

1 **Toll-like receptor 4 -**
2 **a multifunctional virus recognition receptor**

3 Elina Gerber-Tichet¹, Fabien P. Blanchet², Karim Majzoub¹ and Eric J. Kremer^{1*}

4
5 ¹Institut de Génétique Moléculaire de Montpellier, Université de Montpellier,
6 CNRS UMR 5535, 34090 Montpellier, France

7
8 ²Institut de Recherche en Infectiologie de Montpellier, Université de
9 Montpellier, CNRS UMR 9004, 34090 Montpellier, France

10 *Correspondence: eric.kremer@igmm.cnrs.fr

11
12 The authors declare no competing financial interests.

13 **Abstract**

14 Since the initial description of toll receptors in *Drosophila* and their mammalian counterparts
15 Toll-like receptors (TLRs), numerous fundamental and applied studies have explored their
16 crucial role as sensors of pathogen-associated molecular patterns (PAMPs). Among the ten
17 human TLRs, TLR4 is particularly well known for its ability to detect lipopolysaccharides (LPS),
18 a component of gram-negative bacterial cell wall. In addition to its archetypal functions, TLR4
19 is also a versatile virus sensor. This review provides a background on the discovery of TLR4
20 and how this knowledge laid a foundation for characterization of its diverse roles in antiviral
21 responses, examined through genetic, biochemical, structural, and immunological
22 approaches. These advances have led to a deeper understanding of the molecular functions
23 that enable TLR4 to orchestrate multi-nodal control by professional antigen-presenting cells
24 (APCs) to initiate appropriate and regulated antiviral immune responses.

25 **Keywords:** *TLR4, viruses, antigen-presenting cells, immune response, polymorphisms*

26 **Highlights**

- 27 ● TLR4 is a single-pass transmembrane protein whose structure and function as a PRR
28 are highly conserved in mammals
- 29 ● TLR4 can be located on the cell surface and/or within cytoplasmic vesicles
- 30 ● TLR4 participates in direct and indirect interactions with viruses and viral proteins to
31 trigger antiviral responses
- 32 ● In several instances, TLR4 polymorphisms can influence virulence
- 33 ● TLR4 agonist are being used to improve vaccine efficacy
- 34 ● Extracellular proteins, including antimicrobial peptides and danger associated
35 molecular patterns (DAMPs) impact TLR-mediated antiviral responses

36 **Milestones in toll-mediated immunity**

37 Until the end of the 20th century, most immunologists focused on the adaptive arm of the
38 immune system. In 1989, Charles Janeway proposed that in addition to the interaction of
39 specific ligands with somatically rearranged antigen receptors, lymphocytes require another
40 signal from antigen-presenting cells (APCs) [1]. Janeway predicted that such signals must be
41 the result of the recognition of microbial molecules via germline encoded pattern recognition
42 receptors (PRRs), as opposed to the refined antigen receptors of the adaptive immune system.
43 Janeway posited that such microbial components should be **i)** absent from the host to
44 distinguish between self and non-self, **ii)** conserved among a large number of pathogens, and,
45 **iii)** essential components of the microbe's lifecycle, preventing escape from recognition
46 through mutations [1]. The identification of *toll* genes in *Drosophila* and Toll-like receptor genes
47 in mammals opened the gates to understanding an evolutionarily ancient arm of host defense,
48 dubbed "innate immunity" [2,3]. Extensive research following the identification of TLRs
49 deciphered how pathogen-associated molecular patterns (PAMPs) such as bacterial, fungal,
50 viral nucleic acids, proteins or components induce signaling cascades that lead to inflammation
51 in vertebrate and invertebrate animals [4] (**Figure 1**).

52 **Archetypal role in bacterial sensing**

53 LBP, an extracellular LPS-binding protein, facilitates LPS extraction from bacterial outer
54 membrane forming an LPS-LBP complex. This complex is then recognized by soluble or
55 membrane-bound CD14, which shuttles it to an MD-2-TLR4 complex, leading to TLR4
56 dimerization (**Figure 2A**) [5,6]. Mechanistically, the contact of lipid A, a hydrophobic
57 component of LPS, with MD-2 induces the formation of TLR4 homodimers, leading to a
58 multilayered signaling cascade that eventually induces NF- κ B translocation to the nucleus and
59 the transcription of genes encoding pro-inflammatory cytokines (**Box 1**) [7]. In addition to LPS,
60 lipo-oligosaccharides and lipoteichoic acid (an anchor for peptidoglycans on some gram-
61 positive bacteria) interact with the TLR4-MD-2 complex on the surface of human immune cells
62 [8–10].

63 **Genetic evidence of the impact on viral infections**

64 At the turn of the century, Beutler and colleagues linked differences in responses to LPS to
65 **TLR4** single nucleotide polymorphisms (SNPs) [11,12]. These polymorphisms, likely caused
66 by selective pressure from pathogens, are found in the promoter, introns, and exons. Although
67 detailed analyses have not been performed, it is likely that SNPs outside the coding regions
68 modify TLR4 levels in APCs, while those within exons lead to changes in PAMP recognition
69 [13–15] (**Figure 2B**). **TLR4** SNPs, such as RS⁴⁹⁸⁶⁷⁹⁰ and RS⁴⁹⁸⁶⁷⁹¹ cause an aspartic acid to

70 glycine substitution at position 299, and isoleucine to threonine at 399, respectively, which alter
71 the formation of the TLR4/MD-2 complex [16]. The first epidemiological study that identified
72 SNP-virulence links concerned severe forms of bronchiolitis caused by respiratory syncytial
73 virus (RSV) [17] (see **Table 1** for a list of viruses and **Table 2** for virulence-associated SNPs).
74 Of recent interest, SARS CoV-2 and RS⁴⁹⁸⁶⁷⁹⁰ or RS⁴⁹⁸⁶⁷⁹¹ were associated with cytokine
75 storms, possibly via increased spike-TLR4 interactions or dysregulation of the TLR4 signaling
76 cascade [18]. To add another layer of complexity, the impact of RS⁴⁹⁸⁶⁷⁹⁰ and RS⁴⁹⁸⁶⁷⁹¹, may
77 be due to dominance of a guanine or cytosine, as the final base in the respective codon [19–
78 23]. In the case of human papillomavirus (HPV), transient TLR4 engagement leads to the
79 inhibition of the HPV genome integration, while chronic activation by high-mobility group box 1
80 protein (HMGB1), a by-product of HPV infection, may induce HPV-associated tumorigenesis
81 [24]. A study of Han women (the largest ethnic group in China), and women from Gujarat (one
82 of India's western states) showed that the risk of developing HPV-associated cervical cancer
83 was higher in carriers of RS⁷⁸⁷³⁷⁸⁴-G and RS¹⁹²⁷⁹¹¹, respectively [25,26].

84 **Direct or indirect interactions with TLR4/MD-2 complex**

85 The interactions between viruses and TLR4 can be loosely divided into i) direct interactions
86 (**Figure 2C**), ii) interactions via a bridging protein, or iii) detection of viral components during
87 propagation (**Figure 3**). Virus-receptor interactions are often context dependent, and therefore
88 drawing broad conclusions can be challenging because viruses tend to enter different cell
89 types using different receptors and mechanisms. Considering this, one needs to take into
90 account the possibility that virus can use multiple modes of entry, and therefore blocking one
91 pathway may have a modest impact on some readouts. Another caveat is that studies have
92 tended to focus on TLR4-virus interactions at the cell surface, while downplaying TLR4
93 interactions in endosomal vesicles.

94 **Direct interactions**

95 **Coronavirus** (e.g., SARS-CoV-2, NL63, 229E, OC43, and HKU1)

96 The immunopathology of COVID-19, is due, in part, to exacerbated expression of
97 proinflammatory cytokines like interleukin-6 (IL-6) and TNF, which are prototypic products of
98 TLR activation. Using *in silico* studies, Choudhury & Mukherjee suggested that the SARS-CoV-
99 2 spike glycoprotein has stronger interactions with TLR4 compared to TLR1 or TLR6
100 (respective binding energy values of -120 vs -57 or -68 kcal/mol) in docked protein complexes
101 [27]. Interestingly, high valued antigenic epitopes were at the interacting interface of the spike
102 protein and the TLR-binding region. Affinity-based interactions using surface plasmon
103 resonance were consistent with spike interacting with TLR4 with a K_d of 300 nM [28].

104 A handful of labs explored the impact of spike protein on the transcriptome of TLR4-expressing
105 human and rodent APCs [28–31]. RT-qPCR assays showed that SARS-CoV-2 spike induces
106 TLR4-associated transcription. The key assays included the use of TAK-242 or siRNA
107 knockdown of mouse TLR4. In addition to transcriptional responses, biochemical assays
108 showing modification of TNF, phosphorylated NF- κ B p65 and JNK p54 levels also supported
109 the conclusion that spike activates TLR4 [30]. In addition to the TLR-4-mediated response in
110 APCs, Li *et al.* found that seasonal coronaviruses (229E or OC43) seeded an NLRP3
111 inflammasome [32]. An inflammasome is a multiprotein platform containing a PRR that
112 promotes aggregation of ASC (apoptosis-associated speck-like protein containing a caspase
113 activation and recruitment domain). ASC recruits and auto-activates pro-caspase-1, which can
114 be followed by protease cleavage of the N terminus of gasdermin D, which initiates pore-
115 formation leading to the loss of plasma membrane integrity [33] (**Figure 2D**). Canonical
116 NLRP3-based inflammasome formation is typically preceded by a transcriptional priming event
117 that produces mRNAs of inflammasome components and cytokines [34]. However, in human
118 monocytes, LPS-TLR4 engagement induces an alternative inflammasome activation
119 characterized by IL-1 β release in the absence of a transcriptional priming event, inflammasome
120 aggregation, and pyroptotic cell death [33]. Li *et al.* concluded that 229E- and OC43-TLR4
121 priming induced inflammasome activation in PMA-differentiated THP-1 cells (where TAK-242
122 inhibited this cascade) [32].

123 While Zhao *et al.* used THP-1 cells (a human monocyte cell line) to identify SARS-CoV-2 spike-
124 TLR4 interactions (presumably in liaison with CD14 and MD-2 on the cell surface) and
125 signaling [28], van der Donk *et al.* proposed more nuanced conclusions [35]. Donk *et al.* found
126 that primary human monocyte-derived dendritic cells (moDCs) respond to SARS-CoV-2
127 nanoparticles (analyzing IP10 & IL-8 protein levels, **IL6**, **IL10**, **IFN β** mRNA, and phenotypic
128 readouts) only when infected by SARS-CoV-2 via ectopically expressed ACE2. These data led
129 to the conclusion that innate immune sensing in some cells occurs via cytosolic TLRs [35]. In
130 addition, van der Donk *et al.* also suggested that SARS-CoV-2 suppresses TLR4-induced
131 immunity via DC-SIGN (a C-type lectin receptor on DCs) via the Raf-1 pathway [36]. Finally, a
132 SARS-CoV2 spike-TLR4 axis was also challenged by Khan *et al.*, who found that all
133 inflammatory pathways were still activated in Tlr4^{-/-} murine bone marrow-derived macrophage
134 [37]. Importantly, neither TAK-242 nor TLR4 blocking antibodies were used in these latter
135 studies. Given the differences in species (mouse vs human), cell lines vs. primary cells,
136 agonists (spike proteins, nanoparticles, pseudotyped virions), and readouts, distilling a
137 simplified conclusion is unwarranted.

138 **Chikungunya** is a disease caused by the Chikungunya virus (CHIKV). CHIKV is transmitted
139 by *Aedes* mosquitoes, and therefore referred to as an arbovirus (arthropod-borne virus).
140 CHIKV can bind to cells by using several cell surface proteins including Eps15, TIM-1, DC-
141 SIGN, PHB1 & 2, and Mxra8, which may contain glycosaminoglycans as post-translational
142 modifications. Using *in vivo* and *in vitro* murine models, as well as human PBMCs, Mahish *et al.*
143 showed that TAK-242, anti-TLR4 Abs, or knockout of TLR4 prevented CHIKV-induced
144 increase in inflammatory cytokine production (TNF, IL-6, MCP-1) and reduced activation of
145 p38, JNK and NF- κ B signaling pathways and cell maturation, leading to decreased CHIKV
146 infection and prolonged survival of mice [38]. To complement the functional data, Mahish *et al.*
147 also used molecular docking to propose eleven polar interactions between the extracellular
148 domain of TLR4 (from aa 454-592) and CHIKV E2 protein (primarily between aa 303-315).

149 **Respiratory syncytial virus (RSV)**: Upon infection by RSV, syncytia (large multinucleated
150 "cell-like structures") arise due to the effect of RSV fusion protein (F) causing neighboring cell
151 membranes to merge. More than 20 years ago, Kurt-Jones *et al.* reported TLR4 involvement
152 in RSV sensing [39] by using recombinant F protein and neutralizing antibodies binding F
153 protein in TLR4^{-/-} and CD14^{-/-} mice [39]. Using *in silico* molecular docking simulations, Akagawa
154 *et al.* proposed that TLR4 binding affinity increases during pre- to post-fusion conformational
155 changes [40].

156 [Indirect TLR4 interactions via bridging molecules](#)

157 One complex challenge faced when exploring virus-receptor interactions is modelling and/or
158 understanding an *in vivo* environment. Relatively simplified *in vitro* assays, using cultured cells
159 in defined media, poorly take into account the impact of extra-cellular factors that could interact
160 with viral particles and influence binding and uptake.

161 The **Adenoviridae** family contains greater than 300 different types [41]. Much of the impetus
162 for characterizing adenovirus-induced immune responses has been their use as gene transfer
163 vectors and vaccines [42]. An initial clue that some adenovirus types interact with TLR4 came
164 from Amalfitano and colleagues [43]. They used transgenic mice to identify TRIF, and TRIF-
165 interacting TLRs, that differentially modulate vector-induced immune responses. In a
166 syngeneic approach, Doronin *et al.* show that factor X (FX) binding to some human adenovirus
167 types leads to activation of the TLR4/Myd88-TRIF pathway in murine macrophages [44]. Using
168 modified capsids that do not bind FX, they concluded that FX acts as a bridging molecule
169 between the capsid and TLR4 to induce an innate immune response. Paradoxically, human
170 adenovirus type 5, a benchmark of adenovirus-induced immunogenicity, poorly induces human
171 DC maturation and cytokine production [45].

172 In addition to coagulation factors, some adenovirus types interact with antimicrobial peptides
173 (AMPs). AMPs are innate immune effector molecules that can also promote the maturation of
174 APCs and amplify or synergize with innate responses [46]. Neutrophils are a rich source of
175 AMPs and, following a breach in tissue homeostasis, release their cytoplasmic content which
176 can be up to 20% AMPs. Lactoferrin and α -defensins are alarmins (a subset of AMPs) that
177 also impact innate and adaptive immune responses by activating PRRs in APCs [47]. In
178 epithelial cells, α -defensins impairs human adenovirus type 5, 12, and 35 infection by
179 stabilizing their vertices, thus preventing the disassembly of the capsid in endocytic vesicles
180 [48]. In two studies, Eichholz, Cheneau, Tran *et al.* found that lactoferrin and human neutrophil
181 defensin 1 bind, with micro- to nanomolar affinity, to the capsid of adenovirus type 5, 26, and
182 35. Then, via a bridging mechanism, the AMPs bind to TLR4 on the surface of human APCs
183 to facilitate AdV uptake [33,49]. Downstream of the adenovirus-AMP interactions with TLR4,
184 the APCs undergo maturation via the induction of an NLRP3-associated inflammasome.

185 **Detection of secreted viral proteins**

186 Similar to bacteria, virus propagation often involves active or passive release of intracellular
187 pathogen components into the extracellular environment. Consequently, viral proteins that may
188 not be exposed on the surface of viral particles can still act as TLR4 agonists.

189 The five **Dengue virus** (DENV) serotypes cause a spectrum of disease. During propagation,
190 a polyprotein precursor of ~3,400 aa is cleaved into three structural (C, prM and E) and seven
191 nonstructural proteins (NS1, 2A & B, 3, 4A & B, and 5). In 2015, Modhiran *et al.* demonstrated
192 that NS1, released from infected cells as soluble lipid-associated hexamer, activates TLR4 on
193 the surface of endothelial cells to increase vasculature leakage [50]. In addition to the
194 interaction with APCs, NS1 appears to be linked with thrombocytopenia by triggering TLR4-
195 mediated platelet activation [51].

196 There are six **Ebolavirus** (EBOV) species: Bundibugyo, Reston, Sudan, Taï Forest, Bombali,
197 and Zaire, with the latter causing the largest number of outbreaks and highest mortality rate.
198 EBOVs are filamentous-shaped particles with genomes that encode seven proteins: NP
199 (nucleoprotein), VP30, -35, -40 & 42 (matrix proteins), L (an RNA-dependent RNA
200 polymerase), and GP (which encodes two glycosylated spike proteins). NP, L, VP30 and -35
201 form the nucleocapsid containing the genome. During an active EBOV infection, GP, the key
202 protein in EBOV virulence, is shed from both cells and virions (the EBOV envelope is decorated
203 by the membrane-bound trimeric GP [52]) due to cleavage by ADAM17 (*A disintegrin and*
204 *metalloprotease 17*). Due to reading errors during transcription and error-prone post-
205 translational processes due to seven uracil residues, three GP variants are produced: i) full-

206 length type I transmembrane GP (pre-GP1,2), ii) a furin-cleaved pre-GP that creates GP1 and
207 GP2 to form a heterodimer that assembles into the trimeric 450-kDa spike at the surface [53],
208 and iii) a dimeric pre-shed GP, which is generated by “correct” transcription and post-
209 translational processes. Shed GP (sGP) molecules diffuse through the bloodstream and can
210 activate TLR4-expressing cells at distal locations [54]. sGP interacts directly with TLR4/MD-2
211 through the hydrophobic internal fusion loop to activate the signaling pathways leading to a
212 storm of pro-inflammatory and antiviral cytokines [55]. Harty and colleagues used EBOV-like
213 particles, in which they could selectively incorporate proteins, and analyzed cytokine
214 production from human cell lines to screen for an interaction with TLR4 [56].
215 Immunoprecipitation assays suggested an interaction between GP and TLR4, which was
216 consistent with TLR4-associated signaling cascade. Of note, the 150-residue mucin-like
217 domain of GP1,2 is heavily glycosylated, which may be associated with its ability to induce
218 cytokine storms and increase virulence [56]. Volchkov and colleagues found that extensive
219 enzymatic-based deglycosylation decreased GP-induced cytokine production [57], a finding
220 however recently challenged by Scherm *et al.* using non-glycosylated mutants of GP [55].
221 Using mice and recombinant GP as a ligand, Lai *et al.* also found a direct involvement of GP
222 in TLR4 activation [58].

223 Similar to EBOV, **Marburg virus** can cause hemorrhagic fever. Younan *et al.* showed that the
224 TLR4 antagonist eritoran prevented the death of mice following challenge with EBOV and
225 Marburg virus via reduced pro-inflammatory cytokine production and decreased systemic
226 levels of infiltrating neutrophils and CD4⁺ and CD8⁺ T cells [59]. Eritoran also protects EBOV-
227 infected mice by reducing the inflammatory effects of oxidized phospholipids, which interfere
228 with maturation of DCs, resulting in limited lymphocyte activation.

229 Cellular environments and cellular partners

230 Cellular environments

231 Although TLR4 is canonically found on a subset of immune myeloid cells, some tissue-resident
232 epithelial cells express low levels of TLR4 on their surface, which may contribute to organ and
233 tissue immunosurveillance. Bulk transcriptome analyses detect **TLR4** mRNA in many organs
234 and tissues [60], and data suggest that some tissues and cell types modulate TLR4 expression
235 levels in pathophysiological conditions [61]. Of note, the pattern of TLR4 mRNA and protein
236 expression *in situ* parallels those of CD14 and LY96 (MD-2). TLR4 location and levels are
237 relevant when considering the cellular environment and the impact on host inflammation and
238 viral infections. As such, TLR4 engagement by PAMPs or DAMPs can induce a MyD88-
239 dependent inflammatory response, as well as a MyD88-independent antiviral environment,

240 both of which impact host immune responses and virulence [62]. Massive engagement of TLR4
241 complexes should lead to a deleterious response (cytokine storm), while a fine-tuned TLR4-
242 mediated IFN-dependent response favors the host by abrogating virus propagation [63].

243 When TLR4 engagement increases virulence, then strategies to prevent TLR4 signaling might
244 be beneficial [63,64]. For example, blocking TLR4 signaling prior to infection by Tembusu virus
245 (TMUV), a flavivirus causing egg drop disease syndrome in birds, led to replication inhibition
246 [65]. Also, HTLV-I protein p30 may directly impact TLR4-dependent host immune tolerance or
247 anergy by interfering with activation and expression in macrophages [66]. Additionally, Wilks
248 and colleagues showed that mouse mammary tumor viruses (MMTV) can incorporate TLR4
249 into their envelope, which leads to an LPS-dependent enhancement of transmission [67].

250 *Extra- and intracellular partners*

251 An underappreciated aspect of TLR4 versatility is its different subcellular locations. While the
252 recognition of PAMPs by TLR4 certainly occurs at the cell surface, TLR4 can also traffic
253 through endosomal compartments to initiate interactions or signaling events that regulate
254 antiviral or pro-viral pathways via structural and spatiotemporal parameters [35,68]. The TLR4
255 complex can also interact with extracellular alarmins, leading to the amplification of
256 surrounding inflammation upon viral infections. Indeed, alarmins are passively or actively
257 secreted upon cellular stress or viral infections and can interact with inflammatory receptors
258 such as TLR4 or RAGE [69]. Interestingly, viral infections leading to severe inflammatory
259 disorders often rely on uncontrolled and excessive release of alarmins, like HMGB1 or
260 members of the S100 proteins family (which may aid as biomarkers of disease severity) [69].
261 This is the case of viral myocarditis caused by Coxsackievirus B3 and for which HMGB1 and
262 S100A8/S100A9 are involved [70]. Similarly, acute respiratory disease caused by RSV, SARS-
263 CoV1/2, or influenza virus A is associated with elevated levels of HMGB1 [71] and/or
264 S100A8/S100A9 [72], which can exacerbate TLR4-mediated cytokine production. There is
265 therefore a clinical interest to better detect and control the alarmin-TLR4 axis upon virus-
266 induced inflammatory disorders. Therefore, it is not only the TLR4-virus interaction, but also
267 associated elements and parameters such as TLR4 localization and conformation, protein
268 partners, and antigen presentation, that impact the inflammatory response.

269 **Impact on T- and B-cell responses**

270 *TLR4 and host antiviral cellular immunity*

271 TLR4-mediated immune responses are intimately linked to the adaptive immune system of
272 vertebrates. The involvement of TLR4 in adaptive immune responses was thought to mainly
273 rely on the adjuvant capacity of TLR4 activation that enhances functional maturation and

274 migratory capacities of APCs [73]. It became clear that TLR4-mediated control of CD4⁺ T cell
275 priming impacts inflammatory cytokines expression and overcoming suppressive effects from
276 regulatory T cells [74]. Interestingly, the strength of TLR4 stimulation might differentially
277 regulate the profile of T helper 1 cells (T_{h1}) towards T_{h2} responses, which could become
278 deleterious in a context of viral infection [75]. Furthermore, the engagement of TLR4 with
279 natural or engineered ligands influences adaptive immunity against HIV-1 [76,77] and LCMV
280 [78] infections.

281 Importantly, a better understanding of the dynamics between viral infections, TLR4 complex,
282 and host cellular immunity, will help identify strategies to enhance antiviral immunity and design
283 effective vaccines. TLR4 targeting is a potent option in vaccine development strategies against
284 viral infections. Promising *ex vivo* and *in vivo* data suggest that TLR4 engagement potentiates
285 humoral and cellular immune responses upon HBc-AdV-B7-VLPs immunization [79], and
286 confers resistance to HBV [80], and Marek's disease virus [81] infections - as well as inducing
287 robust mucosal immune responses [82] and even improving immunotherapeutic approaches
288 [83].

289 *TLR4 and host antiviral humoral immunity*

290 The interaction between TLR4 and B-lymphocyte responses is multifaceted. A seminal study
291 by Pasare and Medzhitov reported that the addition to CD4⁺ T-cell help, the generation of T
292 cell-dependent antigen-specific antibody responses require TLR4 activation in B cells [84].
293 Similarly, TLR4 is an intermediate immune effector between host innate and adaptive immune
294 responses against RSV [39]. This was demonstrated in a study uncovering that optimal specific
295 antibody responses were dependent on CD4⁺ T-cell help and triggering of TLR4 and TLR7 on
296 B cells and DCs [85]. Indeed, immunization with nanoparticles containing Yellow fever virus-
297 vaccine, plus ligands signaling through TLR4 and TLR7, protected birds from lethal avian,
298 swine influenza virus strains in mice, and induced robust immunity against pandemic H1N1
299 influenza in rhesus macaques [85].

300 These studies highlighted the concept that viral infections and virus-derived vaccines can
301 leverage TLR4-mediated signaling to enhance B-cell activation and promote the production of
302 protective antibodies. This was indeed the case in the context of HBc-HAdV7-VLPs
303 immunization [79], Enterovirus-71 virus-like particles [86], and adenovirus-based anthrax
304 vaccine [87]. A recent study showed that inflammatory signals upon immunization with
305 influenza A virus + LPS functioned as B-cell fate-determinants for the rapid generation of
306 protective antiviral extrafollicular responses [88]. TLR4-B-cell receptor co-engagement also
307 induces T cell-independent hypermutated, class-switched, neutralizing antibody responses

308 which could also be beneficial for antiviral vaccines [89]. Noteworthy, TLR4 effect on B-cell
309 responses could also be indirect and cell-mediated as concluded by Chen *et al.* when finding
310 that the recruitment of conventional type 2 DCs into secondary lymph organs initiated follicular
311 helper T differentiation, thus promoting the proliferation of germinal center B cells and the
312 production of RABV-specific IgGs and neutralizing antibodies [90].

313 **Concluding remarks**

314 TLR4 has yet to reveal all its functional secrets. Future work will need to i) characterize the
315 impact of SNPs on virulence, ii) explore its involvement in detecting other viruses (either
316 directly, indirectly or via bridging factors), iii) untangle the in vivo role DAMPs versus PAMPs
317 plays in inducing an antiviral environment, iv) identify the mechanism by which viral proteins
318 activate a MyD88-dependent or -independent response, and v) determine the most effective
319 ways to stimulate TLR4 with adjuvants in vaccines to generate broad and long-term T_{H1}
320 responses against emerging viruses, thus helping to tackle future pandemics [91].

321 **Acknowledgments**

322 We thank the members of our labs for constructive comments. We thank Carmen S Martin and
323 Pablo Herrera Nieto for help with TLR4 structure. The Kremer lab is supported by the European
324 Innovation Council Pathfinder (iAds, 101098647), Agency National Recherche (ANR-19-
325 CE37-0008-01, ANR-20-CE37-0019) Fondation pour la recherche Medicale
326 (ALZ201912009681), the Dravet Syndrome Foundation, and the Montpellier Université
327 d'Excellence program (MUSE). The Majzoub lab is supported by the European Research
328 Council Starting Grant (101039538 – DELV), the MUSE program and the ANR (DeZincRNA).
329 The Blanchet team is supported by the ANR (ANR-23-CE15-0028-03) and the ANRS (AO1-
330 2019-15236 and AO1-2022-22037). We apologize to our colleagues whose work was not cited
331 due to space limitations. EJK and FPB are Inserm Fellows.

332 **References**

- 333 1. Janeway, C.A. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring*
334 *Harb Symp Quant Biol* 54 Pt 1, 1–13
- 335 2. Hoffmann, J.A. *et al.* (1999) Phylogenetic perspectives in innate immunity. *Science* (1979) 284, 1313–1318
- 336 3. Palm, N.W. and Medzhitov, R. (2009) Pattern recognition receptors and control of adaptive immunity.
337 *Immunol Rev* 227, 221–233
- 338 4. Kawai, T. and Akira, S. (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med* 13, 460–469
- 339 5. Miyake, K. *et al.* (2000) Innate recognition of lipopolysaccharide by Toll-like receptor 4/MD-2 and
340 RP105/MD-1. *J Endotoxin Res* 6, 389–91
- 341 6. Shimazu, R. *et al.* (1999) MD-2, a Molecule that Confers Lipopolysaccharide Responsiveness on Toll-like
342 Receptor 4. *J. Exp. Med* 189, 1777–1782
- 343 7. Saitoh, S.-I. and Miyake, K. (2006) Mechanism regulating cell surface expression and activation of Toll-like
344 receptor 4. *Chem Rec* 6, 311–9
- 345 8. Nosratabadi, R. *et al.* (2017) Toll like receptor 4: an important molecule in recognition and induction of
346 appropriate immune responses against Chlamydia infection. *Comp Immunol Microbiol Infect Dis* 51, 27–33
- 347 9. Tapping, R.I. *et al.* (2000) Toll-Like Receptor 4, But Not Toll-Like Receptor 2, Is a Signaling Receptor for
348 Escherichia and Salmonella Lipopolysaccharides 1. *J Immunol* 165, 5780–5787
- 349 10. Takeuchi, O. *et al.* (1999) Differential Roles of TLR2 and TLR4 in Recognition of Gram-Negative and Gram-
350 Positive Bacterial Cell Wall Components. *Immunity* 11, 443–451
- 351 11. Dai, Q.X. *et al.* (2015) Sequence characterization and phylogenetic analysis of toll-like receptor (TLR) 4
352 gene in the Tibetan macaque (*Macaca thibetana*). *Genet Mol Res.* 14, 1875–1886
- 353 12. Smirnova, I. *et al.* (2001) Excess of rare amino acid polymorphisms in the Toll-like receptor 4 in humans.
354 *Genetics* 158, 1657–1664
- 355 13. Smirnova, I. *et al.* (2000) Phylogenetic variation and polymorphism at the toll-like receptor 4 locus (TLR4).
356 *Genome Biol* 1, 1–10
- 357 14. Figueroa, L. *et al.* (2012) The Asp 299 Gly Polymorphism Alters TLR4 Signaling by Interfering with
358 Recruitment of MyD88 and TRIF. *J Immunol.* 188, 4506–4515
- 359 15. Arbour, N.C. *et al.* (2000) TLR4 mutations are associated with endotoxin hyporesponsiveness in humans.
360 *Nat Genet* 25, 187–191
- 361 16. Yamakawa, N. *et al.* (2013) Human TLR4 polymorphism D299G/T399I alters TLR4/MD-2 conformation and
362 response to a weak ligand monophosphoryl lipid A. *Int Immunol* 25, 45–52
- 363 17. Puthothu, B. *et al.* (2006) TLR4 and CD14 polymorphisms in respiratory syncytial virus associated disease.
364 *Dis Markers* 22, 303–8
- 365 18. Taha, S.I. *et al.* (2021) Toll-like receptor 4 polymorphisms (896A/G and 1196C/T) as an indicator of COVID-
366 19 severity in a convenience sample of Egyptian patients. *J Inflamm Res* 14, 6293–6303
- 367 19. Jedlińska-Pijanowska, D. *et al.* (2020) Association between single nucleotide polymorphisms (SNPs) of IL1,
368 IL12, IL28 and TLR4 and symptoms of congenital cytomegalovirus infection. *PLoS One* 15, e0233096
- 369 20. Posadas-Mondragón, A. *et al.* (2020) Association of Genetic Polymorphisms in TLR3, TLR4, TLR7, and
370 TLR8 with the Clinical Forms of Dengue in Patients from Veracruz, Mexico. *Viruses* 12, DOI:
371 10.3390/v12111230
- 372 21. Sharma, S. *et al.* (2016) Analysis of TLR4 (Asp299Gly and Thr399Ile) gene polymorphisms and mRNA
373 level in patients with dengue infection: A case-control study. *Infect Genet Evol.* 43, 412–417
- 374 22. Ravi P. Arya, Nischay Mishra, Kakali Biswas, V.A.A. (2018) Association of Toll-like receptor 4 polymorphism
375 with hepatitis E virus-infected Indian patients. *J Viral Hepat* 25, 1617–1623
- 376 23. Wujcicka, W. *et al.* (2015) TLR9 2848 GA Heterozygotic Status Possibly Predisposes Fetuses and
377 Newborns to Congenital Infection with Human Cytomegalovirus. *PLoS One* 10, e0122831
- 378 24. Bahramabadi, R. *et al.* (2019) TLR4: An Important Molecule Participating in Either Anti-Human
379 Papillomavirus Immune Responses or Development of Its Related Cancers. *Viral Immunol* 32, 417–423
- 380 25. Jin, Y. *et al.* (2017) Association of toll-like receptor gene polymorphisms and its interaction with HPV
381 infection in determining the susceptibility of cervical cancer in Chinese Han population. *Mamm Genome.*
382 28, 213–219
- 383 26. Pandey, N.O. *et al.* (2019) Association of TLR4 and TLR9 polymorphisms and haplotypes with cervical
384 cancer susceptibility. *Sci Rep* 9, DOI: 10.1038/s41598-019-46077-z
- 385 27. Choudhury, A. and Mukherjee, S. (2020) In silico studies on the comparative characterization of the
386 interactions of SARS-CoV-2 spike glycoprotein with ACE-2 receptor homologs and human TLRs. *J Med*
387 *Viro* 92, 2105–2113
- 388 28. Zhao, Y. *et al.* (2021) Publisher Correction: SARS-CoV-2 spike protein interacts with and activates TLR4.
389 *Cell Res* 31, DOI: 10.1038/s41422-021-00501-0
- 390 29. Tsiloni, I. and Theoharides, T.C. (2023) Recombinant SARS-CoV-2 Spike Protein and Its Receptor Binding
391 Domain Stimulate Release of Different Pro-Inflammatory Mediators via Activation of Distinct Receptors on
392 Human Microglia Cells. *Mol Neurobiol* 60, 6704–6714
- 393 30. Shirato, K. and Kizaki, T. (2021) SARS-CoV-2 spike protein S1 subunit induces pro-inflammatory responses
394 via toll-like receptor 4 signaling in murine and human macrophages. *Heliyon* 7, e06187
- 395 31. Olajide, O.A. *et al.* (2022) SARS-CoV-2 Spike Glycoprotein S1 Induces Neuroinflammation in BV-2
396 Microglia. *Mol Neurobiol* 59, 445–458

397 32. Li, Y. *et al.* (2023) Seasonal coronavirus infections trigger NLRP3 inflammasome activation in macrophages
398 but is therapeutically targetable. *Antiviral Res* 216, 105674

399 33. Eichholz, K. *et al.* (2016) Immune-Complexed Adenovirus Induce AIM2-Mediated Pyroptosis in Human
400 Dendritic Cells. *PLoS Pathog* 12, e1005871

401 34. Eichholz, K. *et al.* (2022) Adenovirus- α -Defensin Complexes Induce NLRP3-Associated Maturation of
402 Human Phagocytes via Toll-Like Receptor 4 Engagement. *J Virol* 96, e0185021

403 35. van der Donk, L.E.H. *et al.* (2022) SARS-CoV-2 infection activates dendritic cells via cytosolic receptors
404 rather than extracellular TLRs. *Eur J Immunol* 52, 646–655

405 36. van der Donk, L.E.H. *et al.* (2023) SARS-CoV-2 suppresses TLR4-induced immunity by dendritic cells via
406 C-type lectin receptor DC-SIGN. *PLoS Pathog* 19, e1011735

407 37. Khan, S. *et al.* (2021) SARS-CoV-2 spike protein induces inflammation via TLR2-dependent activation of
408 the NF- κ B pathway. *Elife* 10, DOI: 10.7554/eLife.68563

409 38. Mahish, C. *et al.* (2023) TLR4 is one of the receptors for Chikungunya virus envelope protein E2 and
410 regulates virus induced pro-inflammatory responses in host macrophages. *Front Immunol* 14,
411 DOI: 10.3389/fimmu.2023.1139808

412 39. Kurt-Jones, E.A. *et al.* (2000) Pattern recognition receptors TLR4 and CD14 mediate response to
413 respiratory syncytial virus. *Nat Immunol* 1, 398–401

414 40. Akagawa, M. *et al.* (2022) Detailed Molecular Interactions between Respiratory Syncytial Virus Fusion
415 Protein and the TLR4/MD-2 Complex In Silico. *Viruses* 14, DOI: 10.3390/v14112382

416 41. Kremer, E.J. (2021) What is the risk of a deadly adenovirus pandemic? *PLoS Pathog* 17, DOI:
417 10.1371/journal.ppat.1004821

418 42. Kremer, E.J. and Nemerow, G.R. (2015) Adenovirus tales: from the cell surface to the nuclear pore
419 complex. *PLoS Pathog* 11, 10.1371/journal.ppat.1004821

420 43. Appledorn, D.M. *et al.* (2009) TRIF, and TRIF-interacting TLRs differentially modulate several adenovirus
421 vector-induced immune responses. *J Innate Immun* 1, 376–388

422 44. Doronin, K. *et al.* (2012) Coagulation factor X activates innate immunity to human species C adenovirus.
423 *Science* (1979) 338, 795–798

424 45. Eichholz, K. *et al.* (2015) Human Coagulation Factor X-Adenovirus Type 5 Complexes Poorly Stimulate an
425 Innate Immune Response in Human Mononuclear Phagocytes. *J Virol* 89, 2884–2891

426 46. Spadaro, M. *et al.* (2008) Lactoferrin, a major defense protein of innate immunity, is a novel maturation
427 factor for human dendritic cells. *The FASEB Journal* 22, 2747–2757

428 47. Spadaro, M. *et al.* (2014) Recombinant human lactoferrin induces human and mouse dendritic cell
429 maturation via Toll-like receptors 2 and 4. *The FASEB Journal* 28, 416–429

430 48. Smith, J.G. and Nemerow, G.R. (2008) Mechanism of adenovirus neutralization by Human alpha-defensins.
431 *Cell Host Microbe* 3, 11–19

432 49. Chéneau, C. *et al.* (2021) Lactoferrin Retargets Human Adenoviruses to TLR4 to Induce an Abortive
433 NLRP3-Associated Pyroptotic Response in Human Phagocytes. *Front Immunol* 12, DOI:
434 10.3389/fimmu.2021.685218

435 50. Modhiran, N. *et al.* (2015) Dengue virus NS1 protein activates cells via Toll-like receptor 4 and disrupts
436 endothelial cell monolayer integrity. *Sci Transl Med* 7, DOI: 10.1126/scitranslmed.aaa3863

437 51. Chao, C.-H. *et al.* (2019) Dengue virus nonstructural protein 1 activates platelets via Toll-like receptor 4,
438 leading to thrombocytopenia and hemorrhage. *PLoS Pathog* 15, DOI: 10.1371/journal.ppat.1007625

439 52. Lee, J.E. and Saphire, E.O. (2009) Ebola virus glycoprotein structure and mechanism of entry. *Future Virol*
440 4, 621–635

441 53. Jeffers, S.A. *et al.* (2002) Covalent modifications of the ebola virus glycoprotein. *J Virol* 76, 12463–12472

442 54. Jain, S. *et al.* (2021) Structural and Functional Aspects of Ebola Virus Proteins. *Pathogens* 10, DOI:
443 10.3390/pathogens10101330

444 55. Scherm, M.J. *et al.* (2022) Activation of Toll-like receptor 4 by Ebola virus-shed glycoprotein is direct and
445 requires the internal fusion loop but not glycosylation. *Cell Rep* 41, 111562

446 56. Okumura, A. *et al.* (2010) Interaction between Ebola virus glycoprotein and host toll-like receptor 4 leads to
447 induction of proinflammatory cytokines and SOCS1. *J Virol* 84, 27–33

448 57. Escudero-Pérez, B. *et al.* (2014) Shed GP of Ebola Virus Triggers Immune Activation and Increased
449 Vascular Permeability. *PLoS Pathog* 10, DOI: 10.1371/journal.ppat.1004509

450 58. Lai, C.Y. *et al.* (2017) Ebola virus glycoprotein induces an innate immune response in vivo via TLR4. *Front*
451 *Microbiol* 8, 271734

452 59. Younan, P. *et al.* (2017) The toll-like receptor 4 antagonist eritoran protects mice from lethal filovirus
453 challenge. *mBio* 8, DOI: 10.1128/mBio.00226-17

454 60. Zarembek, K.A. and Godowski, P.J. (2002) Tissue Expression of Human Toll-Like Receptors and
455 Differential Regulation of Toll-Like Receptor mRNAs in Leukocytes in Response to Microbes, Their
456 Products, and Cytokines. *J Immunol* 168, 554–561

457 61. Hu, J. *et al.* (2021) Differential Expression of the TLR4 Gene in Pan-Cancer and Its Related Mechanism.
458 *Front Cell Dev Biol* 9, DOI: 10.3389/fcell.2021.700661

459 62. Gauthier, A.E. *et al.* (2022) Lipopolysaccharide detection by the innate immune system may be an
460 uncommon defence strategy used in nature. *Open Biol* 12, DOI: 10.1098/rsob.220146

- 461 63. Olejnik, J. *et al.* (2018) Toll-like receptor 4 in acute viral infection: Too much of a good thing. *PLoS Pathog*
462 14, e1007390
- 463 64. Shirey, K.A. *et al.* (2021) Targeting TLR4 Signaling to Blunt Viral-Mediated Acute Lung Injury. *Front*
464 *Immunol* 12, 10.3389/fimmu.2021.705080
- 465 65. Wu, Z. *et al.* (2022) Toll-like receptor 4 and lipopolysaccharide from commensal microbes regulate
466 Tembusu virus infection. *J Biol Chem.* 298, DOI: 10.1016/j.jbc.2022.102699
- 467 66. Datta, A. *et al.* (2006) The HTLV-I p30 Interferes with TLR4 Signaling and Modulates the Release of Pro-
468 and Anti-inflammatory Cytokines from Human Macrophages*. *J Biol Chem.* 281, 23414–23424
- 469 67. Wilks, J. *et al.* (2015) Mammalian Lipopolysaccharide Receptors Incorporated into the Retroviral Envelope
470 Augment Virus Transmission. *Cell Host Microbe* 18, 456–462
- 471 68. Gangloff, M. (2012) Different dimerisation mode for TLR4 upon endosomal acidification? *Trends Biochem*
472 *Sci* 37, 92–98
- 473 69. Yang, D. *et al.* (2017) Alarmins and immunity. *Immunol Rev* 280, 41–56
- 474 70. Müller, I. *et al.* (2017) Pathogenic Role of the Damage-Associated Molecular Patterns S100A8 and S100A9
475 in Coxsackievirus B3-Induced Myocarditis. *Circ Heart Fail* 10, e004125
- 476 71. Chen, R. *et al.* (2020) HMGB1 as a potential biomarker and therapeutic target for severe COVID-19. *Heliyon*
477 6, e05672
- 478 72. Guo, Q. *et al.* (2021) Induction of alarmin S100A8/A9 mediates activation of aberrant neutrophils in the
479 pathogenesis of COVID-19. *Cell Host Microbe* 29, 222–235.e4
- 480 73. Medzhitov, R. *et al.* (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive
481 immunity. *Nature* 388, 394–397
- 482 74. Pasare, C. and Medzhitov, R. (2004) Toll-Dependent Control Mechanisms of CD4 T Cell Activation.
483 *Immunity* 21, 733–741
- 484 75. Sacramento, L.A. *et al.* (2020) TLR4 abrogates the Th1 immune response through IRF1 and IFN- β to
485 prevent immunopathology during L. infantum infection. *PLoS Pathog* 16, e1008435
- 486 76. Blanchet, F.P. *et al.* (2010) Human immunodeficiency virus-1 inhibition of immunoamphisomes in dendritic
487 cells impairs early innate and adaptive immune responses. *Immunity* 32, 654–669
- 488 77. Cardinaud, S. *et al.* (2017) Triggering of TLR-3, -4, NOD2, and DC-SIGN reduces viral replication and
489 increases T-cell activation capacity of HIV-infected human dendritic cells. *Eur J Immunol* 47, 818–829
- 490 78. Siddiqui, S. *et al.* (2011) TLR engagement prior to virus infection influences MHC-I antigen presentation in
491 an epitope-dependent manner as a result of nitric oxide release. *J Leukoc Biol* 89, 457–468
- 492 79. Li, Y. *et al.* (2023) Human adenovirus type 7 virus-like particle vaccine induces Dendritic cell maturation
493 through the TLR4/NF- κ B pathway and is highly immunogenic. *Antiviral Res* 212, 105559
- 494 80. Wang, Q. *et al.* (2022) Proliferation of CD11b+ myeloid cells induced by TLR4 signaling promotes hepatitis
495 B virus clearance. *Cytokine* 153, 155867
- 496 81. Parvizi, P. *et al.* (2014) The effects of administration of ligands for Toll-like receptor 4 and 21 against Marek's
497 disease in chickens. *Vaccine* 32, 1932–1938
- 498 82. Bakkari, M.A. *et al.* (2021) Toll-like Receptor-4 (TLR4) Agonist-Based Intranasal Nanovaccine Delivery
499 System for Inducing Systemic and Mucosal Immunity. *Mol Pharm* 18, 2233–2241
- 500 83. Wang, Y. *et al.* (2019) TLR4 signaling improves PD-1 blockade therapy during chronic viral infection. *PLoS*
501 *Pathog* 15, e1007583
- 502 84. Pasare, C. and Medzhitov, R. (2005) Control of B-cell responses by Toll-like receptors. *Nature* 438, 364–
503 368
- 504 85. Kasturi, S.P. *et al.* (2011) Programming the magnitude and persistence of antibody responses with innate
505 immunity. *Nature* 470, 543–547
- 506 86. Lin, Y.-L. *et al.* (2014) Enterovirus-71 virus-like particles induce the activation and maturation of human
507 monocyte-derived dendritic cells through TLR4 signaling. *PLoS One* 9, e111496
- 508 87. Li, R. *et al.* (2018) Toll-like receptor 4 signalling regulates antibody response to adenoviral vector-based
509 vaccines by imprinting germinal centre quality. *Immunology* 155, 251–262
- 510 88. Lam, J.H. and Baumgarth, N. (2023) Toll-like receptor mediated inflammation directs B cells towards
511 protective antiviral extrafollicular responses. *Nat Commun* 14, 3979
- 512 89. Rivera, C.E. *et al.* (2023) Intrinsic B cell TLR-BCR linked coengagement induces class-switched,
513 hypermutated, neutralizing antibody responses in absence of T cells. *Sci Adv* 9, eade8928
- 514 90. Chen, C. *et al.* (2021) Toll-Like Receptor 4 Regulates Rabies Virus-Induced Humoral Immunity through
515 Recruitment of Conventional Type 2 Dendritic Cells to Lymph Organs. *J Virol* 95, e0082921
- 516 91. Sartorius, R. *et al.* (2021) Exploiting viral sensing mediated by Toll-like receptors to design innovative
517 vaccines. *NPJ Vaccines* 6, 1–15
- 518 92. Martínez-Gómez, L.E. *et al.* (2023) Implication of myddosome complex genetic variants in outcome severity
519 of COVID-19 patients. *J Microbiol Immunol Infect.* DOI: 10.1016/j.jmii.2023.06.002
- 520 93. Sahanic, S. *et al.* (2023) SARS-CoV-2 activates the TLR4/MyD88 pathway in human macrophages: A
521 possible correlation with strong pro-inflammatory responses in severe COVID-19. *Heliyon* 9, e21893
- 522 94. Kagan, J.C. *et al.* (2008) TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-
523 beta. *Nat Immunol* 9, 361–368
- 524 95. Li, K. *et al.* (2005) Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-
525 like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci U S A.* 102, 2992–2997

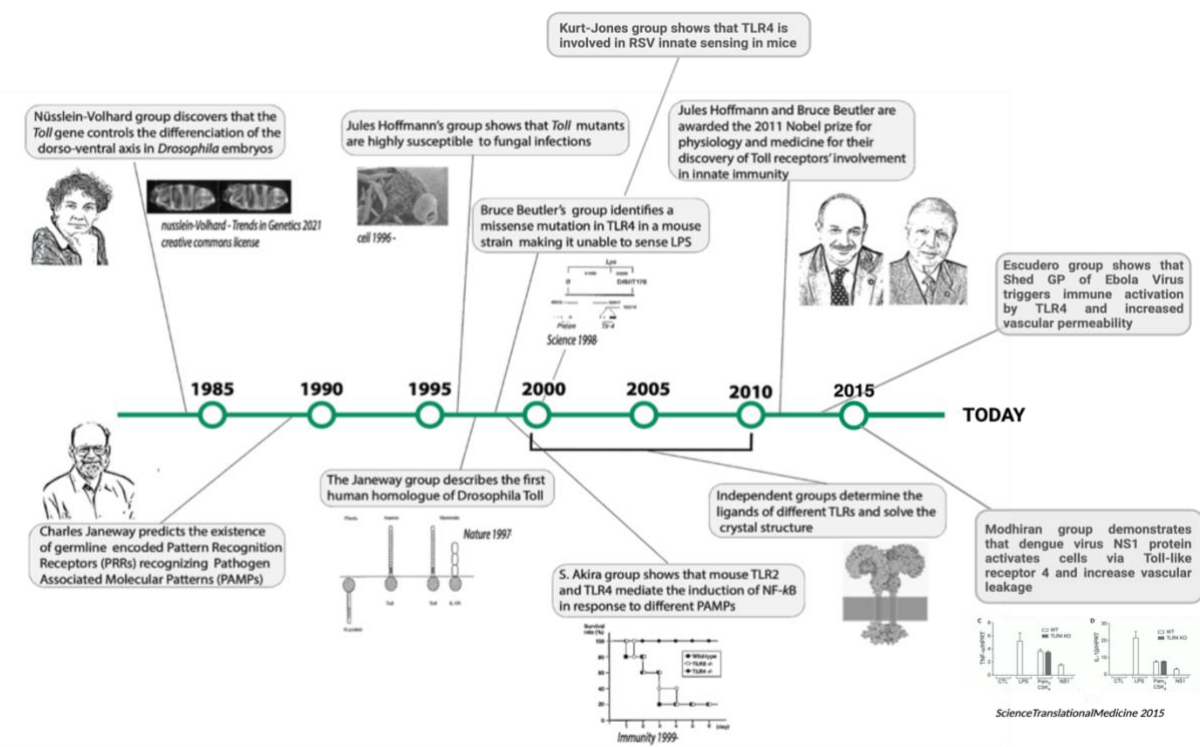
- 526 96. Lysakova-Devine, T. *et al.* (2010) Viral Inhibitory Peptide of TLR4, a Peptide Derived from Vaccinia Protein
527 A46, Specifically Inhibits TLR4 by Directly Targeting MyD88 Adaptor-Like and TRIF-Related Adaptor
528 Molecule. *J Immunol.* 185, 4261–4271
- 529 97. Bonham, K.S. *et al.* (2014) A Promiscuous Lipid-Binding Protein Diversifies the Subcellular Sites of Toll-
530 like Receptor Signal Transduction. *Cell* 156, 705–716
- 531 98. Chen, Z. *et al.* (2020) Correlation between TLR4 gene polymorphism and severe enterovirus 71 infection.
532 *J Infect Chemother.* 26, 1015–1020
- 533 99. Vidyant, S. *et al.* (2019) A single-nucleotide polymorphism in TLR4 is linked with the risk of HIV-1 infection.
534 *Br J Biomed Sci* 76, 59–63
- 535 100. Kim, Y.-C. and Jeong, B.-H. (2020) Strong Association of the rs4986790 Single Nucleotide Polymorphism
536 (SNP) of the Toll-Like Receptor 4 (TLR4) Gene with Human Immunodeficiency Virus (HIV) Infection: A
537 Meta-Analysis. *Genes (Basel)* 12, DOI: 10.3390/genes12010036
- 538 101. Pandey, N.O. *et al.* (2019) Association of TLR4 and TLR9 polymorphisms and haplotypes with cervical
539 cancer susceptibility. DOI: 10.1038/s41598-019-46077-z
- 540 102. Al-Qahtani, A.A. *et al.* (2014) The Association of Toll-Like Receptor 4 Polymorphism with Hepatitis C Virus
541 Infection in Saudi Arabian Patients. *Biomed Res Int* 2014, 1–9
- 542 103. Sghaier, I. *et al.* (2019) TLR3 and TLR4 SNP variants in the liver disease resulting from hepatitis B virus
543 and hepatitis C virus infection. *Br J Biomed Sci* 76, 35–41

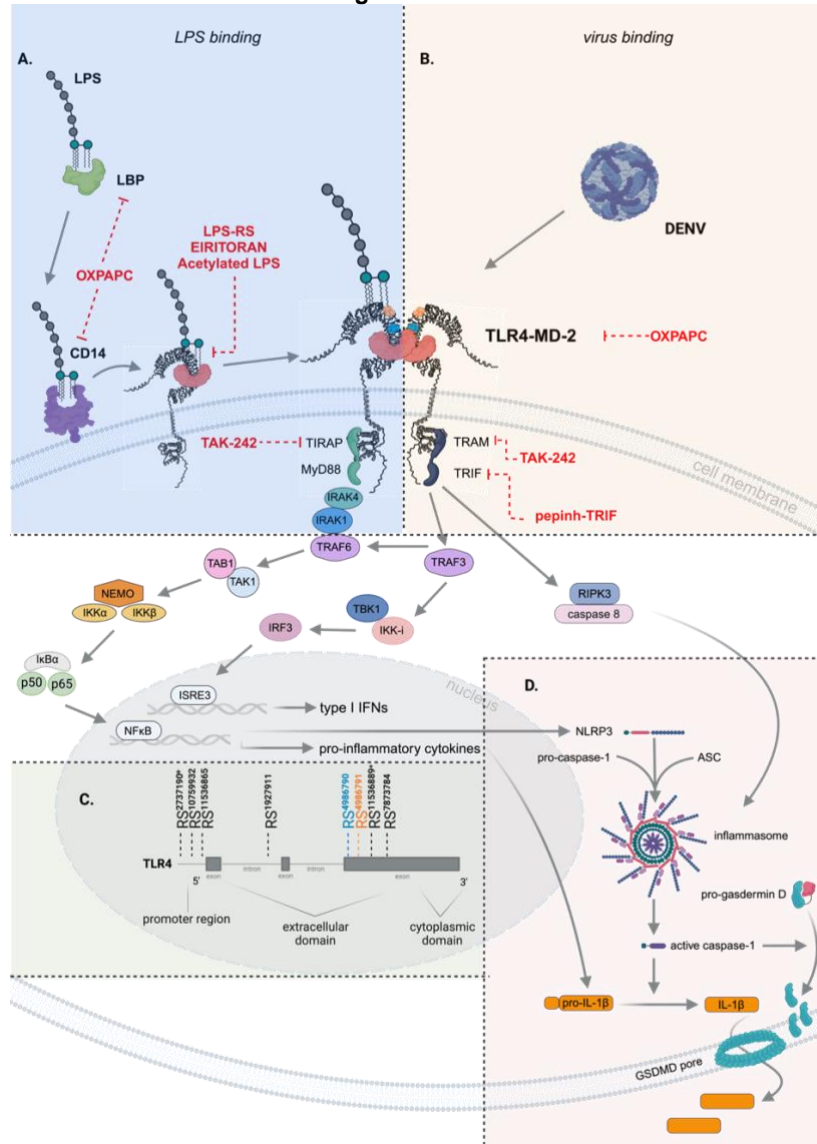
544

545

546 **Figure 1. toll and the Toll-like receptors milestones**

547 In the early 1980's Nüsslein-Volhard and colleagues found that the toll is involved in the differentiation of the dorso-
 548 ventral axis of the drosophila embryo. In 1989, Janeway posited the "pattern recognition hypothesis," suggesting
 549 that the immune system distinguishes between self and non-self through the recognition of conserved molecular
 550 patterns on pathogens through PRRs. In the mid-1990's, Hoffmann and colleagues showed that toll mutant flies
 551 succumb rapidly to challenge with *Aspergillus fumigatus*, an opportunistic fungus. The first mammalian Toll-like
 552 gene was described in 1997. Shortly thereafter, Beutler and colleagues demonstrated that TLRs carry immune
 553 functions, linking them to the sensing of PAMPs. Beutler and colleagues showed that mice that harbor missense
 554 mutations in TLR4 are unable to sense LPS. A year later, further generation and characterization of knockout mutant
 555 mice for TLR4 by Akira and colleagues showed that mammalian TLRs induce NF-κB-dependent responses to
 556 different PAMPs. In the decade that followed, the "innate immunity" field accelerated the pace of discovery, as the
 557 microbial ligands of several mammalian TLRs were identified. This decade included the seminal study by Kurt-
 558 Jones and colleagues showing that TLR4 is involved in the detection of RSV. The Nobel prize in Physiology and
 559 Medicine in 2011 "for the discoveries concerning the activation of innate immunity" recognized not only fundamental
 560 research, but also its far-reaching implications concerning vaccine design against infectious agents and
 561 development of therapies against cancer and immune-related diseases. Parallel to functional data, the atomic
 562 structure led to a molecular understanding of the different modes of PAMP binding to the ectodomains of TLRs,
 563 their effect on the conformation of the cytoplasmic TIR domain, and subsequent signaling cascades. Over the last
 564 10 years, TLR4 interactions with viruses (including Dengue, Ebola, SARS-CoV-2 and adenovirus) have helped us
 565 understand virulence and pathogenicity.





567

568 **A)** The archetypal role of TLR4: LPS detection is initiated by LBP and then LPS is transferred to membrane-bound
 569 CD14, prior to engaging the TLR4-MD2 complex and inducing TLR4 dimerization, which activates the TIRAP-
 570 MyD88 signaling cascade. This cytoplasmic signaling pathway (from IRAK4-p65) eventually induces the
 571 translocation of NF-κB and the expression of proinflammatory cytokines and, in some cases, **D)** inflammasome
 572 components (e.g., NLRP3, caspase-1, ASC).

573 **B)** While TLR4-virus (e.g., DENV) interactions also activate the TIRAP-MyD88 signaling cascade, the bivalent
 574 TRAM-TRIF pathway also leads to the upregulation of type-I IFNs upon IRF3 activation, which may be more relevant
 575 for antiviral responses. The formation of an inflammasome leads to caspase-1 autoactivation, cleavage of pro-
 576 GSDMD to GSDMD to create oligomeric pores, and cleavage of pro-IL1β to release IL-1β via the pores.
 577 Pharmacological inhibitors (in red) can be used to identify and inhibit TLR4 interactions or the downstream signaling
 578 cascade. TAK-242 is a cyclohexane derivative that efficiently inhibits the TLR4 pathway. TAK-242 binds cysteine
 579 residue 747, located intracellularly, and prevents TIRAP and TRAM engagement. Other TLR4 antagonists include
 580 acetylated LPS and LPS-RS from O111:B4, which bind to the hydrophobic pocket of MD-2. Similarly, eritoran
 581 tetrasodium is a lipid A analog that binds to MD-2, blocking the interaction of LPS with the TLR4-MD-2 complex
 582 and subsequent dimerization and activation. OxpAPC competes with LPS-binding protein (LBP), CD14, and MD-
 583 2. Finally, pepinh-TRIF interferes with TLR4-TRIF binding.

584 **C)** Single nucleotide polymorphisms (SNPs) in *TLR4*, located in the enhancer/promoter, coding, or noncoding
 585 regions, can influence TLR4 expression levels, its conformation, and binding partners. As examples, RS⁴⁹⁸⁶⁷⁹⁰ (blue)
 586 and RS⁴⁹⁸⁶⁷⁹¹ (orange) are illustrated because their locations in the coding region have been linked to altered
 587 virulence of some microbes.

588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629

Box 1. Divergent versus conventional pathways

TLR4-mediated signaling can transit through two distinct pathways involving TIRAP/MyD88 and TRIF/TRAM (TICAM1/2) adaptor proteins, which induce the production of proinflammatory cytokines and antiviral type-I interferon responses respectively. A simplistic interpretation suggests that the conventional TLR4-TIRAP-MyD88 pathway leads to uncontrolled NF- κ B-dependent inflammation, while the TLR4-TRIF-TRAM pathway initiated through endosomal trafficking leads to IRF3-dependent transcription of antiviral genes.

The TLR4-TIRAP-MyD88 pathway

Upon ligand binding to TLR4, MyD88 is recruited via the bridging factor TIRAP (Mal) to the TLR4 TIR domain which associates and transiently activates dynamic and concerted signalling through factors like interleukin-1 receptor-associated kinase 4 (IRAK1) and IRAK1/2, nucleating the formation of a supramolecular organizing center (SMOC) coined "myddosome". The myddosomes then co-ordinate TLR-mediated inflammatory response upon association with TNF receptor-associated factor 6 (TRAF6). Then, polyubiquitination allows TRAF6 to activate the I κ B complex and transforming growth factor- β -activated kinase 1 (TAK1) leading to the activation and nuclear translocation of NF- κ B and AP-1 transcription factor subunits Jun/Fos and subsequent induction of expression of proinflammatory cytokines and other immune response-related genes. Conventional TLR4-mediated signaling pathway has been most often tagged for its implication in virus-associated severe pathogenesis whether due to genetic regulations or polymorphisms of myddosome-associated factors [92], or to a direct effect of viral proteins on this pathway [93].

The TLR4-TRIF-TRAM pathway

TLR4 can also drive a MyD88-independent TRAM/TRIF-mediated pathway upon endocytosis and alternative dimerization state of the receptor for which CD14 is essential. The involvement of the TRIF-related adaptor molecule (TRAM) is essential to recruit the adapter protein TRIF to the TLR4-TIR domain and form another SMOC called "Trifosome". The particularity of the TRIF-dependent pathway relies on its connection to other signaling kinases like TANK-binding kinase 1 (TBK1) and inducible I κ B kinases (I κ B α , I κ B β and I κ B γ) leading to the phosphorylation and nuclear translocation of the activated interferon regulatory factor 3 (IRF3) required to induce type-I IFN thus establishing an antiviral state [94].

Overlapping pathways

These pathways are not mutually exclusive and some viruses impact both (e.g., Tat of HIV-1 [120]) or induce non-canonical or alternative pathways: e.g., VSV glycoprotein-G induces a TRIF-independent IFN-I response in DCs and macrophages, and SARS-CoV M protein promotes IFN- β response in immortalized epithelial cells in a TRAF3-independent manner. Interestingly, because TRAM and TRIF may regulate the NF- κ B pathway and control pro-inflammatory cytokines expression upon TLR4 stimulation, this pathway appears central in fine-tuning the amplitude of inflammatory and antiviral responses. As such, the TLR4-TRIF-TRAM pathway needs to be tightly regulated as reported for cellular factors like Sterile Alpha and TIR Motif Containing 1 (SARM1), or TRAM adaptor with GOLD domain (TAG). Given its critical role in establishing the antiviral response, this pathway is also impacted during viral infections as shown for hCMV, HCV [95] or the vaccinia virus [96].

The balance between TLR4-mediated proinflammatory and antiviral signaling is crucial to mount effective host defense, but also to limit virus-driven immunopathologies caused by excessive inflammation. It remains to better determine the mechanisms at play upon viral infections leading to selective modulation of TLR4-mediated signaling hubs as well as potential viral components able to modify one pathway over the other and therefore impacting innate and adaptive antiviral responses. In this context, Bonham *et al.* concluded that the promiscuity of lipid binding of the sorting adaptor TIRAP could explain the diversity in subcellular localization of TLR4 signaling hubs and virus detection via endosomal TLRs [97].

630 **Table 1. Glossary of viruses**

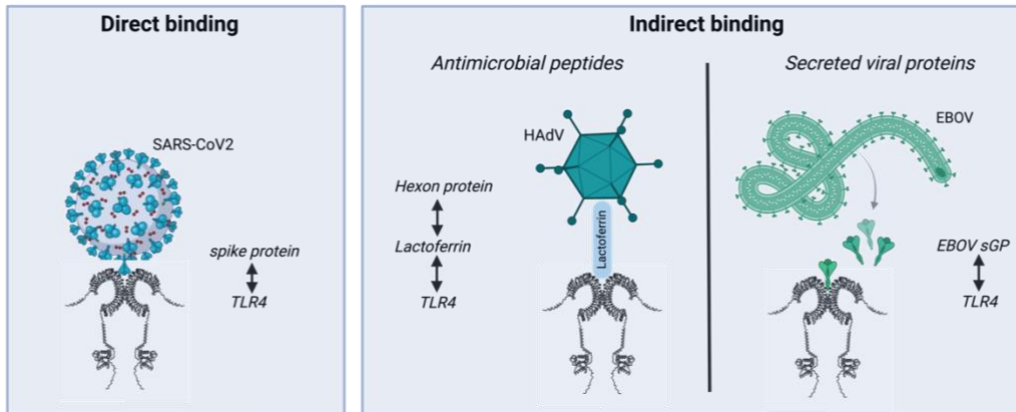
Genus	Family	Genome*	~size (kb)	Structure
Adenovirus	<i>Adenoviridae</i>	ds DNA	28 to 42	non-enveloped
Chikungunya virus	<i>Togaviridae</i>	+ss RNA	11.7	enveloped
Coxsackievirus B3	<i>Picornaviridae</i>	+ss RNA	7.3	non-enveloped
Dengue virus	<i>Flaviviridae</i>	+ss RNA	11	enveloped
Ebolavirus	<i>Filoviridae</i>	-ss RNA	19	enveloped
Enterovirus A71	<i>Picornaviridae</i>	+ss RNA	7.5	non-enveloped
Foot & mouth disease virus	<i>Picornaviridae</i>	+ss RNA	8.3	non-enveloped
Human T-lymphotropic virus 1	<i>Retroviridae</i>	+ss RNA	9	enveloped
Hepatitis B virus	<i>Hepadnaviridae</i>	ds DNA	3.2	enveloped
Hepatitis C virus	<i>Flaviviridae</i>	+ss RNA	9.6	enveloped
Hepatitis E virus	<i>Hepeviridae</i>	+ss RNA	7.2	non-enveloped
Human cytomegalovirus	<i>Herpesviridae</i>	ds DNA	235	enveloped
Human immunodeficiency virus 1	<i>Retroviridae</i>	2 copies +ss RNA	9.7	enveloped
Human papillomavirus	<i>Papillomaviridae</i>	ds DNA	8	non-enveloped
Influenza H1N1	<i>Orthomyxoviridae</i>	segmented -ss RNA	13.5	enveloped
Marburg virus	<i>Filoviridae</i>	-ss RNA	19	enveloped
Marek's disease virus	<i>Orthoherpesviridae</i>	ds DNA	177	enveloped
Mouse mammary tumor virus	<i>Retroviridae</i>	+ss RNA	10	enveloped
Murine norovirus	<i>Caliciviridae</i>	+ss RNA	7.5	non-enveloped
Rabies virus	<i>Rhabdoviridae</i>	-ss RNA	12	enveloped
Respiratory syncytial virus	<i>Pneumoviridae</i>	-ss RNA	15.2	enveloped
SARS-CoV-2, NL63, 229E, OC43 & HKU1	<i>Coronaviridae</i>	+ss RNA	26 to 32	enveloped
Tembusu virus	<i>Flaviviridae</i>	+ss RNA	10.9	enveloped
Yellow fever virus	<i>Flaviviridae</i>	+ss RNA	11	enveloped

631 *ds = double stranded, ss = single stranded, "+" = positive sense, "-" = negative sense

Table 2. Examples of TLR4 Single nucleotide polymorphisms (SNPs) in selected populations that impact virulence

SNP	Population	Virulence	Viruses	References
RS ⁷⁸⁷³⁷⁸⁴	Chinese	↗	HPV	[25]
RS ¹⁰⁷⁵⁹⁹³²	Chinese	↗	EV71	[98]
RS ⁴⁹⁸⁶⁷⁹⁰	Indian	↗	HPV; DENV; HIV	[21,99–101]
	Egyptian		SARS-CoV2	[18]
	Caucasian		HIV	[99]
	Saudi Arabian		HCV	[102]
	Tunisian		HCV; HBV	[103]
RS ⁴⁹⁸⁶⁷⁹¹	Polish	↗	HCMV	[23]
	Indian		HEV; DENV	[21,22]
	Saudi Arabian		HCV	[102]
	Egyptian		SARS-CoV2	[18]
	Mexican		DENV	[20]
RS ¹⁹²⁷⁹¹¹	Polish	↗	HCMV	[19]
	Indian		HPV	[26]
RS ¹¹⁵³⁶⁸⁶⁵	Mexican	↘	DENV	[20]
RS ²⁷³⁷¹⁹⁰	Mexican	↘	DENV	[20]

633 **Figure 3.** Indirect and direct TLR4 engagement
634 A schema of the means by which TLR4 is engaged by viruses: the left panel depicts direct detection of
635 intact SARCoV-2, the middle panel depicts the detection of an adenovirus capsid via an AMP bridge
636 (lactoferrin in this case), and the in the right panel the detection of viral proteins.



637