed in several papers[11–14]. The binding of ligands to a TLR either induces the formation of a receptor dimer or changes the conformation of a preexisting dimer **(Figure 1D)** which subsequently allows adaptor proteins to bind and trigger an immune response[15]. TLRs can recruit various adaptor proteins, however, myeloid differentiation primary-

response protein 88 (MyD88) and TIR domain-containing adaptor protein inducing interferon-β (TRIF) are the most important ones. Two distinct signalling pathways used by TLRs start from them - MyD88-dependent and TRIF-dependent pathways. In general, the MyD88-dependent pathway is utilised by all TLRs, except TLR3, and leads to the production of various proinflammatory cytokines. The TRIF-dependent pathway is utilised by TLR3 and 4 and is associated with the stimulation of type-I interferon[16–19] **(Figure 1E)**.

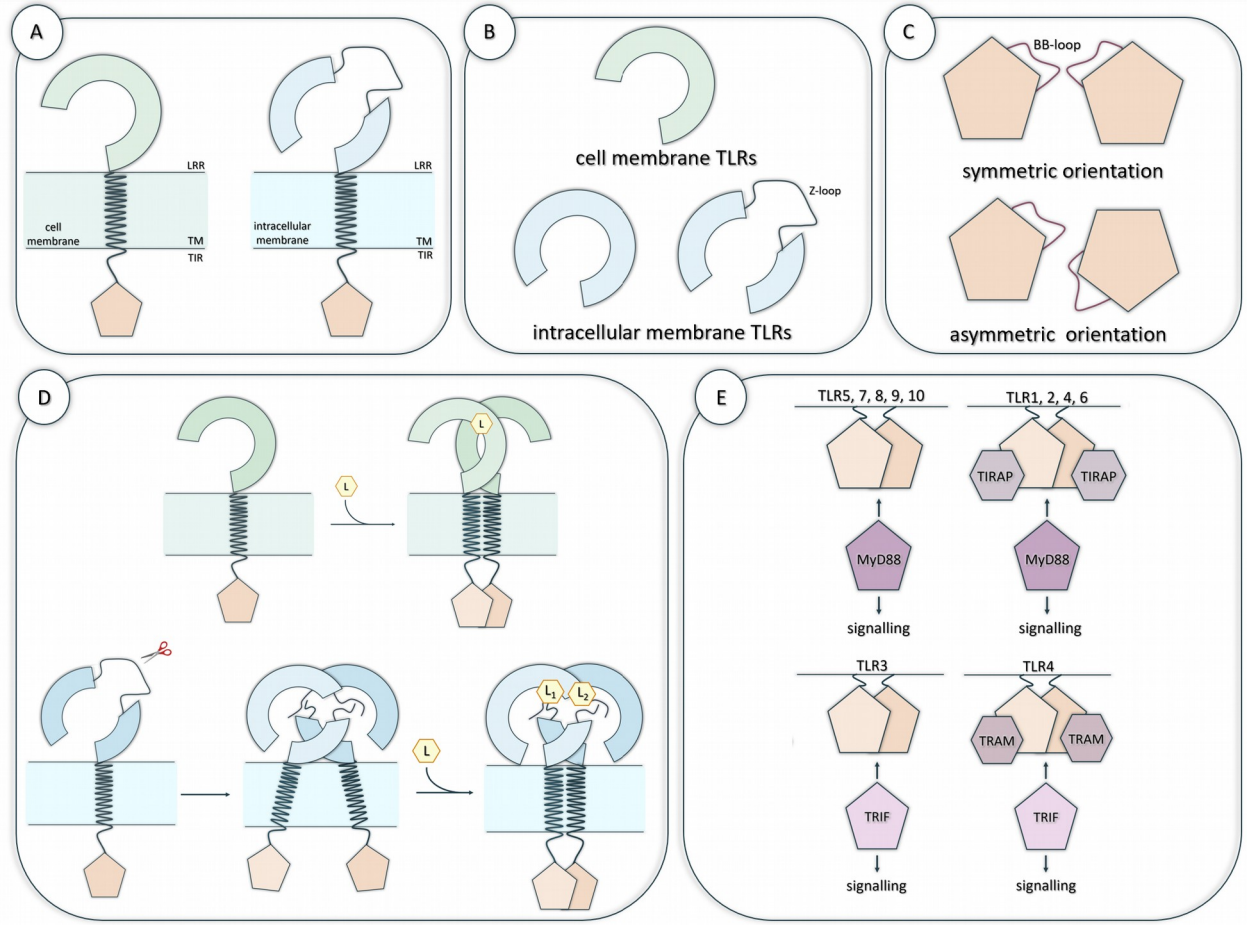

Figure 1. Structural organisation and potential Toll-like receptors (TLRs) mechanism of action. **(A)** The general structure of the TLRs' monomers. **(B)** Differences in the TLRs' LRR domains

between the cell membrane and intracellular membrane TLRs. **(C)** Various orientations (symmetric and asymmetric) of the TIR domain subunits in the TLRs' TIR dimer. **(D)** Potential mechanisms of the TLRs activation. The upper panel shows the mechanisms of the cell membrane TLRs activation, while the lower panel presents the mechanisms of the intracellular membrane TLRs containing a Z-loop. (L) indicates the ligand, while the scissors symbol indicates the proteolytic cleavage of the Z-loop. **(E)** Binding of the adaptor proteins - MyD88 and TRIF to the respective TLRs' TIR dimer.

Toll-like receptors are a potential therapeutic target in various diseases and conditions. Thus, searching for and designing compounds which can act as agonists or antagonists is the objective of many studies. The distinction between agonists and antagonists for TLRs is crucial since they are used to treat different conditions. For instance, TLR agonists have been developed to treat allergies, asthma, different types of cancer, and chronic infections by upregulating the innate immune system. Moreover, since TLRs induce the response of the body's defences, they are also promising targets for designing vaccines. On the other hand, TLR antagonists have been used to treat many inflammatory conditions such as acute/chronic inflammation, sepsis, chronic obstructive pulmonary diseases, cardiovascular diseases, neuropathic and chronic pain and various autoimmune diseases[20–23].

In recent years, multiple studies have been published, in which TLRs were the main object of research. Particular studies were focused on the following aspects regarding Toll-like receptors: their structure, ligand recognition, signal transduction, and modulator design. Some of these works were done with the use of *in silico* methods. Due to the increase in the use of

computational techniques, it was our goal to summarise how various *in silico* solutions have contributed to a better understanding of TLRs. More than five years have passed since the last published reviews on this topic[24–26], and we decided to gather the latest relevant results in this paper. We summarised the research conducted so far, while also emphasising in which areas we still lack knowledge or solutions. In this work, we focused exclusively on research on human Toll-like receptors (hTLRs).

Available structures of TLRs

The first solved structures of hTLRs - TIR domains of TLR1 and TLR2 have been available since 2000[27], while the LRR domain of TLR3 has been available since 2005[28,29]. In the case of the TM helix, the first structures were elucidated in 2014 as the result of an NMR experiment[30]. The vast majority of available structures have been deposited in the Protein Data Bank (PDB)[31] in the past decade **(Supplementary Table S1)**. However, almost all are single domains of TLRs. Obtaining full-length structures of TLRs remains a challenge. So far, only the LRR and TM domains of TLR3 and TLR7 have been determined together as a result of the Cryo-EM experiment[32]. Furthermore, there is a large disproportion in the number of structures between the individual members of the TLRs family. The biggest number of structures has been deposited for the LRR domain of TLR8. In contrast, other TLRs have very few (or none) representative structures of their particular domains. Investigation of the available structures revealed that a part of them miss a number of residues, which worsens their overall quality. Moreover, some deposited LRR domains of TLR1, TLR2, and TLR4 are hybrids of human TLR with hagfish variable lymphocyte receptor B. Those factors make not only the structural analysis but also

studies on ligand binding, receptor activation, signal transduction and modulator design not trivial. An interesting combination of computational and experimental approaches was applied for the identification and understanding of the Zn binding to the TIR domain[33]. Lushpa et al. proposed a hypothesis in which Zn^{2+} ions can bind to the TLR1 TIR domain BB-loop and stabilise the conformation of the domain, which interact with TLR2 TIR domain or adaptor proteins. With the use of the NMR experiment, the authors confirmed that the computationally-obtained two modes correspond to distinct conformations of the BB-loop and that Zn binding may affect the dynamics and conformational landscape of the BB-loop in the TIR domain.

Recently, we have entered an era where we have gained relatively straightforward access to the prediction of structures. Models of full-length TLRs structures in their monomeric form can be found in the repository of the AlphaFold Protein Structure Database[34,35]. Still, one needs to remember that in the case of the predicted structures, they need to be carefully assessed in terms of their quality, and usability.

Computational studies on TLRs

Review articles on computational methods applied in the Toll-like receptors research published before 2017 cover mostly the topics related to designing small-molecule modulators of TLRs[24–26]. For instance, Murgueitio et al.[24] described three main application areas of computational methods to the discovery of TLR modulators: i) exploration of the structure and function of the receptor, ii) analysis of receptor-ligand interactions, and iii) rational design of novel TLR agonists and antagonists by virtual screening (VS). In another work, Pérez-Regidor et al.[25], focused almost

exclusively on the search for novel chemical modulators for TLRs employing VS techniques. Not only did the authors provide information about the available results for five members of the TLRs family - TLR2, 3, 4, 7, and 8, but also they described the available information about the databases, protocols and techniques used in virtual screening. In their review, Billod et al.[26] focused on TLR4 exclusively and summarised the following aspects: a perspective of the TLR4/MD2/ligand recognition and dimerisation, mutant studies, binding mode modulators analysis and VS strategies for various types of modulators. In 2020 Wang et al. published an article aimed at the progress in developing TLR signalling pathway modulators[36]. They mainly focused on the results provided by Yin and Wang laboratories and discussed the identification and characterisation of new chemical entities, their modes of action, and further applications. For works that used computational methods, they provided such information in the paper. Based on the results summarised in those reviews, it is clear that almost all the studies focused on finding small-molecule modulators for the LRR domain of the TLRs. As rightly noted by Wang et al.[36], TM domains are usually considered 'undruggable' and TIR domains among TLRs are highly conserved, which is why most modulators are designed to target the LRR domain of TLRs.

Below, we summarised substantial studies that have been published in recent years in which computational methods have been employed. First, we gathered the recent works which focused primarily on designing modulators for TLRs. In particular, we focused on two types of modulators: small-molecule and vaccine components. While small-molecule compounds have been extensively studied, vaccine components have not been reviewed in detail. Second, we reviewed studies principally focused on the investigation of the dynamic nature of TLRs, which is crucial for understanding their function and mechanism of action.

Modulators of TLRs

The search for new chemical entities as potential TLRs modulators is an ongoing process, especially because relatively few compounds with therapeutic potential have been tested in clinical trials. Additionally, the use of a strategy involving the TLRs as a driving force for the design of next-generation vaccines has become increasingly popular recently. Since different types of modulators (small-molecule or part of the vaccines, e.g. epitopes), requires various methods and techniques for their identification, we reviewed both classes separately.

Novel potential small-molecule agents

The general protocol used for the search for novel small-molecule TLRs modulators has remained the same in most of the studies conducted so far. It consists of the following steps: i) preparation of the target structure, ii) preparation of small molecules from available libraries, iii) structure-, ligand, and/or pharmacophore-based virtual screening combined with molecular docking, iv) selection of best candidates, v) experimental testing, vi) identification of potential drug candidates. Before the selection of the best candidates, more advanced computational methods are sometimes used, e.g molecular dynamics (MD) simulations, MM-PBSA, MM-GBSA binding free energy analysis, combined with receptor-ligand interaction network analysis **(Figure 2)**. By applying those advanced methods it is possible to gain better insight into the molecular basis of ligand recognition. Usually, all-atom MD simulations of the receptor-ligand complex are performed.

For VS, scientists have various commercial, public or in-house databases at their disposal. Many groups have concentrated on modifying the previously identified small-molecule compounds or mimicking the native ligands within known binding sites. Nevertheless, there are also examples revealing novel chemical classes of potential modulators. Studies conducted so far are still mainly focused on targeting the LRR domain of TLRs. There has been no noticeable progress in the design of modulators for the TIR domain.

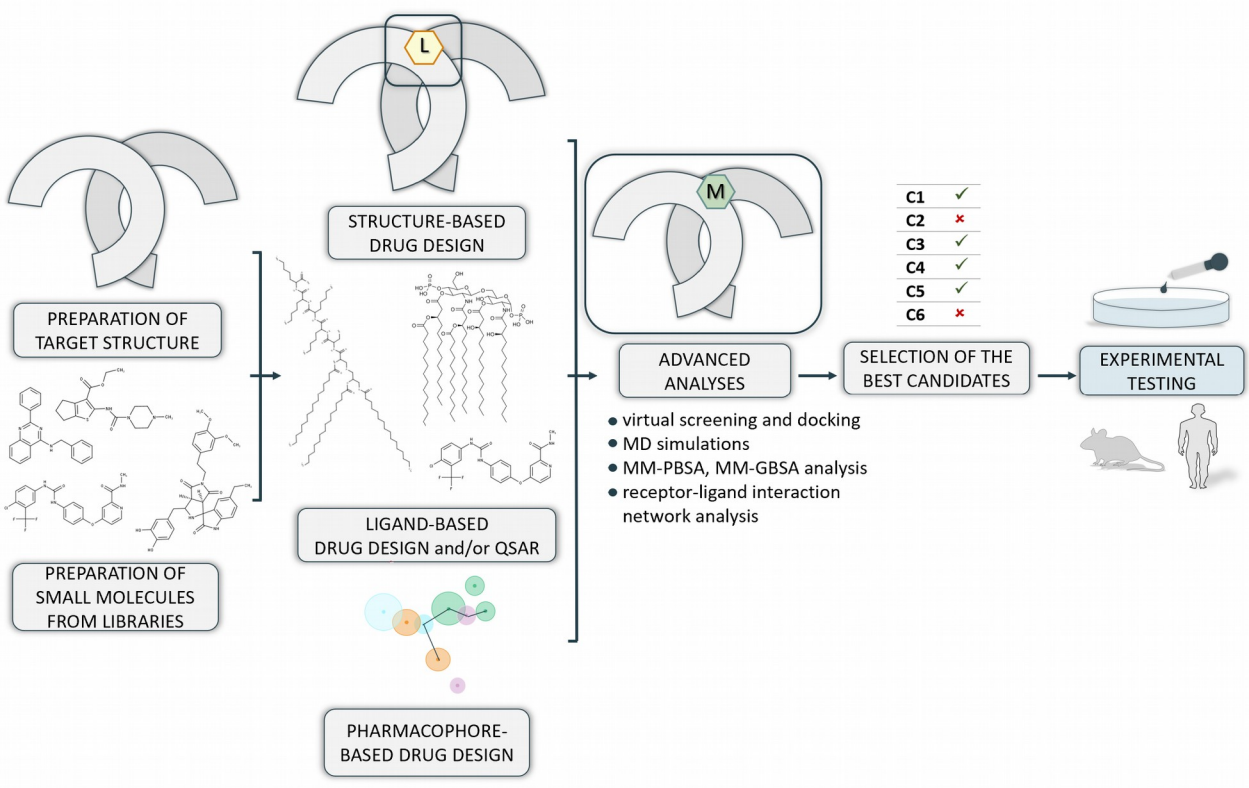

Figure 2. A general protocol for small-molecule modulators design targeting the LRR domain of TLRs. The subunits of the LRR domain are coloured grey, indicating both TLRs located in the cell membrane and TLRs located in the intracellular compartments. (L) indicates the location of

the ligand binding site, while (M) points out the designed modulator and (C) the selected candidate(s).

Many recent studies have been carried out on TLR2. For instance, Murgueitio et al.[37] used a shape- and feature-based similarity VS to screen some commercially available databases. For similarity search, they used the previously discovered TLR modulators from Guan et al.[38] and Liang et al.[39]. The authors tested selected hits and four (AG1-AG4) were found to synergistically increase the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation induced by the known lipopeptide ligand Pam3CSK4. Further studies indicated that the tested compounds could act as ago-allosteric modulators of TLR2.

Durai et al.[40] used receptor–ligand- and ligand-based VS to screen in-house libraries. They focused on the non-peptide TLR2 antagonists, distinct from several known inhibitors with fatty acid chains. For the receptor-ligand-based model, they prepared the protein–lipopeptide complex (PDB ID: 2Z7X)[41], while for the ligand-based model, they selected compounds from Guan et al.[38]. The authors evaluated the best hits for their ability to bind directly to human recombinant TLR2. Furthermore, they tested the compounds' ability to inhibit the synthesis and secretion of IL-8 in human embryonic kidney cells overexpressing TLR2. Two molecules - C11 and C13 displayed both direct binding to TLR2 extracellular domain and reduced IL-8 production. The results supported the possibility that C11 and C13 can disrupt TLR2/1 heterodimerisation.

Chen et al.[42] performed a structure-based VS of the ZINC database. Based on the scoring results, including shape, chemical-feature, and drug-like properties, they identified potential agonists targeting the TLR2 heterodimer and modulating the TLR2/1 response. For the most promising

candidates, which shared a motif of an amine conjugated with an acid substituent, they tested their activity *in vitro*. The results revealed that two compounds showed a high TLR2 activation effect and that one compound - ZINC6662436 (SMU127) - stimulated the NF-κB and promoted tumour necrosis factor-α in human macrophage and mononuclear cells. Also, the *in vivo* results showed signs of inhibition of breast cancer tumour growth in BABL/c mice. In a later study, Chen et al.[43] improved the potency of the SMU127 by modifying the ring system, while keeping all other structural features. One of the modified compounds - SMU-C13 possessed the highest TLR2 activity. Also, the *in silico* simulation indicated a tight fit into the known binding site. Based on the structure-activity relationship (SAR) results, the authors concluded that the introduced piperidine ring contributed to the increased activity against TLR2. Grabowski et al.[44] performed both ligand- and structure-based VS using commercial databases of nearly six million compounds. The authors selected two well-characterised chemotypes of small-molecule modulators to build their models - i) m1 proposed in previous work by Murgueitio et al.[45] and (ii) CU-CPT22 and the other benzotropolones discovered by Yin et al.[46]. They carried out docking, rescoring, and visual inspection analyses and selected the best hits for biological testing to confirm their ability to inhibit TLR2-mediated responses. The most active compound, a pyrogallol derivative named MMG-11 inhibited both TLR2/1 and TLR2/6 signalling. It also showed a higher potency than the previously discovered CU-CPT22. Additionally, in a subsequent paper[47], Grabowski et al. confirmed that the identified compound was also able to selectively inhibit TLR7 and TLR8 signalling. Encouraged by these results, they applied a computationally-guided synthesis approach to get an analogue of that compound which showed dual inhibition of TLR2/8. Also, in another work, Bermudez et al.[48] explored the chemical space around the pyrogallol-containing antagonists to improve synthetic accessibility and chemical

stability.

Boger's lab proposed a new and potent class of TLRs agonists - diprovocims[49]. They obtained results from a compound library designed to promote cell surface receptor dimerisation. The revealed mechanism of action was that diprovicims act by inducing cell surface TLR2 dimerisation and activation with TLR1. Later, the basis of TLR2/TLR1 activation by Diprovocim was studied by Su et al.[50]. They combined structural data with MD simulations, MM-PBSA, MM-GBSA binding free energy and mutagenesis analyses, and they showed that the new modulator interacts with TLR2/TLR1 at the same binding pocket as Pam3CSK4. However, the observed conformations around the ligand binding sites were different. The authors noticed the widespread hydrophobic interactions and a hydrogen-bonding network between the receptor and Diprovocim molecules within the ligand binding pocket, which is probably in correlation with the high potency of the discovered compound.

For the TLR4 receptor associated with myeloid differentiation factor 2 (MD2), Mishra and Pathak[51] aimed at the identification of small-molecule protein-protein inhibitors based on a pharmacophore mapping-based approach. For that, they used information about the hot-spot residues and their corresponding pharmacophoric features on the protein-protein interaction interfaces in the TLR4/MD2 homodimer complex. The authors performed extensive post-VS filtration (based on ADME/T properties, oral bioavailability, and possible side effects - off-targeting and environmental hazard) to propose novel small-molecule inhibitors. From selected hits, two (C11 and C15) with the predicted best inhibitory concentration were confirmed to form a stable complex with the target protein. In other studies, Facchini et al.[52] and Cochet et al.[53] focused on designing the monosaccharide mimetics of lipid A, which is a known agonist. The authors successfully designed mimetics through docking with MD2 and confirmed the

stability of the modulators by performing MD simulations. Subsequently, compounds were synthesised and tested to confirm their ability to bind to MD2 and inhibit LPS-stimulated TLR4 activation. In a very recent study, Pérez-Regidor et al.[54] focused on a different strategy of finding non-LPS-like modulators among the approved drugs and drug-like molecules from commercial, public, and in-house libraries of compounds. Based on the structure- and ligand-based VS, combined with docking and biological results, the authors presented a common scaffold consisting of two hydrophobic moieties separated by a polar linker. They showed that one large hydrophobic moiety occupies the hydrophobic MD2 cavity, while the second moiety is associated with the same hydrophobic region as one of the lipid A alkyl chains, and the polar linker occupies the entrance to the pocket.

An interesting study was performed by Borges et al.[55]. The authors investigated the effect of the natural limonoid gedunin on different TLRs (2, 3, and 4) activation. They performed *in vitro*, *in vivo* and *in silico* studies. The experimental results confirmed that gedunin is able to impair inflammasome activation, and cytokine production and induce anti-inflammatory factors in macrophages. The *in silico* studies revealed that the investigated compound can efficiently bind to the TLR2, TLR3, MD2 protein of TLR4 and also to the caspase-1, making gedunin considered a multitarget compound. For both TLR2 and TLR4, gedunin bound within the known ligand binding site, while for TLR3 two distinct binding sites were predicted. The authors pointed out that one of the predicted regions for TLR3 is involved in the dimerisation of TLR3 and is considered the dsRNA binding site, thus it might be the most prominent. Still, as pointed out by the authors, further biochemical assays are required to confirm gedunin binding.

For endosomal TLRs - TLR3 and TLR7-9, Talukdar et al.[56] recently published a perspective paper regarding the structural evolution of their small-molecule agonists and antagonists. They concluded in detail information about structural features around binding sites of both types of modulators, and their evolution and provided information about the development of various chemotypes. Here we wanted to highlight a few studies not included in the above-mentioned publication.

One example is the work performed by Gupta et al.[57]. They used the known ligand-based pharmacophore modelling approach to find novel human TLR7 modulators based on the set of TLR7 agonists with confirmed experimental activity. They created a pharmacophore model and screened the natural hit compounds from the InterBioScreen Natural product database. They filtered the screened compounds and based on molecular docking and interaction analyses, they selected the most interesting compound - STOCK1N-65837 (an indoline derivative natural alkaloid). The compound was further validated with MD simulation. Authors underlined that further experimental validation is necessary to confirm the activity of the compound, however, their results already provided a basis for further designing of natural modulators targeting TLRs.

Šribar et al.[58] used the previously established approach consisting of structure- and pharmacophore-based computational studies followed up by experimental validation to find novel inhibitors of TLR8. They performed two rounds of VS. The authors used the best hit from the first round of VS and performed its optimisation by shape- and chemistry-based screening. Later, they prioritised them according to their diversity and physicochemical properties. Based on that approach, they found a novel pyrimidine scaffold for TLR modulators. Experimental validation of the most promising compounds from the second round of VS revealed their low cytotoxicity, suggesting that they are relevant for further lead optimisation.

Recently, Wang et al.[59] focused on revealing the mechanism of action of known agonists for TLR7 and TLR8 - imidazoquinoline derivatives (Resiquimod (R), Hybrid-2 (H), Gardiquimod (G)). They carried out MD simulations for both TLR7 and TLR8 apo structures and TLR7 and TLR8 with bound antagonists, followed by the MM-GBSA calculations. Their analysis showed that TLR7-R and TLR7-G complexes formed open conformations during the simulation, while the others were kept in closed conformations. They found that the binding pocket of TLR7 was less flexible than in TLR8, thus, the binding of the antagonist was tighter. Moreover, these *in silico* predictions were in agreement with the experimental data.

In **Figure 3** we presented examples of scaffolds of both agonists and antagonists targeting the LRR domain of TLRs proposed in reviewed publications. Also, we showed the localisation of the designed small-molecule modulators in relation to the subunits of the TLRs. In **Supplementary Table S2** we gathered the structures of all the best hits from the reviewed research papers.

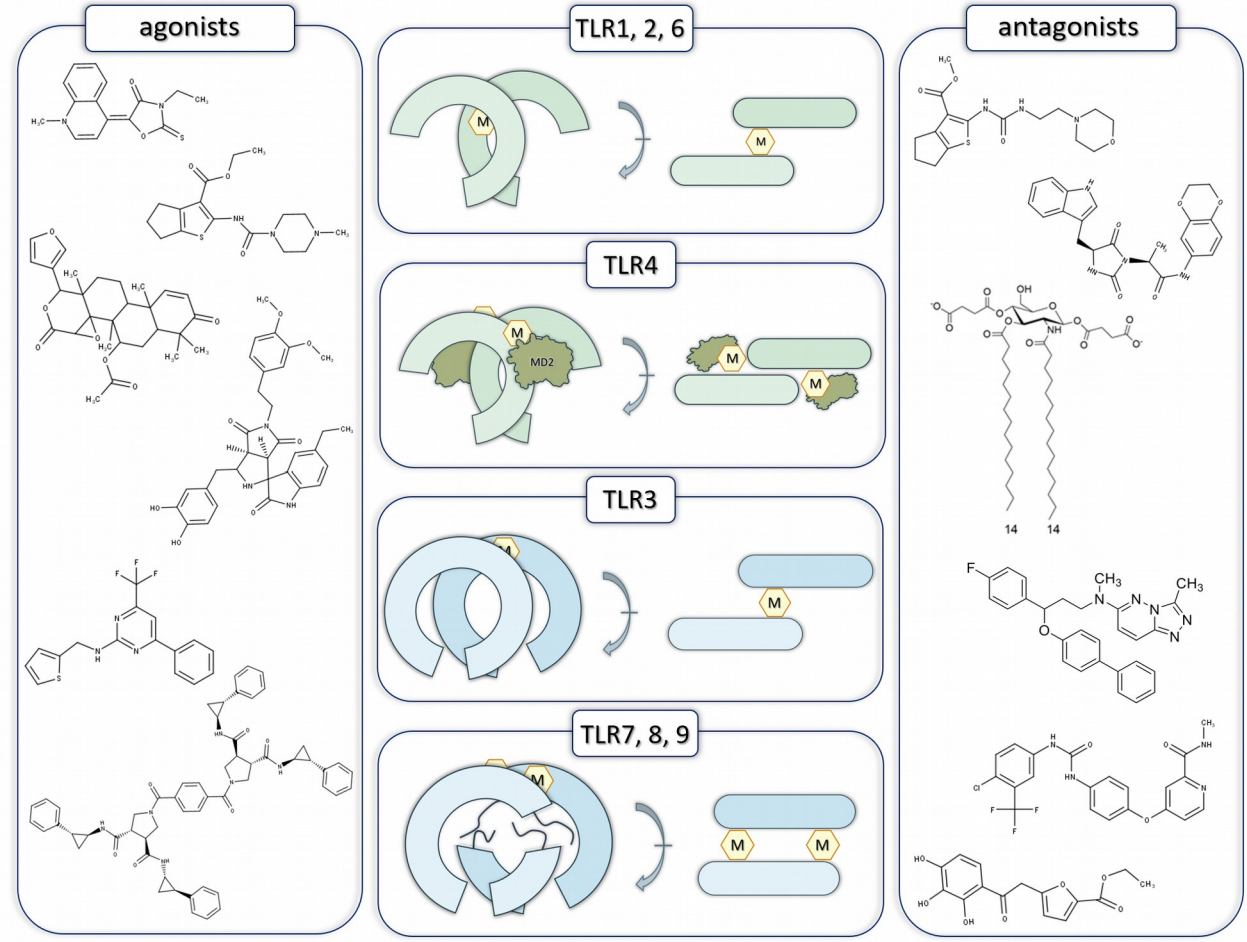

Figure 3. Examples of scaffolds of small-molecule modulators targeting the LRR domain of TLRs. Agonists are presented on the left panel, while antagonists are on the right panel. The middle panel shows the approximate location of small-molecule modulators (M) with respect to the LRR subunits of the TLR dimers described in this review. TLR4 was shown with the associated myeloid differentiation factor 2 (MD2).

As can be seen from the above-mentioned studies, many groups used the information from the previously designed modulators either for introducing some modifications aimed at increasing their activity or for obtaining models for VS and further studies. In the reviewed papers we encountered both the strategy to design modulators structurally similar to known ligands and

compounds with a completely different structure. Interestingly, the targeting sites remain the same, which highlights the challenges in reconstruction of TLRs structure and difficulties with the identification of other potential binding sites which could affect TLRs function. We could also notice that some of the proposed modulators were able to influence the signalling pathways in various TLRs. Nevertheless, the molecular basis of their selectivity have not been thoroughly examined. Therefore, one needs to keep in mind that we still need in-depth studies revealing the differences in the mechanism of action in relation to different receptors. We believe that in the coming years, more groups will include analyses related to potential off-targeting effects, as well as that there will be an increase in interest in the screening of natural compounds databases for proposing novel small-molecule modulators. Regarding methods, we are expecting an increased contribution of AI-supported screening, especially in ligand-based screening.

Next-generation vaccines

Subunit vaccines are considered one of the next-generation vaccines. They consist of pieces of a pathogen, instead of the whole organism. Evidently, this also means they do not contain any live pathogen and thus show significantly lower immunogenicity. The immunogenicity of the subunit vaccines can be improved by several factors, e.g. addition of adjuvants, choice of different delivery systems, usage of multiple antigens or epitopes, and optimisation of vaccine dosage. TLRs are excellent targets for such multi-epitope vaccines to provide a signal to induce an effective immune response that in turn leads to long-lasting protection[23,60,61]. The protocol used for the search for multi-epitope modulators is substantially different from the one used for small-molecule modulators. The general protocol consists of multiple steps: i) retrieval of target

proteins sequences, ii) evaluation of antigenic and physicochemical properties of the target proteins, iii) epitopes prediction, iv) multi-epitope vaccine construction, v) evaluation of antigenicity and allergenicity of the vaccine combined with the exploration of the physicochemical parameters, vi) prediction of secondary and tertiary structure, vii) molecular docking to the immune receptors, and viii) dynamics' analysis of the complexes. Some studies also include further computational immune simulation to assess the vaccine's ability to stimulate the immune response **(Figure 4)**.

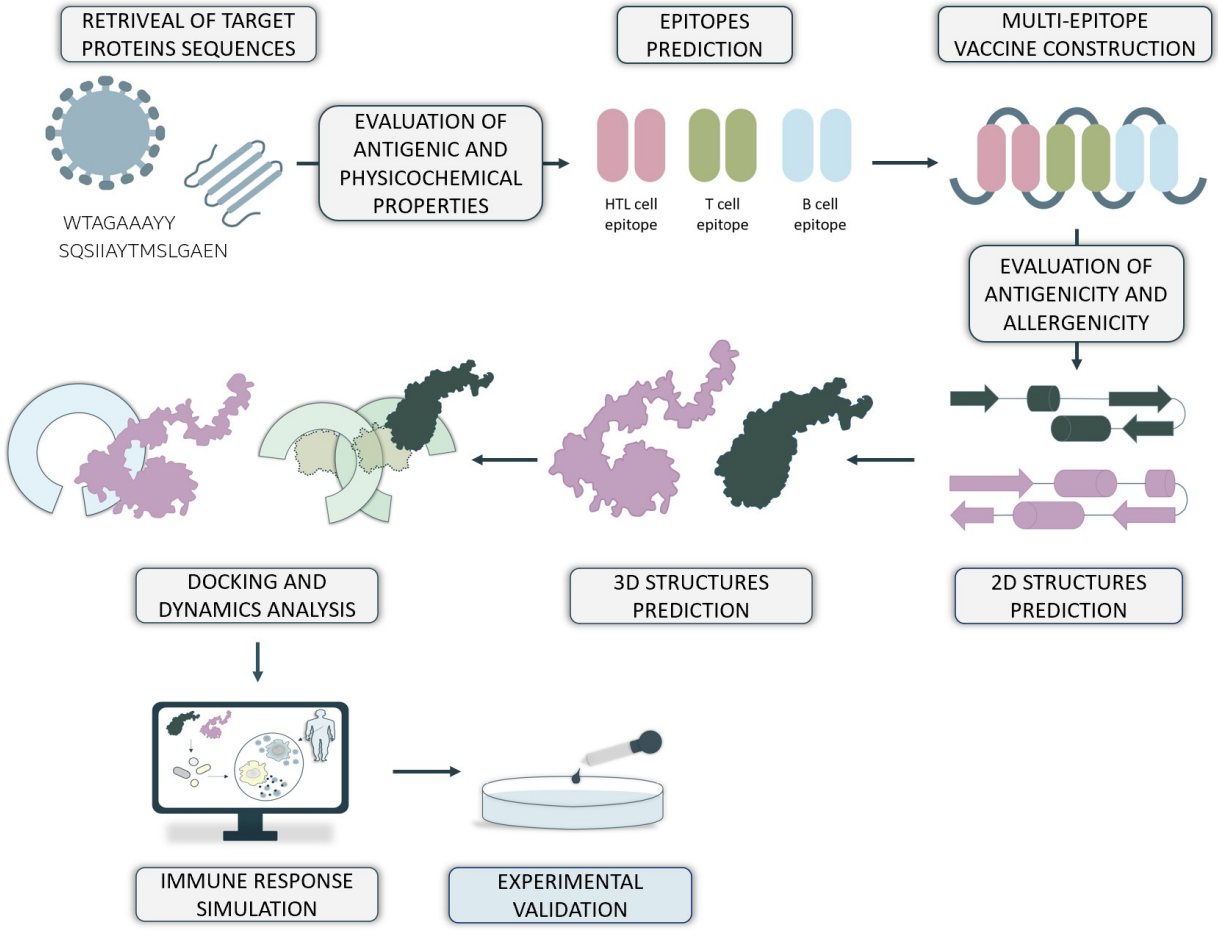

Figure 4. General protocol for next-generation multi-epitope vaccine design. The ability of binding different epitopes (shown dark green and pink shapes, respectively) to LRR subunits of the TLRs located both in the cell membrane (light green) and in the intracellular membrane (light blue) has been shown.

Each step of this protocol is quite elaborate and usually requires the usage of several tools/servers. As information about vaccine construction has not previously been addressed in computational reviews about TLRs, a brief summary is given here. Target sequences might be

obtained from databases like PDB or UniProt[62]. Then, they are submitted e.g. to the VaxiJen[63] to check the antigenicity and to ExpasyProtParam[64] to investigate the physicochemical properties. Multiple servers can be used to predict the epitopes, depending on the type. Among them, there are NetCTL[65], NetMHCIIpan[66], Immune Epitope Database[67], BepiPred[68] and BCPREDS[69]. Antigenicity, promiscuity, and allergenicity of epitopes can be evaluated with the use of AllerTop[70], AlgPred[71,72], VaxiJen, and ToxinPred[73,74] servers. Structural evaluation of the vaccine begins with the prediction of secondary structure, which is usually done by the SOPMA server[75]. Later, the tertiary structure can be predicted, often by the I-TASSER[76]. However, the obtained models still need further refinement. For that, ModRefiner[77] and GalaxyRefine[78] are common choices. At this stage, it is evident that the way to obtain a structure of this type of modulator is quite demanding. Molecular docking to the immune receptors is similar to blind docking, meaning that there is a predefined binding site. The ClusPro server[79] is able to perform such computations. Further investigation of the dynamical properties is usually performed using Normal Mode Analysis (NMA) rather than all-atom MD simulations. However, the latter one (if used) can provide better and more detailed insight. A simulation of a possible immune response, which usually concludes the *in silico* part, is often performed using the C-ImmSim tool[80].

In studying TLRs, molecular docking, combined with the investigation of the dynamical stabilities and prediction of the vaccine's ability to stimulate the immune response are the most crucial. The above-mentioned protocol and its variations have been used multiple times for vaccine design. Undoubtedly, vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have received the most attention in recent years[81–84]. However, studies on other vaccine designs have also been carried out, both before and after the outbreak of COVID-19. The following examples are studies focused on designing vaccines against Middle East respiratory

syndrome (MERS)[85], Hepatitis C virus (HCV)[86], human immunodeficiency virus (HIV)[87], Neo-Coronavirus (NeoCoV)[88], Human cytomegalovirus (HCMV)[89], Kaposi Sarcoma[90], as well as infections like dengue[91], chikungunya[92] or those caused by *Taenia solium*[93], *Klebsiella oxytoca*[94], *Klebsiella pneumoniae*[95] or *Mycobacterium tuberculosis*[96,97]. What is also worth mentioning in the context of next-generation vaccine design is the potential use of TLR agonists as vaccine adjuvants. Since TLR agonists are capable of stimulating innate immune responses, which also trigger adaptive immune responses, they can likewise be used to improve vaccine efficacy[60,98,99]. For instance, monophosphoryl lipid A (MPL) and CpG-1018 have been used as adjuvants in licensed vaccines, and other TLR agonists are under the investigation. Below, we want to elaborate more on vaccines against SARS-CoV-2, although the ultimate goal remains similar in all the studies - to get a stable protein-vaccine complex which triggers the immune response.

Different groups focused on studies of multi-epitope vaccines against various TLRs. For instance, Oladipo et al.[81] studied the TLR2, TLR3, TLR4 and TLR9, while Rafi et al.[82] focused on TLR2 and TLR4, and Ysrafil et al.[83] investigated TLR3, TLR4 and TLR8, as well as angiotensin-converting enzyme 2 (ACE2) as the entry receptors of SARS-CoV-2. Drawing upon the structure of the SARS-CoV-2 spike (S) glycoprotein (and nucleocapsid (N) protein and open reading frame 1a (ORF1a) protein in the case of Ysrafil et al.[83]), the authors tried to develop a potent multi-epitope subunit vaccine. Another interesting study was proposed by Pitaloka et al.[84]. The authors focused on designing a vaccine for protection against *Mycobacterium tuberculosis* (MTB) and SARS-CoV-2 coinfections. The potential epitopes were screened from outer membrane protein A Rv0899 (OmpATb) of MTB and S protein of SARS-CoV-2 and were further combined with adjuvants. In general, all the results showed that the proposed multi-

epitope vaccine candidates were non-toxic, capable of initiating the immunogenic response and not inducing an allergic reaction. Also, the results revealed rather strong and stable interactions between the vaccines and receptors within their LRR domains. During the simulations of the potential immune response, the authors noticed a rise in the production of immune defences, i.a. rise in the HTL cell population with memory T and B cells development, an increase in IgM, IgG1 + IgG2, and IgG + IgM antibodies levels. The stability of the complexes was confirmed by studying their dynamic properties. For instance, Oladipo et al.[81] and Pitaloka et al.[84] [58] used NMA to study the stability and mobility of selected receptor-vaccine complexes. In the first study, as a result, the vaccine protein and its receptor were predicted to spin towards each other. In the second study, based on the detected correlations in the covariance matrix between pairs of residues, the authors confirmed the stability of the vaccine candidate model. Rafi et al.[82] performed classical MD simulations to check the stability of the constructed vaccine with the extracellular subunit of TLR2 and TLR4/MD2. The results indicated that the TLR-vaccine complexes were both stable and compact during the simulations. Especially for the TLR4-vaccine complex, a strong hydrogen bond network was pointed out, suggesting reduced flexibility of the vaccine when bound to the receptor, improved binding strength, and increased vaccine–receptor stability. Furthermore, the authors expanded their analysis by using the full-length heterodimer TLR4/MD2-vaccine complex which was placed in a membrane to imitate the dynamic behaviour during the MD simulation of the vaccine in biological systems. This study is one of the first where the full-length models of TLR receptors from the AlphaFold Protein Structure Database were used. For both TLR2 and TLR4 complexes, significant structural transitions toward membrane bilayer were observed, but the crucial interactions between the vaccine and the extracellular domain of receptors remained stable. Based on the observations

made in the above-mentioned papers, one can speculate that during the binding, potentially well-designed vaccines may have a stabilising effect on the TLRs in the system.

Although at first glance, epitopes may be treated similarly to modulators, the specificity of their search is quite different. It takes into account not only the process of binding to the TLR but also the stability and specificity of the epitope. Research on epitopes has the potential to reveal the mechanism of action of TLRs and their specificity to a greater extent. In the near future, this type of research can contribute to a much better understanding of the functioning of our immune system and the recognition of threats. We also anticipate that the contribution of AI-based methods will allow for a better understanding of the signalling pathways and their interrelations.

Dynamic nature of TLRs

The complexity of TLRs has consequences in the relatively weak understanding of the structural basis of their modes of action. Therefore, significant effort is required to comprehend TLR dynamics, both at the level of particular domains, full-lenght receptor and the dimerisation process. Here, in the first part, we gave an outline of the studies that examined the effect of certain mutations on the receptor's dynamics. In the second part, we summarised the works that focused on the characterisation of the dynamical properties and conformational changes of full-length TLRs.

Mutations' effects on the TLRs dynamics

It is known that even a single mutation can induce substantial changes in terms of the macromolecule's structure and function. For TLRs, one can hypothesise that depending on the mutation location, the ligand recognition or the adaptor protein binding could be disturbed. Below, we summarised studies focused on examining the effect of various mutations on TLRs. Those studies have usually focused on the analysis of individual domains of TLRs - the LRR or TIR domains.

Regarding the LRR domain, Anwar and Choi[100] examined the structure-activity relationship in TLR4 mutants by the application of MD simulations together with principal component (PCA) and residue interaction network (RIN) analyses. To evaluate the influence of single nucleotide polymorphisms (SNPs), they examined four different models: i) wild-type TLR4 (TLR4WT); ii) a double mutant - aspartic acid-to-glycine at position 299 and threonine-to-isoleucine at position 399 (TLR4GI); iii) the aspartic acid-to-glycine mutant (TLR4G299); and iv) the threonine-to-isoleucine mutant (TLR4I399). Those mutations were classified as eliminating signalling activity, however, they did not disturb the ligand recognition nor did they establish contact with the associated MD2 protein. Computational studies revealed differences in the dynamic properties of the analysed variants. The authors pointed out that the mutated complexes were less cohesive and displayed both local and global variation in the secondary structure, which could affect the proper exploration of conformational phase space. They also showed that decay in the rotational correlation function together with the observed density distributions and alteration of the number of hydrogen bonds between the protein and ligand could result in the loss of function.

Gosu et al.[101] performed MD simulations of human wild-type and mutant TLR3 to get insights into the dynamic nature of the dsRNA-bound TLR3 complex. They investigated several complexes: dsRNA-unbound TLR3 wild-type dimer (apo_dTLR3WT), dsRNA-bound TLR3 wild-type dimer (dTLR3WT-dsRNA), dsRNA-bound TLR3 dimer with a leucine-to-phenylalanine mutation at position 412 (dTLR3L412F-dsRNA), and dsRNA-bound TLR3 dimer with a proline-to-leucine mutation at position 680 (dTLR3P680L-dsRNA). In TLR3, L412F polymorphism was associated with several human diseases, while the P680L mutation was found as one which reduces the binding affinity of dsRNA to TLR3 and affects subsequent signalling. The authors performed MD simulations together with PCA, RIN, hydrogen bond and protein-nucleic acid interaction analyses to investigate the global motions and the distribution of crucial residues for signal transduction. They claimed that apo wild-type pre-formed dimer is unlikely to be stable in physiological conditions. Thus, they proposed that TLR3 might exist as a monomer in a solution. Further, the interaction energies and hydrogen bonds analyses indicated that the mutations induced certain conformational changes which could disturb the TLR3 signalling. The interaction sites between TLR3 and dsRNA were observed at both the N-terminal and C-terminal ends of TLR3 LRR, while the dimerisation interface was confirmed at the C-terminal site but only for dTLR3WT-dsRNA and dTLR3L412F-dsRNA. It might suggest that P680 is crucial for maintaining the dimer interface for ligand binding.

Regarding the TIR domain, Mahita and Sowdhamini investigated the effect of key mutations on the conformational dynamics, based on TLR2 and TLR3[102]. For that, they used a combination of MD simulations, protein-protein interaction and protein structure network analyses. They carried out the analyses for eight different complexes, including not only wild-type and mutant dimers, but also wild-type and mutant trimers (TIR dimers with different adaptor proteins). They

highlighted the significant differences between the dimer interfaces of the wild type and mutant forms and also provided a possible explanation of how the introduced mutations may affect adaptor binding to the receptor. For the proline-to-histidine (P681H) mutation in the TIR domain of TLR2, they observed an increase in the stability of the TLR1-TLR2 heterodimer. This mutation also affected the surface of the putative adaptor-binding platform causing it to become slightly more curved. For the alanine-to-proline (A795P) mutation in the TIR domain of TLR3, they pointed out that individual subunits in a mutant tilt slightly more toward each other in comparison to the wild type. Such a subtle change may influence the orientation of the BB-loops (important for mediating interactions between dimer subunits) on the homodimer, and thus also the binding of the adaptor proteins - MyD88 and TRIF. The authors pointed out that the obtained results were based on the assumption that TLR2 and TLR3 TIR dimer adopt a similar conformation as that of the TLR10 TIR dimer crystal structure. As they admitted, this does not rule out the possibility of the dimers adopting a different TIR dimer conformation during signal transduction, e.g. an asymmetrical arrangement.

Ghosh et al.[103], showed that by applying the random alanine scanning mutation, it was possible to validate how much the residues from the BB- and DD-loops of the TIR domain contribute to TLR2 heterodimer complex formation. For that, the binding free energy (ΔΔGbinding) of the interface residues was computed. The residues with positive cut-off values > 0.5 kcal/mol were accepted as the residues of importance in the dimer stability for human TLR1-2 and TLR2-6. The authors concluded that for the hTLR1-TLR2 complex, three residues - Q97, N99, Y136 of TLR1, and two residues - E55, K62 of TLR2 impact the binding energy of the complex. For the hTLR2-TLR6 complex, the following residues were predicted to have a significant role: Y44, W45 of TLR2 and E159, K160 of TLR6. While combining the results of alanine scanning

mutation studies with sequence alignment, structure prediction and superimposition, molecular docking, and MD simulations, the authors presented two key conclusions. The first was that the subtle conformational variations in the TLR structures might play a crucial role during special circumstances. The second was that the role of TLR2 BB-loop residues and TLR1/TLR6 near-DD-loop residues is important for the process of heterodimerisation and for initiating differential downstream signalling.

In the summarised studies[100–103] authors showed that the analysis of mutations' effect can be helpful not only in studying the TLRs' structural dynamics but also in uncovering their mechanism of action, especially in the context of ligand or adaptor protein binding. However, we still have limited knowledge regarding the particular TLRs. Given the fact that many more mutations in TLRs are reported (e.g. in the UniProt or ClinVar[104] databases), more research should be carried out to clarify the effect of those substitutions.

Full-length TLRs

Due to the complexity of the TLR structure and the presence of the lipid bilayer, the study of the dynamics of the full-length receptor is difficult. However, some studies have been published in recent years and they provided important insights, especially regarding the possible structure rearrangement and mechanism of action of TLRs.

One of the first extensive studies of full-length TLR in a membrane-aqueous environment was the work by Patra et al.[105]. The authors focused on TLR4 (TLR4/MD2/LPS homo-heterodimer; TLR4 associated with MD2 protein and lipopolysaccharide LPS) and provided key insights into

the orientation and interaction of LRR (named ECD in the paper), TM, and TIR domains with respect to the dipalmitoylphosphatidylcholine (DPPC) bilayer. To reach these results, they successfully applied homology modelling methods, followed by protein-protein docking and MD simulations. Additionally, they used molecular docking and binding free energy calculations to get insight into the binding of the TAK-242 ligand with the TLR4-TIR dimer. They showed that each domain of TLR4 exhibits several structural transitions. The results revealed that LRR and TIR domains may be partially immersed in the membrane bilayer and that the TM domain tilts and bends to overcome the hydrophobic mismatch with the bilayer core. The authors claimed that the dynamic properties of TLR4-LRR had little effect on the interactions between LPS and MD2. For the TLR4-TM, the authors pointed out the possibility of an alternate dimerisation or a potential oligomerisation interface, as previously found for TLR3-TM[30]. Patra et al. also observed that the gradual absorption of the TLR4-TIR domain to the membrane leaflet could be a consequence of the electrostatic interactions and the bending/twisting actions of the LRR and TM domains. Their analyses indicated that even though TLR4-TIR surfaces are potentially membrane-absorbed, they also include the solvent-exposed part dedicated to interactions with other proteins. Thus, such a partial immersion is unlikely to prevent these segments from contacting the adaptor or other binding components. In the case of TLR4, the MyD88 adaptor protein is guided to TLR4-TIR by the membrane-anchored adaptor, TIR domain-containing adaptor protein (TIRAP). Hence, it is probable that the activated receptor complex TLR4/TIRAP/MyD88 is close to the membrane. For TAK-242, Patra et al. constructed two possible homodimerisation interfaces - first, where helix αC and the BB loop of both TIR subunits form the dimer interface, and second, where helix αC is exposed toward the solvent and places helix αE and the BB loop in between the dimer interface. Results obtained from estimated

binding free energy revealed that the first model - the αC-αC dimer had a greater binding affinity and that the affinity of TAK-242 for the αC-αC dimer was stronger than for αE-BB dimer. This could be an indication that the αC-αC/BB-BB model might represent the physiological dimeric interface of TLR4. However, the TAK-242 binding inside the TIR dimer cavity remains speculative, since in the case of separate simulation of full-length TLR4 as well as simulation of full-length TLR4 with TAK-242, the binding cavity of the ligand was partially blocked due to the rotation and upward movement of the TIR dimer.

In the following years, Matamoros-Recio et al.[106] also studied the full-length model of the agonist LPS-bound TLR4. They combined *ab initio* calculations with molecular docking, all-atom MD simulations, and thermodynamics calculations to provide the complete 3D models of the active TLR4 complex embedded into a membrane system. They showed that the interactions on different interfaces - TLR4/TLR4*, TLR4/MD-2*, and TLR4*/MD2 were kept within the simulations and that both subunits in the dimeric complex show a mutual stabilising role. Also, they confirmed that the transmembrane domain and the following hydrophobic region (HR) indicate plasticity, depending on the membrane composition. Such plasticity may determine the dimerisation of the intracellular domain. The authors proposed a few models of TM-TM* (named TD-TD* in the paper) and pointed out that TM-HR can adopt different conformations, thus changing the mode of dimerisation depending on the environment, regulated by TLR4 localisation. Matamoros-Recio et al. described two models for the TIR-TIR* dimer (named ID-ID* in the paper) - symmetrical and asymmetrical. In the first model, αC helix and the BB-loop in TIR domains were facing the dimerisation interface, while in the second model, the dimerisation interface was preserved in a head-to-tail way. The authors pointed out that both models were capable of binding the adaptor proteins. It could mean that the dimerisation

mechanism, and thus the receptor's activation depends on (among others) the membrane composition (localisation of TLR4) and structural rearrangement. They also showed that both symmetric and asymmetric TIR-TIR* models are suitable for MyD88-adapter-like (MAL) binding, supporting the hypothesis that both models could co-exist, and have a direct implication in the activation of distinct TLR4 pathways.

In their other work, Patra et al. studied the structure and dynamics of a full-length dimer of TLR3 immersed in a bilayer of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)[107]. They used a similar set of molecular modelling methods as in the case of TLR4 [69]. They studied three membrane-solvated complexes of the TLR3 homodimer bound with the dsRNA. Their analyses indicated that the TLR3-TIR homodimer built from the TLR6-TIR structure led to obtaining a full-length receptor structure with the stability necessary to maintain key intermolecular interactions with the ligand and with the membrane. Furthermore, they showed that flexible juxtamembrane loops of TLR3 allow for the simultaneous bending of the LRR and TIR domains on both surfaces of the membrane. They also observed that the complex immersed in the bilayer progressively tilted on the bilayer surface due to the electrostatic attraction between the charged parts of both the protein and phospholipids from the bilayer. In that case, the LRR-NT was only partially absorbed by the lipid headgroups. That was in contrast to the LRR-NT from their previously reported TLR4 model which was completely buried in the bilayer surface. They assumed that it is possible that the negatively charged dsRNA restricted the insertion of LRR-NT into the membrane surface. During the simulations, the dsRNA kept its structural integrity while bound to TLR3. The observed distortions in the TLR3-TM domain were distinct from the previously reported TLR4-TM. Thus, the authors concluded that the orientation and conformational changes of each TLR type may vary, depending on their location in the cell or

the lipid composition in the membrane. Based on the MD simulations analysis, Patra et al. indicated the probable interface involving residues from the αC and αD helix and the CD and DE loops of both TIR monomers. The BB-loop of one subunit was completely solvent-exposed, while the other was partially involved in dimer packing. The solvent-exposed part confirmed the importance of this segment in TRIF recruitment by the activated receptor.

The reviewed papers revealed important insight into TLRs dynamics. In summarised studies, authors presented relevant information on possible changes in position and conformation that receptors embedded in the cell membrane or intracellular compartments may undergo. Also, an important message regarding the potential mechanism of TIR domain dimerisation and binding of the adaptor protein came from the analysed models of both symmetrical and asymmetrical domains. This may be helpful for designing new types of TLR modulators, especially those targeting the TIR domain. One should remember that the presented studies referred only to TLR3 and TLR4, which means that for now, the conclusions can not be unified for all other receptors. As in the case of studying the effect of mutations, it seems that research regarding the dynamics of TLRs is just beginning. Considering the differences in TLR structure, substrate recognition, dimerisation requirements, and association with adaptor proteins, along with the importance of understanding the TLR signal transduction pathway, we can expect a significant increase in interest in this field in the coming years.

Conclusions

Toll-like receptors are one of the most crucial components of the immune system. Given their importance, it was not a surprise that the 2011 Nobel Prize in Physiology or Medicine was awarded to Dr Jules A. Hoffmann and Dr Bruce A. Beutler for their discoveries of the role of TLRs in innate immunity. It happened relatively quickly after the discovery of TLRs, only within 15 years. Since that time, tens of thousands of papers have been published in which TLRs have been the main subject of research. TLRs are complicated in terms of their structure, dynamics, and functioning, and this complexity is a challenge despite the enormous progress in the development of both experimental and computational methods. In our review, we aimed to highlight the progress made in recent years with the use of *in silico* methods for TLRs studies. Also, we wanted to point out the areas that still await their discoverers. One of the main limitations in understanding the function of TLRs is difficulty in the proper characterisation of receptor structure at various stages of signal transduction. Even the latest breakthrough in AI-based structure prediction is not yet widely used in research aimed at revealing the mechanism of action of TLRs.

Based on the results presented in the reviewed papers, we can conclude that still, the most attention is paid to the use of computational solutions for the design of small-molecule modulators. The use of *in silico* methods to design other types of modulators, such as multi-epitope vaccines, is gaining more popularity, but yet, it is not as common as in the case of small-molecule compounds. Both small-molecule and multi-epitope modulators are designed in such a way as to target the LRR of TLRs. There was no breakthrough in the design of small-molecule modulators targeting the TIR domain. Among things that scientists will want to keep improving

is obtaining the best binding affinity and stability of the modulators. Regarding the dynamics of TLRs, scientists have shown that studying the mutations' effect can contribute to a better understanding of the potential mechanism of action of the receptors. That is of special interest for both ligand and adaptor protein binding. More demanding, both in terms of system preparation and computing power, is the analysis of the dynamics of the full-length TLR complex. So far, only TLR3 and 4 have been built as full-length models embedded in the lipid bilayer. Those studies presented relevant information on possible conformational changes that may occur in the receptor's structure. Thus, it would be very important to perform similar studies for members of the TLR family. Since now we have easier access to the predictions of large macromolecule structures, we expect that in the coming years, we will witness progress in research on the TLRs' dynamics and mechanism of action.

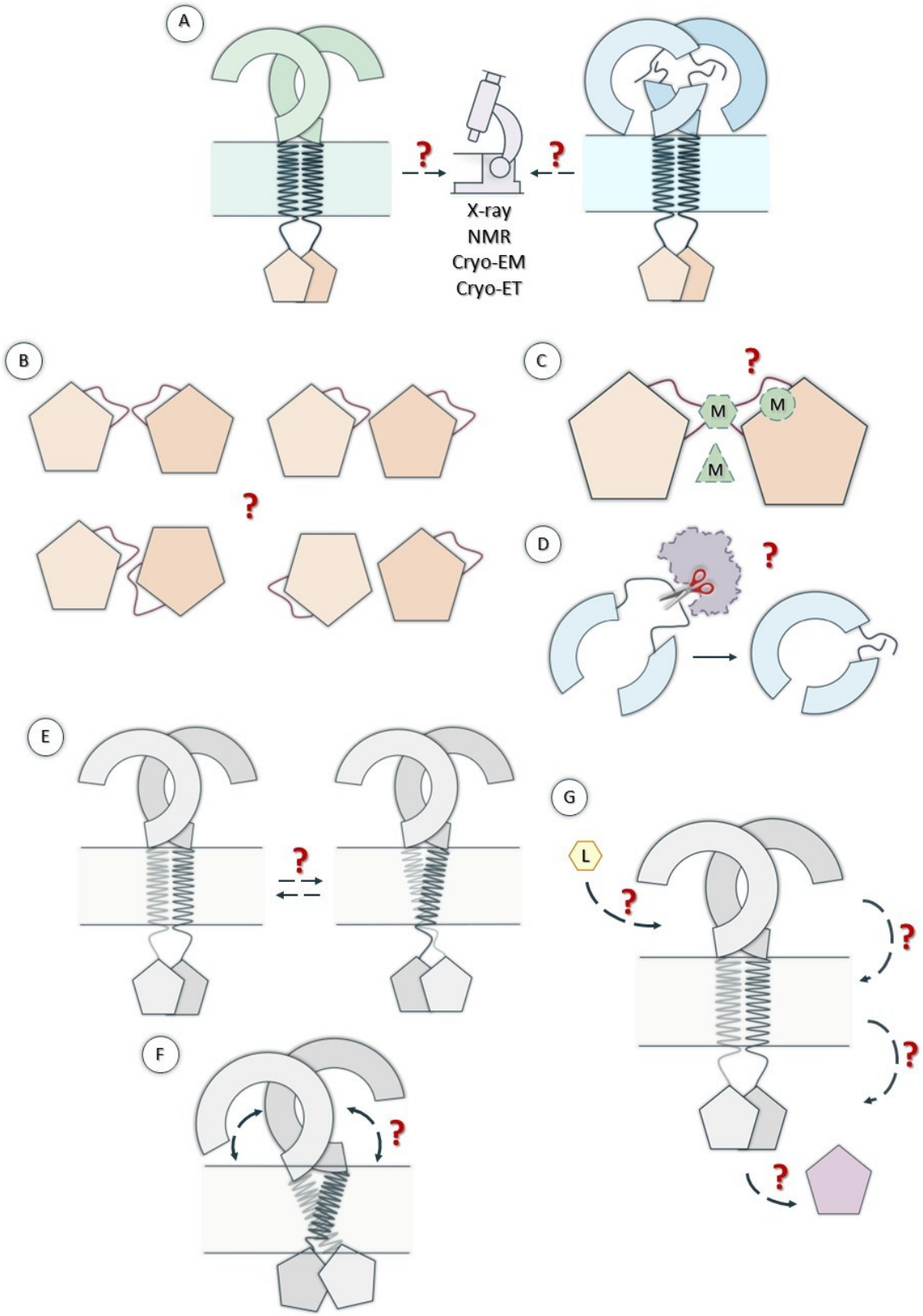
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Figure 5. Areas in TLR research that still require further development. **(A)** Experimental verification of the predicted structures **(B)** Studying the orientation of the subunits of the TIR domain dimers of TLRs. **(C)** Designing small-molecule modulators (M) targeting the TIR domain of TLRs. **(D)** Studying the proteolytic cleavage of the Z-loop in TLR7-9. **(E)** Analysing potential changes in the subunits dynamics in TLRs **(F)** Analysing the conformational changes and structural rearrangements in both TLR receptors and bilayer membrane. **(G)** Studying the whole process of ligand recognition through the signalling cascade to the immune response.

In **Figure 5** we presented the main areas in TLR research which still require further studies. **Figure 5A** illustrates the necessity of the experimental verification of the predicted structures. Despite the great progress in AI-based methods to predict the tertiary structures of macromolecules, experimental validation is a must to confirm the compliance of the obtained predictions. Access to experimentally-solved structures of transmembrane proteins is also important in order to confirm the orientation of individual domains or subunits of the structure towards each other. Obtaining information about the orientation of the subunits of the TIR domain dimers of TLRs is of special interest **(Figure 5B)**. So far, we have information about possible symmetrical or asymmetric orientations. However, we lack a systematic review of what orientations are preferred by specific receptors and how the orientation of the subunits can determine the binding of the adaptor proteins and the initiation of the signal cascade. This issue is also related to the design of small-molecule modulators targeting the TIR domain **(Figure 5C)**. Without details about the orientation of the subunits, it is difficult to properly select the best binding site for modulators.

As we mentioned in the introduction of this review, some TLRs (7-9) require the proteolytic cleavage of the Z-loop in their LRR domain **(Figure 5D)**. It is needed to allow ligands to bind and to further activate the receptor. Very little is known about the molecular basis of this process. Basically, only the information about the examples of proteases potentially involved in cleavage is available. To our best knowledge, there are no *in silico* studies attempting to explain this process. We are aware that one of the obstacles may be the size of the system and that no accurate structure predictions of the TLR-protease complex have been available so far. However, we hope that with the increase of the computational resources and the possibility to predict the structure of complexes using e.g. AlphaFold Multimer, this issue will be soon addressed.

In **Figures 5E** and **5F** we wanted to highlight the importance of conducting further research on the dynamics and conformational changes of TLRs. As we mentioned, studies presented to date have mainly focused on TLR3 and TLR4. Very little is known about other receptors, e.g. how the conformational changes occur in individual subunits or how full-length receptors behave in relation to the membrane in which they are immersed. In particular, we would like to know whether the location of the receptor (cell membrane or intracellular compartments) determines the TLRs' dynamics and the subsequent ability to bind the adaptor proteins. **Figure 5G** illustrates the ultimate goal of studying the Toll-like receptors with the use of computational methods which is to get deep insight into each stage of the receptor functioning. Thus, the challenge is to combine all the information, starting from the recognition of the ligand by the receptor, through the triggering of the signalling cascade, to the immune response.

Supporting Information

Supplementary Table S1. Overview of human Toll-like receptors domains deposited in the Protein Data Bank.

Supplementary Table S2. Chemical structures of the best hits (small-molecule agonists and antagonists) from the reviewed research papers.

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Notes

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Abbreviations

TLRs, Toll-like receptors; PRRs, pattern recognition receptors; MPs, molecular patterns; DAMPs, damage/danger-associated molecular patterns; MAMPs, microbial/microbe-associated molecular pattaerns; PAMPs, pathogen-associated molecular patterns; XAMPs, xenobiotic-associated molecular patterns; LRR, leucine-rich repeats domain; TM, transmembrane domain; TIR, Toll-interleukin-1 receptor domain; MyD88, myeloid differentiation primary-response protein 88; TRIF, TIR domain-containing adaptor protein inducing interferon-β; hTLRs, human Toll-like receptors; PDB, Protein Data Bank; VS, virtual screening; MD, molecular dynamics; MM-PBSA, Molecular Mechanics Poisson-Boltzmann Surface Area; MM-GBSA, Molecular Mechanics with Generalised Born and Surface Area; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; SAR, structure-activity relationship; MD2, myeloid differentiation factor 2; ADME/T, absorption, distribution, metabolism, excretion, toxicity properties; NMA, Normal Mode Analysis; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; MERS, Middle East respiratory syndrome; HCV, Hepatitis C virus; HIV, human immunodeficiency virus; NeoCoV, Neo-Coronavirus; MPL, monophosphoryl lipid A; S protein, SARS-CoV-2 spike glycoprotein; N protein, nucleocapsid protein; ORF1a, open reading frame 1a protein; MTB, *Mycobacterium tuberculosis*; OmpATb, outer membrane protein A Rv0899; HTL, human thymus lymphoid; IgM, immunoglobulin M; IgG, immunoglobulin G; PCA, principal component analysis; RIN, residue interaction network; SNPs, single nucleotide polymorphisms;

WT, wild type; LPS, lipopolysaccharide; DPPC, dipalmitoylphosphatidylcholine bilayer; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; TIRAP, TIR domain-containing adaptor protein;

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