Targeting the TLR signalosome with TIR domain-derived cell-permeable decoy peptides: the current state and perspectives

Vladimir Y Toshchakov ⓘ and Artur Javmen

Abstract

The ability to engineer pharmaceuticals that target the signal-dependent interactions of signaling proteins should revolutionize drug development. One approach to the rational design of protein interaction inhibitors uses decoy peptides, i.e. segments of protein primary sequence, which are derived from interfaces that mediate functional protein interactions. Decoy peptides often retain the ability of the full-length prototype to bind the docking site of the folded protein and thereby block the signal transduction. This review summarizes advances made in the last decade in the development of cell-permeable decoy peptide (CPDP) inhibitors to target the Toll/IL-1R resistance (TIR) domain-mediated protein interactions in TLR signaling, in connection with the recent progress in understanding of the TLR signalosome assembly mechanisms. We present a large collection of currently available, TIR-targeting CPDPs and propose their classification based on the types of TIR–TIR interactions they target. The binding behavior of different CPDP-TIR pairs, studied in cell-based assays and in binary *in vitro* systems using recombinant TIR domains, is also reviewed. The available affinity data provide benchmarks for rapid preliminary evaluation of future inhibitors. We review literature that evaluates the *in vivo* potency of select CPDPs and attempt to outline the areas of forthcoming progress, towards the development of CPDP-based TLR inhibitors of pharmaceutical grade.

Introduction

Detection of pathogens and activation of host defense is the primary function of innate immunity. TLRs are front-line host defense molecules that detect invading pathogens. TLRs are type I transmembrane receptors consisting of a leucine-rich repeat-containing ectodomain, single transmembrane helix, and cytosolic Toll/IL-1R resistance (TIR) domain.[1] A TIR domain, an α/β protein domain, typically comprises five parallel β-strands alternating with five α-helices (Figure 1a).[1–3] TLR activation induces host antimicrobial defenses primarily through the induction of pro-inflammatory cytokine secretion.[4,5] When TLR signaling is not properly regulated, the excessive activation can cause negative effects and lead to inflammatory disease.[6–9] The dysregulated TLR response is implicated in the pathogenesis of rheumatoid arthritis, atherosclerosis, systemic lupus erythematosus, and systemic sclerosis.[10–18] In light of this, specific TLR inhibitors should be useful in the treatment of many inflammatory conditions.

The activated TLRs initiate intracellular signaling through dimerization of their TIR domains. The emerged TIR dimers nucleate a formation of larger protein complexes through multiple interactions of

Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA

Corresponding author:
Vladimir Y Toshchakov, Department of Microbiology and Immunology, University of Maryland School of Medicine, 685 W. Baltimore St., Rm. 380, Baltimore, MD 21201, USA.
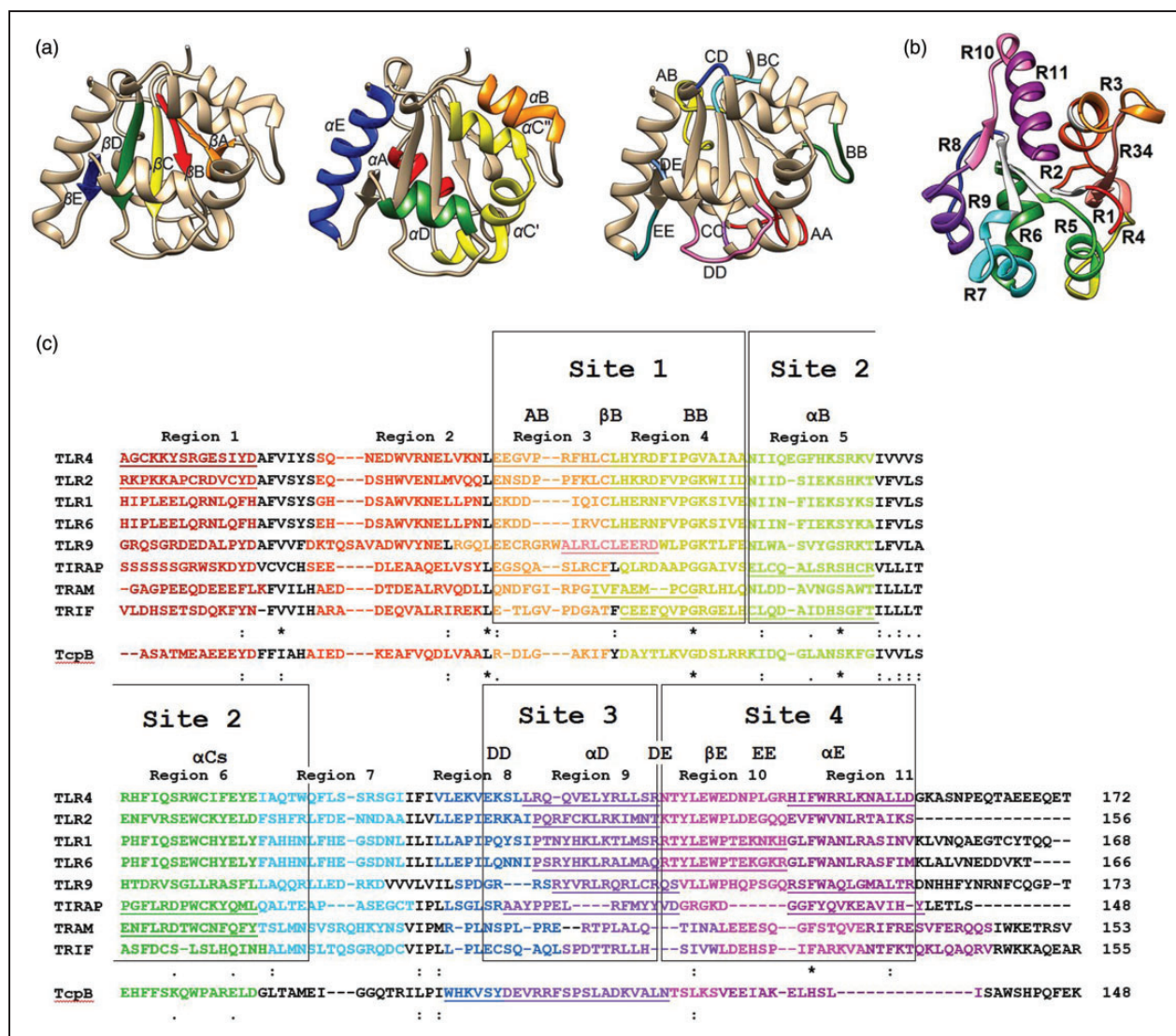Email: vtoshchakov@som.umaryland.edu

(a)

(b)

(c)

	Region 1	Region 2	**Site 1** AB βB BB Region 3 Region 4	**Site 2** αB Region 5
TLR4	AGCKKYSRGESIYD	AFVIYSSQ---NEDWVRNELVKNL	EEGVP--RFHLCLHYRDFIPGVAIAA	NIIQEGFHKSRKVIVVVS
TLR2	RKPKKAPCRDVCYD	AFVSYSEQ---DSHWVENLMVQQL	ENSDP--PFKLCLHKRDFVPGKWIID	NIID-SIEKSHKTVFVLS
TLR1	HIPLEELQRNLQFH	AFVSYSGH---DSAWVKNELLPNL	EKDD-----IQICLHERNFVPGKSIVE	NIIN-FIEKSYKSIFVLS
TLR6	HIPLEELQRNLQFH	AFVSYSEH---DSAWVKNELLPNL	EKDD-----IRVCLHERNFVPGKSIVE	NIIN-FIEKSYKAIFVLS
TLR9	GRQSGRDEDALPYD	AFVVFDKTQSAVADWVYNELRGQL	EECRGRWALRLCLEERDWLPGKTLFE	NLWA-SVYGSRKTLFVLA
TIRAP	SSSSSSGRWSKDYD	VCVCHSEE----DLEAAQELVSYL	EGSQA--SLRCFLQLRDAAPGGAIVS	ELCQ-ALSRSHCRVLLIT
TRAM	-GAGPEEQDEEEFLK	FVILHAED---DTDEALRVQDLL	QNDFGI-RPGIVFAEM--PCGRLHLQ	NLDD-AVNGSAWTILLLT
TRIF	VLDHSETSDQKFYN	-FVVIHARA---DEQVALRIREKL	E-TLGV-PDGATFCEEFQVPGRGELH	CLQD-AIDHSGFTILLLT
	: *	:	*:	*
TcpB	--ASATMEAEEEYD	FFIAHAIED---KEAFVQDLVAAL	R-DLG---AKIFYDAYTLKVGDSLRRKIDQ	-GLANSKFGIVVLS

	Site 2 αCs Region 6	Region 7	**Site 3** DD αD Region 8	**Site 4** DE βE EE αE Region 9 Region 10 Region 11	
TLR4	RHFIQSRWCIFEYEIAQTWQFLS-SRSGI	IFIVLEKVEKSLLRQ-QVELYRLLSR	NTYLEWEDNPLGRHIFWRRLKNALLDGKASNPEQTAEEEQET		172
TLR2	ENFVRSEWCKYELDFSHFRLFDE-NNDAA	ILVLLEPIERKAIPQRFCKLRKIMNT	KTYLEWPLDEGQQEVFWVNLRTAIKS----------------		156
TLR1	PHFIQSEWCHYELYFAHHNLFHE-GSDNL	ILILLAPIPQYSIPTNYHKLKTLMSR	RTYLEWPTEKNKHGLFWANLRASINVKLVNQAEGTCYTQQ--		168
TLR6	PHFIQSEWCHYELYFAHHNLFHE-GSDNL	ILILLEPILQNNIPSRYHKLRALMAQ	RTYLEWPTEKGKRGLFWANLRASFIMKLALVNEDDVKT----		166
TLR9	HTDRVSGLLRASFLLAQQRLLED-RKD	VVVLVILSPDGR---RSRYVRLRQRLCRQ	SVLLWPHQPSGQRSFWAQLGMALTRDNHHFYNRNFCQGP-T		173
TIRAP	PGFLRDPWCKYQMLQALTEAP---ASEGCT	IPLLSGLSRAAYPPEL----RFMYY	VDGRGKD------GGFYQVKEAVIH-YLETLS----------		148
TRAM	ENFLRDTWCNFQFYTSLMNSVSRQHKYNS	VIPMR-PLNSPL-PRE--RTPLALQ	--TINALEEESQ--GFSTQVERIFRESVFERQQSIWKETRSV		153
TRIF	ASFDCS-LSLHQINHALMNSLTQSGRQDC	VIPLL-PLECSQ-AQLSPDTTRLLH	---SIVWLDEHSP--IFARKVANTFKTQKLQAQRVRWKKAQEAR		155
	. . :	: :	:	* :	
TcpB	EHFFSKQWPARELDGLTAMEI---GGQTRILP	IWHKVSYDEVRRFSPSLADKVALNTSLKSVEEIAK-ELHSL--------------ISAWSHPQFEK			148

Figure 1. Main structural elements of a TIR domain and the sequences of TIR-derived CPDPs. (a) The structural elements of TIR domains. Three images of the panel show in different colors the strands (left image), helices (the image in the middle), and loops of a TIR. (b) Design of a TIR-derived CPDP library. Segments of the primary sequence that correspond to Regions 1–11 are shown in different colors. (c) The sequence alignment of TIR-derived CPDP libraries screened up to date. Segments shown in different colors correspond to different decoy peptides. The color coding corresponds to the color coding of TIR Regions in panel 1B. Amino acids shown in black were not included into decoy peptides because these residues have no or minimal surface exposure. The Region 7 TcpB peptide was not tested due to poor solubility.[19] The sequences of inhibitory decoys are underlined. The black boxes marked Site 1–4 show the approximate positions of four functional TIR interfaces that mediate the recruitment of TIR-containing adapters to activated TLRs. These sites are also shown in Figure 2.

All TIR peptide libraries screened by our group were designed in a similar way, such that each peptide corresponded to a particular structural region of TIR domain (Figure 1b and c). Peptides were numbered consecutively, starting from the N-terminal peptide. TLR peptides were named as XRY, were X indicates TLR "number" and Y indicates peptide number. Adapter peptides were designated as TRY, TMY, TFY, and MRY, were TR ... indicates TIRAP peptides, TM-TRAM peptides, TF-TRIF peptide, MR-MyD88 peptides, and Y indicates peptide number, so that the same Y number for peptides from different libraries suggest that these peptides are from structurally homologous regions.

the dimer with the TIR domains of downstream adapters. Thus, multiple interactions of TIR domains are the key for TLR adapter recruitment and initiation of intracellular TLR signaling. Blocking the intracellular TIR-mediated protein interactions is therefore a valid approach to targeting the TLR signaling. The transitory protein interactions may be blocked by short peptides derived from the protein interfaces that mediate

these interactions.[7] Such peptides are often called decoy peptides. The cytosolic location of the signaling events that involve TIR domains implies that the TIR-targeted decoy peptides should be delivered into cytoplasmic space.

There are several approaches to the intracellular delivery of biologically active macromolecules. One such approach uses cell-permeable peptides (CPPs).[6,20] The CPPs are a class of short peptides, often derived from a natural protein that can translocate across cell membranes and carry the "cargo" macromolecules inside cells.[21,22] The CPPs do not require a protein carrier to translocate and therefore are effective in a large variety of cell types.[6,21] The first CPPs were discovered in the late eighties when two independent research groups reported that exogenously added HIV transcription factor Trans-activator of transcription (TAT) can enter cells and induce expression of viral genes.[23,24] Soon after, it was discovered that *Drosophila melanogaster* protein *Antennapedia* homeodomain also can translocate across cell membranes without participation of a transporter.[25] The same group identified a short peptide within *Antennapedia* homeodomain responsible for cell permeability. This peptide was named penetratin.[26] The discovery of first cell-permeable proteins (and short peptides within them responsible for cell permeation) have laid a foundation for a new approach to the targeted delivery of diverse biologically active molecules inside cells.[20] Approximately 1000 CPPs have been reported to date.[27] The length of the CPPs varies from 5 to 30 amino acids. The CPPs have been used for biological delivery of diverse substances including peptides, nucleic acids, proteins, drugs, and imaging agents, and also large multi-molecular structures, such as liposomes or nanoparticles.[21,28,29]

One particular CPP application is the intracellular delivery of decoy sequences, short peptides derived from protein interaction sites to block protein functions dependent on the cognate interaction.[6] This application uses the ability of many decoy peptides to bind and obstruct the protein docking sites.[6] The described approach has been successfully used for specific inhibition of diverse signaling pathways, including the TLR signaling. This review summarizes available literature on the TLR-targeted cell-permeable decoy peptides (CPDPs) and assesses these molecules as potential therapeutics and tools to study TLR physiology.

Decoy peptide inhibitors of TLR signaling

Mammalian TIR domain-derived CPDPs

First TIR-derived CPDPs were developed in the early 2000s.[30–32] All peptides tested then were derived from the BB loop of TLR adapters (Figure 1a). This region was selected because it is the only surface-exposed epitope among three conserved sequence motifs identified in the TIR domains at that time.[2,33] Additional rationale for selection of the BB loop as the decoy sequence in first experiments was that this region is the site of the TLR4 Pro712His mutation, i.e. the LPS[d] mutation that renders TLR4 nonfunctional.[2,34,35] The homologous *TLR2* mutation, Pro681His, impaired the TLR2 signaling,[36] without a significant effect on TLR2 TIR structure.[2] The first study that tested a TIR-derived CPDP for TLR inhibition demonstrated that the 14 amino acid-long TIRAP BB loop peptide fused with penetratin inhibited the LPS-induced NF-κB activation in RAW264.7 cells.[30] Toshchakov et al. later confirmed that the TIRAP BB loop peptide inhibited the LPS-induced signaling; however, this peptide was the weakest TLR4 inhibitor of four adapter-derived BB loop peptides tested.[31,37] Same group later found that the MyD88-derived BB loop peptide, unlike CPDPs derived from TIRAP, TRAM, or TRIF BB loop, significantly suppressed both TLR2 and TLR4 signaling.[31] Loiarro et al. reported that a shorter MyD88 BB loop CPDP reduced the NF-κB-dependent signaling.[32,38] Peptides derived from TLR4 and TLR2 BB loops cross-reacted and inhibited both receptors, though both peptides were more potent towards the corresponding prototype receptor.[39]

Toshchakov et al. tested the first library of peptides derived from different structural elements of a TIR.[40] The library included 12 peptides, each of which represented a non-fragmented surface patch formed by a particular segment of TLR4 primary sequence (Figure 1c). The library was designed to represent the entire TIR surface and included two presumably unstructured segments immediately before and after the TIRA (Figure 1b). The screening of TLR4 library has identified five peptides, which potently inhibited TLR4 when used at 40 μM.[40] This finding was in line with the notion that TIR domains are involved in multiple simultaneous interactions during the formation of primary signaling complexes.[41] In addition to the BB loop peptide, peptides derived from the presumably unstructured segment that connects TIR with the transmembrane region (4R1), AB loop (4R3), and α-helices D and E (4R9 and 4αE) also inhibited the LPS-induced macrophage activation (Table 1; Figure 1c).[40]

The same research group later screened analogously designed peptide libraries derived from other TLRs.[42–44] Screening of each library produced several inhibitory peptides. The TLR2 library yielded three new inhibitory peptides, each of which suppressed the TLR2 activation by Pam2Cys or Pam3Cys.[44] Inhibitory peptides corresponded to TLR2 TIR Regions 1, 3, and 9, i.e. the region that connects TIR

Table 1. TIR-derived inhibitory decoy peptides.

	Peptide	Source TIR	Corresponding structural region	Sequence	TLR inhibition[a]	Verified binding partners	TIR site
1	2R1	TLR2	N-Terminal segment preceding the TIR	RKPKKAPCRDVCYD	TLR2[44]	TLR1, TLR6, MyD88[44]	Site 1
2	2R3	TLR2	AB loop	ENSDPPFKLC	TLR2[44]	TLR1, TLR6[44]	Site 1
3	2BB	TLR2	BB loop	LHKRDCFVPGKWIID	TLR2, TLR4[39]	–	Site 1
4	4R1	TLR4	N-Terminal segment preceding the TIR	AGCKKYSRGESIYD	TLR4[40]	TLR2 and TLR4[40]	Site 1
5	4R3	TLR4	AB loop and βB	EEGVPRFHLC	TLR4[40]	–	Site 1
6	4BB	TLR4	βB, BB, and αB	LHYRDFIPGVAIAA	TLR2, TLR4[39,40]	TLR4[40]	Site 1
7	9R3	TLR9	αA, AB	RGQLEECRGRWALR	TLR9[42]	–	Site 1
8	9R34-ΔN	TLR9	AB, βB, BB	ALRLCLEERD	**TLR9[42]**	TLR9, TIRAP[42]	Site 1
9	TR3	TIRAP	αA, AB, βB	EGSQASLRCF	TLR2, TLR4[45]	–	Site 1
10	TM4-ΔC	TRAM	BB, αB	IVFAEMPCG	**TLR4[46]**	–	Site 1
11	TF4	TRIF	BB, αB	CEEFQVPGRGELH	TLR4[47]	TLR4[47]	Site 1
12	MyD88-BB	MyD88	BB	SDRDVLPGTCVWS	TLR4[39]	TLR9, TIRAP, MyD88[38, 43,44]	Site 1
13	TR5	TIRAP	αB, BC	ELCQALSRSHCR	**TLR4[45]**	–	Site 2
14	TR6	TIRAP	CC, αC	PGFLRDPWCKYQML	**TLR2[45]**, TLR4[46]	MyD88[43]	Site 2
15	TM6	TRAM	αC, CD	ENFLRDTWCNFQFY	**TLR4[46]**	–	Site 2
16	TF5	TRIF	αB, BC	CLQDAIDHSGFT	**TLR4[47]**	TLR4, TRAM[47]	Site 2
17	1R9	TLR1	αD	PTNYHKLKTLMSR	TLR2[43]	MyD88[43]	Site 3
18	2R9	TLR2	DD, αD	PQRFCKLRKIMNT	**TLR2[45]**, TLR4, **TLR7[45], TLR9[44]**	TIRAP[44]	Site 3
19	4R9	TLR4	αD	LRQQVELYRLLSR	TLR2, TLR4[40]	TIRAP[47]	Site 3
20	6R9	TLR6	αD	PSRYHKLRALMAQ	TLR2[43]	TIRAP[43]	Site 3
21	9R9	TLR9	DD, αD	RYVRLRQRLCRQS	TLR9[42]	–	Site 3
22	TR9	TIRAP	DD, αD, DE, βE	AAYPPELRFMYYVD	TLR4[45]	TLR2[43]	Site 3
23	1R10	TLR1	DE, βE, EE	RTYLEWPTEKNKH	TLR2[43]	–	Site 4
24	4αE	TLR4	αE	HIFWRRLKNALLD	TLR4[40]	TLR4[40]	Site 4
25	6R10	TLR6	DE, βE, EE	RTYLEWPTEKGKR	TLR2[43]	–	Site 4
26	9R11	TLR9	αE	RSFWAQLGMALTRD	TLR9[42]	TLR9, TIRAP, MyD88[42]	Site 4
27	TR11	TIRAP	αE	GGFYQVKEAVIHY	TLR4[45]	–	Site 4

[a]**Bold** highlight indicates peptides and TLR, the inhibitory activity for which is confirmed in the *in vivo* experiments; the structural regions and peptide sequences are also shown in Figure 1c as a multiple sequence alignment.

with the transmembrane region (peptide 2R1), the AB loop (2R3), and α-helix D (2R9), respectively (Table 1; Figure 1).[44] Notably, 2R9 demonstrated a broad specificity, as this peptide inhibited the TLR4, TLR7, and TLR9 signaling, in addition to TLR2 signaling.[44]

Screening results for TLR1 and TLR6 libraries were similar. In both libraries, the Region 9 and 10 peptides inhibited the signaling mediated by cognate receptors.[43] Region 9 corresponds to α-helix D; whereas Region 10 includes the DE and EE loops, and β-strand E (Table 1; Figure 1).[43]

Several peptides from the TLR9 TIR library inhibited TLR9.[42] The ODN 1668-induced macrophage activation was inhibited by the AB loop peptide 9R3, the overlapping peptide 9R34 that included β-strand B and parts of neighboring loops, and by peptides derived from α-helices C, D, and E (i.e. peptides 9R6, 9R9, and 9R11) (Table 1; Figure 1a to c).[42]

CPDP libraries derived from TLR adapters TIRAP, TRAM, and TRIF were screened analogously to the TLR peptide libraries.[45–47] The TIRAP peptide library yielded 5 CPDPs, each of which suppressed TLR4 (Table 1).[45] The inhibitory peptides corresponded to the AB loop, and α-helices B, C, D, and E, i.e. peptides TR3, TR5, TR6, TR9, and TR11, respectively (Table 1; Figure 1).[45] Two of five TIRAP-derived peptides that inhibited TLR4, peptides TR3 and TR6, also inhibited TLR2.[45] Screening of TRAM and TRIF CPDP libraries produced two TLR4-inhibitory peptides in each library.[46,47] The BB loop peptides from both adapters (TM4 and TF4) inhibited. The second inhibitory peptide in the TRAM library peptide was from α-helix C

(TM6); whereas the second inhibitory TRIF peptide, TF5, was from α-helix B (Table 1; Figure 1c).[46,47]

CPDPs derived from microbial proteins

Microbial pathogens use a variety of strategies to offset host antimicrobial defenses.[48] One such strategy is the production of proteins that can bind components of TLR signaling pathways and thereby inhibit the TLR-induced immune response. Some proteins of this class contain TIR domains and many target the host's TIR domains.[48] Thus, vaccinia virus (VACV) uses A52R and A46 proteins to block TLR signal transduction.[49–51] McCoy et al. screened the A52R-derived peptides and demonstrated that peptide P13 reduced the cytokine secretion caused by TLR3, TLR4, and TLR9 activation in murine RAW264.7 cells at concentrations of 5–10 μM.[52] The follow-up study of Tsung et al. later reported that P13 inhibited the LPS-induced production of inflammatory cytokines in human HC/NPC and murine bEND cells at significantly higher concentrations of 50 and 100 μM.[49]

Lysakova-Devine et al. identified the viral inhibitor peptide of TLR4 (VIPER) derived from A46; this peptide potently inhibited TLR4 signaling in RAW264.7 cells, immortalized murine BMDMs, and human THP-1 cells, when used at low doses of 1 or 5 μM.[50] Snyder et al. demonstrated that BB and DD loop peptides (2.5 μM) from TIR domain-containing protein TcpC of uropathogenic *Escherichia coli* diminished the murine macrophage activation by LPS.[53] The DD peptide also inhibited TLR3, TLR7, and TLR9 signaling in BMDMs.[53] Ke et al. screened a peptide library derived from the TIR domain of *Brucella* TIR-containing protein (TcpB) for TLR4 inhibition.[19] Two peptides, TB-8 from the DD loop and TB-9 from the region containing α-helix D, DE loop, and β-strand E inhibited the LPS-induced activation of RAW264.4 cells, when used at 20 μM (Figure 1c).[19]

In summary, available studies provide strong evidence that the approach to the development of TLR inhibitors using CPDPs, derived from either mammalian proteins that mediate the targeted signaling or microbial proteins that target the signaling, is robust. Except for a limited sequence similarity within the group of BB loop-derived peptides, inhibitory CPDPs have no common sequence motifs that would correlate with activity. Despite significant differences in sequences, all peptides are effective in a quite narrow concentration range in cell culture experiments and, as will be discussed in the next section, *in vivo*, most likely due to a common cell permeation mechanism. Different authors have reported that the effective concentrations of diverse penetratin-fused CPDPs in cell culture experiments are generally in the range 5–40 μM.

Our *in vivo* studies have demonstrated that peptides with different TLR specificities are effective systemically in small animal models at the dose of 10 nmol/g.[42,44,45,47]

In vivo efficacy of CPDPs

CPDPs inhibit the *in vivo* response to purified TLR agonists

CPDPs were tested for TLR inhibition in a variety of *in vivo* models. First studies were focused on the inhibition of TLR4 responses induced by LPS administration. Tsung et al. demonstrated that the intraperitoneally administered P13 peptide at the dose of 1.55 nmol/g decreased the systemic LPS-induced TNF-α levels in mice by approximately 50%.[49] Lysakova-Devine et al. reported that the viral peptide VIPER (0.11 nmol/g) administered i.v. as a single bolus together with LPS reduced the IL-12p40 levels in the LPS-treated mice by ∼50%.[50] Couture et al. were first to test the mammalian TIR-derived CPDPs in a small animal model.[45] Authors administered the peptides i.p. at the dose of 10 nmol/g as a pre-treatment for a non-lethal i.p. dose of LPS. The systemic cytokine levels were monitored for 4 h following LPS administration. Both TIRAP peptides tested, TR5 and TR6, nearly abolished the LPS-induced production of TNF-α and significantly reduced the late activation of IL-6.[45] A detailed study of Piao et al. tested two TRAM peptides, TM4 and TM6, and confirmed the high potency of TIR-derived peptides as systemic inhibitors of LPS-induced cytokine activation in mice. Intraperitoneal administration of either peptide reduced serum TNF-α and IL-6 by approximately 90% of their peak levels.[46] Intravenous administrations of TM4 or TM6 also significantly reduced both cytokines but were slightly less effective than the i.p. administration.[46] The TRIF-derived CPDP TF5 was tested in similar experiments and demonstrated a comparable *in vivo* efficacy to the TRAM peptides.[47] Intraperitoneal TF5 (10 nmol/g) reduced serum TNF-α levels by ∼85% and IL-6 by ∼80% of their respective peak levels; whereas the i.v. administered TF5 was slightly less effective.[47]

Two TcpB peptides, TB-8 and TB-9, inhibited the systemic LPS-induced cytokines in mice.[19] TB-8 reduced TNF-α by ∼70%, IL-1β by ∼50%, IFN-β by ∼60%, and IL-6 by ∼70%, compared to the levels without peptide treatment. TB-9 acted similarly, reducing circulating TNF-α by ∼40%, IL-1β by ∼80%, IFN-β by ∼70%, and IL-6 by ∼90%.[19]

Piao et al. tested the TLR2-derived peptide 2R9 for inhibition of systemic cytokine activation elicited by Pam3Cys, a TLR2 agonist, and R848, a TLR7 agonist, in mice.[44] This peptide inhibits several TLRs due to its

ability to bind and sequester TIRAP, an adapter common for the targeted TLRs.[44] 2R9 significantly reduced circulating TNF-α, IL-12p40, and IL-6 induced by a Pam3Cys administration to mice. This peptide also inhibited the systemic IFN-α and IL-12p40 elicited by R848 administration.[44] Javmen et al. later expanded these findings by demonstrating that 2R9 potently inhibits the TLR9 signaling in mice.[42] Thus, the i.p. injection of 2R9 (10 nmol/g) completely abolished the TNF-α and IL-12p40 augmentation in ODN 1668-stimulated mice.[42] Authors also reported that the TLR9-derived peptide 9R34-ΔN selectively inhibits the ODN 1668-induced TNF-α and IL-12p40, but not the Pam3Cys-induced TNF-α and IL-12p40.[42]

Efficacy of CPDPs in animal models of TLR-induced lethality and infectious diseases

Experiments reviewed in the previous section provide direct evidence that CPDPs are effective systemic inhibitors of cytokine response elicited by purified TLR agonists. The TLR-targeted CPDPs were also tested in more complex disease models. Several groups have demonstrated that CPDPs protect mice against lethality caused by LPS or a combination of a TLR agonist with D-galactosamine (D-GalN). Tsung et al. reported that the i.p. administered P13 peptide (0.31 nmol/g) improved survival of mice sensitized to LPS challenge by the D-GalN pre-treatment.[49] Piao et al. tested the adapter-derived peptides TM4, TR6, and TM6 for protection against a lethal dose of LPS. TM4 (10 nmol/g) fully protected mice challenged with a lethal LPS dose, while TR6 and TM6 provided ~70% protection (Table 1).[46] The same group later reported that the TRIF-derived peptide TF5 (10 nmol/g) rescued ~80% of mice that received a lethal LPS dose.[47]

TLR9-derived peptide 9R34 and its modification 9R34-ΔN (Table 1) protected the D-GalN pretreated mice against the ODN 1668-induced lethality.[42] Intraperitoneal administration of either 9R34 or 9R34-ΔN completely protected mice challenged with 3.93 nmol of ODN 1668.[42] The survival rate, however, dropped to ~60% when ODN 1668 dose was doubled.[42] Surprisingly, TM4, a CPDP specific for TLR4, also provided a partial protection against the ODN 1668-induced lethality, although less than the TLR9-specific inhibitor, 9R34-ΔN.[42] Thus, TM4 (10 nmol/g) rescued ~50% of mice challenged with 3.93 nmol of ODN 1668 and ~20% after the 7.86 nmol ODN 1668 dose.[42] The partial protection exhibited by TM4 in these experiments was attributed to suppression of injurious effects of TLR4 activation that was elicited by the endogenous danger-associated

molecules (DAMPs) produced consequently to the initial TLR9-induced damage.[42]

CPDPs were tested in several other models of inflammatory diseases. McCoy et al. demonstrated that P13 significantly reduced inflammation in the murine model of *otitis media* with effusion induced by administration of heat-inactivated *Streptococcus pneumoniae*.[52] Hu et al. evaluated the anti-inflammatory potential of TR6 in the mouse model of LPS-induced mastitis.[54] TR6 in a dose-dependent manner (2.5–10 nmol/g) suppressed the biochemical and histological manifestation of mastitis.[54] Hu et al. later tested homologous TRAM peptide, TM6, (2.5–10 nmol/g) for the alleviation of LPS-induced acute lung injury.[55] TM6 treatment decreased the cytokine production and myeloperoxidase activity, and lessened lung edema and histological changes in a dose-dependent manner.[55] Allette et al. demonstrated that rat pretreatment with 4BB peptide (3.2 nmol/g) diminished the LPS-induced allodynia to tactile stimuli.[56]

Piao et al. were the first to use a TIR-targeted CPDP in a model of infectious disease that involves a replicationally competent pathogen.[44] This study tested the multispecific TLR inhibitor 2R9 in a model of lethal flu caused by the mouse-adapted PR8 strain of H1N1 influenza virus.[44] The rationale for this study was the expectation that 2R9 should mitigate the cytokine storm often concomitant to acute, life-threatening infections and thereby might improve survival. In a series of preliminary experiments, authors demonstrated that 2R9 (20 and 40 μM) potently inhibited the TNF-α, IL-1β, and IFN-β production by cultured murine macrophages infected with the PR8 strain.[44] The treatment of PR8-infected mice with 2R9 (mice received 2R9 at the dose of 200 nmol/mouse once daily for 5 consecutive d starting from d 2 post infection) significantly improved the survival rate; thus, 78% of treated mice survived, versus 10% survival in the control group.[44]

Nonetheless, the fact that the *in vivo* studies were conducted in mice only, the available results firmly establish that the TIR-targeted CPDPs are effective systemic TLR inhibitors in small animal models. Because CPDPs can suppress inflammatory response to many PAMPs and DAMPs, the CPDPs (or analogous molecules) might be useful in treatments of various inflammatory conditions of diverse etiologies.

CPDP binding specificities

A TIR domain typically has multiple binding sites, each of which may bind several different TIR domains and also interact homotypically. Nevertheless this ability of TIR domains to interact with several binding partners, the TIR–TIR interactions are not altogether

general. The specificity of TIR–TIR interactions underlies, for example, the ability of activated TLRs to engage specific pathways.[57] The multivalency of interactions mediated by a particular TIR, together with the multispecificity of interactions mediated by individual sites, are fundamental for the key function of TIR domains, i.e. their ability to form oligomeric, signal-dependent protein complexes.[42,58,59] This general feature of the TIR domains entails that CPDP's binding partners are generally not known a priori and should be determined experimentally. Identification of TIR domains targeted by individual CPDPs is necessary for understanding of molecular mechanisms by which CPDPs inhibit TLRs and engineering of improved inhibitors.

In a pilot study, Loirarro et al. used immunoprecipitation to demonstrate that ST2825 peptide from MyD88 BB loop prevents dimerization of MyD88 TIRs.[38] Piao et al. using a dot blot assay later demonstrated that the MyD88 BB loop peptide also binds TIRAP and TLR9 TIR.[44] These findings have verified that a CPDP may have multiple binding partners among TIR domains, analogously to the whole length TIR domains.

Our group utilizes a cell-based FRET (Förster resonance energy transfer) assay for identification of CPDP binding partners.[60] In this assay, host cells are transfected with an expression vector that encodes a TIR fused with Cerulean fluorescent protein (Cer)[61] and the cells that express the fusion protein are treated with a CPDP labeled with a fluorescent dye that can quench Cer fluorescence. Peptide–TIR binding is measured by a decrease in Cer fluorescence lifetime due to quenching. FRET data suggested that TLR4 peptides 4BB and 4αE selectively bound TLR4, but not TLR2; whereas 4R1 interacted with both TLR2 and TLR4 TIRs (Table 1; Figure 1).[40] In contrast, a peptide from the α-helix D of TLR4 TIR, 4R9, did not bind TIR of either receptor. Piao et al. later demonstrated that 4R9 interacts with TIRAP.[45] Using the FRET FLIM (fluorescence lifetime imaging) approach and co-immunoprecipitation, we demonstrated that the structurally homologous TLR2 CPDP 2R9, similarly to 4R9, also binds TIRAP, not TLR1, TLR6, TLR4, or TLR9.[44] Javmen et al. used cell-based FRET FLIM to study the binding of TLR9-derived peptides.[42] 9R34-ΔN bound TLR9 and TIRAP but did not interact with MyD88; whereas 9R11 interacted with all three of these TIRs with comparable affinities (Table 1).[42] Importantly, for all peptides tested, the apparent K_D of TIR–peptide complexes determined in the cell-based FRET assay well corresponded to the apparent inhibition constants measured based on peptide effects on signaling. Apparent inhibition and dissociation constants measured in cell-based assays were in the low micromolar range 1–20 μM for all inhibitory TIR peptides tested. Such a similarity of effective concentrations for a broad range of inhibitory peptides, with presumably different mechanisms of action, may be due to a common cell permeation mechanism.

Additional data on peptide–TIR binding are available from immunoprecipitation experiments, dot blot assays, and peptide interference with co-immunoprecipitation of two TIRs. Thus, Piao et al. determined that TLR2 peptides 2R1 and 2R3 bind TLR2 coreceptors, TLR1 and TLR6 (Table 1).[44] 2R1 also bound MyD88.[45] Interestingly, peptides from α-helix D of TLR1 and TLR6, 1R9 and 6R9, followed the pattern demonstrated by the TLR2 and TLR4 peptides from same structural regions and bound the adapter TIR domains, not the TIRs of related coreceptors.[43] 1R9 bound MyD88; 6R9 – TIRAP TIR (Table 1).[43]

Data on the binding of adapter-derived CPDPs are fragmental at this time. Thus, TIRAP peptide TR6 bound MyD88 (Table 1).[44] TIRAP peptide TR9 interacted with TLR2 TIR (Table 1).[43] Study of TRIF peptides TF4 and TF5 demonstrated that TF4 binds TLR4, while TF5 interacts with both TLR4 and TRAM (Table 1).[47] Neither TRIF peptide interacted with adapters of the MyD88-dependent pathway, MyD88 or TIRAP.[47]

Data on the CPDP binding to recombinant TIR domains in the binary *in vitro* systems are also limited at this time. The current best-studied example is the 2R9-TIRAP TIR pair.[44] The fluorescence polarization (FP) assay estimated that the K_D for 2R9 binding with TIRAP TIR in solution is ∼40 nM.[44] Not surprisingly, the *in vitro* K_D is ∼100 times lower than the apparent K_D obtained in the cell-based FRET assay.[44] Such a significant difference may be accounted by an unspecific peptide binding to intra- or extracellular proteins. Indeed, the FP assay detected that 2R9 binds BSA with K_D of ∼1.5 μM.[44] This low-affinity BSA binding might be important for the *in vivo* activity of 2R9 because binding to albumin increases the lifetime of drugs and peptides in serum.[62] Moreover, peptide modifications aimed to increase their albumin binding is an established strategy in the development of peptidic pharmaceuticals.[62,63] Surface plasmon resonance (SPR) experiments confirmed the high affinity of 2R9-TIRAP binding.[44] The binding depended on the TIRAP folding state because 2R9 did not bind denatured TIRAP.[44] As often observed in cases of peptide binding to a structured protein domain, the high affinity of binding is due to a fairly fast association rate and slow dissociation of peptide-protein complexes.[44]

Ample evidence now indicates that the TIR-derived inhibitory CPDPs do indeed target the TIR domains of

TLR pathway proteins. Although many CPDPs demonstrate multi-specificity manifested by the ability to interact with several TIR domains, many interactions are selective. One notable example of selective CPDP-TIR interactions is the binding of peptides from α-helix D of TLRs to adapters of the MyD88-dependent pathway, not to the TIR domains of TLRs (Table 1).

Spatial relationships of inhibitory segments on the TIR surface and architecture of primary TLR signaling complexes

Screenings of peptide libraries completed to date have identified a large collection of inhibitory peptides (Table 1). In fact, each library of TIR-derived peptides screened by our group produced several TLR inhibitors. Due to low local sequence conservation in TIR domains, identified inhibitory peptides are dissimilar (Table 1). It may be noted that all TLR inhibitory peptides were derived from four topologically distinct, structural regions, which are common for all mammalian TIR libraries tested to date (Table 1; Figures 1c and 2a). Intriguingly, these TIR regions generally correspond to four types of TIR–TIR interfaces in the filamentous complexes spontaneously formed in solution by recombinant TIRAP TIR (Figure 2b).[42,59] This correspondence suggested that the structure of TIRAP homopolymeric, reversibly self-assembled, filamentous complexes resembles the structures of physiological TIR heterocomplexes assembled upon TLR activation. The elementary unit of the self-assembled TIRAP TIR homopolymers is a double-stranded, open-ended filament (Figure 2b and c).[59] The interactions of monomers within both strands of the filament are nearly identical so that the BB loop of each TIR interacts with the opposite area of other TIR without a significant axial rotation (Figure 2b). Interactions between filament strands are through three helical regions located on the convex side of the β-sheet, i.e. helices B, C, and D (Figure 1a and Figure 2c).

One non-fragmented TIR area that has produced many inhibitory CPDPs is formed by the AB and BB loops, and β-strand B (Figure 1a and c). This TIR region is defined as Site 1 (S1) (Figures 1C and 2A).[42] S1 corresponds to two peptides of TIR libraries, i.e. Region 3 (mostly AB loop) and Region 4 peptides (mostly BB loop) (Table 1, Figure 1c). Peptides from both regions were inhibitory in TLR2, TLR4, and TIRAP libraries.[40,44,45] Peptides derived from the BB, but not AB loop, were inhibitory in TRAM and TRIF libraries (Table 1; Figure 1a and c);[46,47] whereas the AB loop peptide and the peptide centered on β-strand B, but not the BB loop peptide were inhibitory in the

TLR9 library (Table 1; Figure 1c).[42] Studies of TIR–peptide binding have demonstrated that CPDPs from Site 1 are often multispecific and may interact with receptor and adapter TIR domains (Table 1). The observed multi-specificity of the S1 CPDPs suggested that this TIR area may be involved in the receptor dimerization, recruitment of adapters to activated receptors, and the adapter-adapter interactions (Table 1).[40,42,44,45] The multispecific binding through S1 may be in part explained by significant conformational flexibility of this area,[64] and by its large size, which is sufficient so that the overlapping, but slightly different segments of S1 mediate interactions for different TIRs. The partly structured TLR segment that links the TIR with TLR transmembrane region (Region 1 peptides; Fig 1B) often produced inhibitory peptides. Peptides from this region were active or partly active in the TLR2, TLR4, and TLR9 libraries.[40,42,44] Because this linker is spatially close to S1 in the 3D TIR structure, it was proposed that this TLR segment also participates in the formation of TLR signaling complexes, serving as an extension of the S1 interface.[40,44]

The TIR surface, which is opposite to S1 in the 3D structure, is formed by β-strand E, loops adjacent to β-strand E, and *a*-helix E (Figures 1a and 2a).[42] CPDPs derived from this site, which was defined as Site 4 (S4),[42] were inhibitory in TLR1, TLR4, TLR6, TLR9, and TIRAP peptide libraries (Table 1).[40,42–44] Similarly to peptides from S1, the S4-derived inhibitory peptides stem from slightly different segments in different TIR domains. Thus, peptides from α-helix E were inhibitory in TLR4, TLR9, and TIRAP libraries (Table 1; Figure 1c),[40,42,45] whereas TLR1 and TLR6 inhibitory sequences were derived from the β-strand E and flanking DE and EE loops (Table 1; Figure 1c).[43] The S4-derived inhibitory CPDPs, similarly to the S1-derived peptides, demonstrated a multispecific binding and interacted with receptor and adapter TIRs (Table 1).[40,42]

Because S1 and S4 correspond to sites, which mediate the intra-strand interactions in the TIRAP protofilaments (Figure 2b), and peptide binding data, it was proposed that S1 and S4 of different TIRs mutually interact in the TLR signaling complexes.[42,59] The S1–S4 interaction of receptor TIR domains mediates dimerization of receptor TIRs; whereas analogous receptor-adapter and adapter-adapter interactions promote elongation of the initial complex (Figure 2b and 2c).[42,57]

α-Helix D and DD loop form the third TIR site, which produced a significant number of inhibitory CPDPs (Table 1; Figure 1c). Peptides from this site (Region 9 peptides) were inhibitory in all TLR libraries screened to date, i.e. TLR1, 2, 4, 6, and 9, and also in
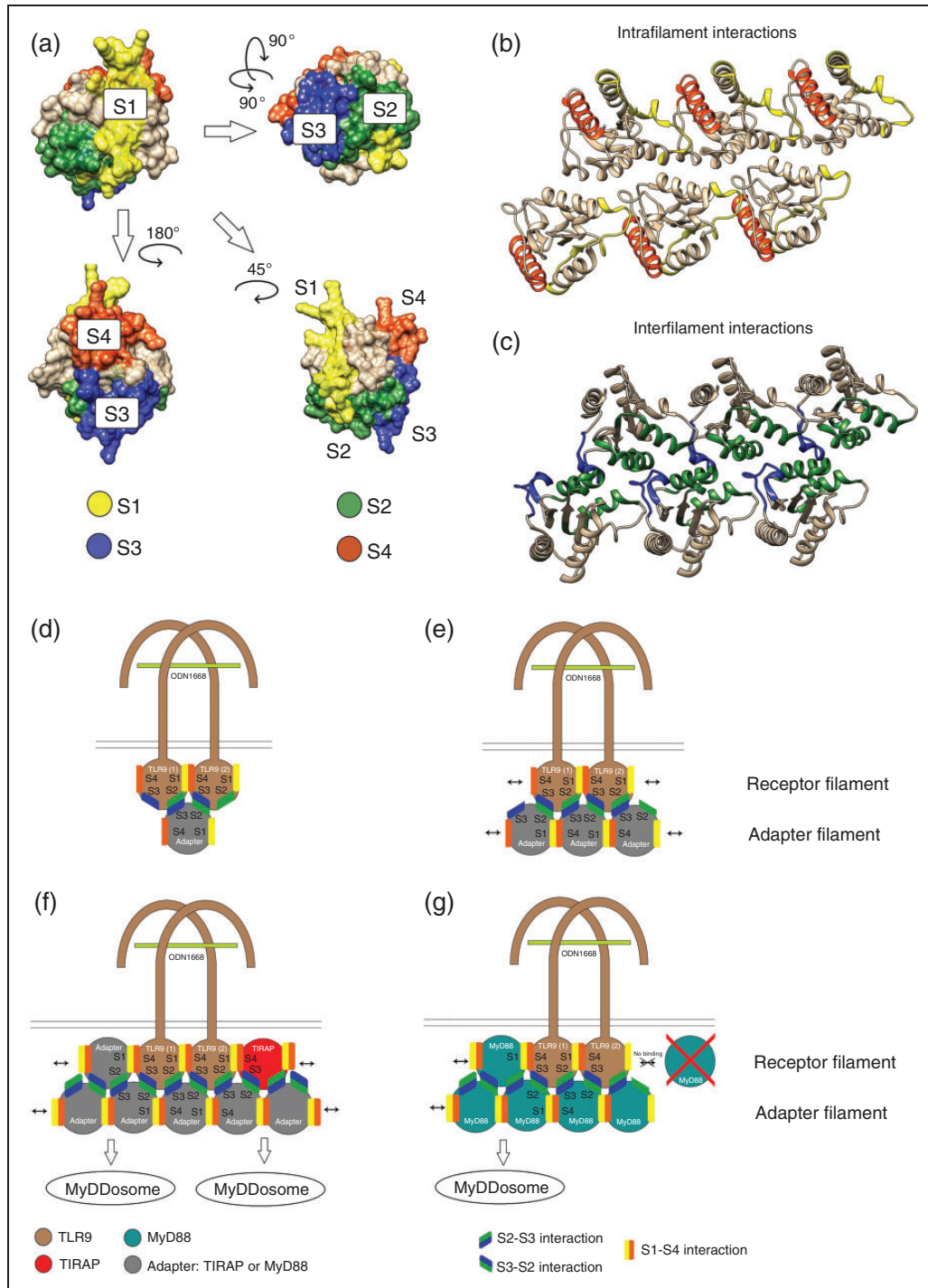
Figure 2. TIR domain interfaces that mediate the assembly of TLR signaling complexes and the mechanism of TIR signalosome formation. (a) Four TIR sites that mediate the assembly of primary TLR signaling complexes. Site 1 (S1) (yellow) and Site 4 (S4) (orange) are located on the opposite sides of a TIR domain, near β-strands B and E, respectively. Site 2 (S2) (green) is formed by α-helices B and C, whereas Site 3 (dark blue) is formed by α-helix D and may include adjacent loops.[42] (b) The intra-filament interactions within the TIRAP filament are mediated by Sites 1 and 4. Images in panels B and C were generated using the coordinate file 5uzb. [59] (c) The inter-filament interactions within the TIRAP filament are formed through mutual interaction of Sites 2 and 3. (d–g) Mechanism of adapter recruitment to activated TLRs. (d) Stage 1 of the TLR signalosome assembly. TLR TIRs dimerize through an asymmetrical S1–S4 interaction. Dimerization of receptor TIRs creates a composite binding site for adapter recruitment through two S2–S3 interactions. (e) Stage 2: Recruitment of the first adapter creates two additional sites for adapter recruitment through simultaneous S1–S4 adapter–adapter and S2–S3 receptor–adapter interactions. (f) Stage 3: Bidirectional elongation of TLR signalosomes through simultaneous intra-filament and inter-filament interactions. (g) Unidirectional elongation of TLR9 signalosome in the absence of TIRAP. TIRAP is required for elongation of TLR9 signalosomes in the S1 direction.

the TIRAP library (Figure 1c).[40,42–45] This TIR site, defined as Site 3 (S3),[42] similarly to S1 and S4, also corresponded to a TIR–TIR interaction site in the TIRAP filament.[59] Unlike mutually interacting S1 and S4 (Figure 2b), S3 mediated the interactions between TIRs of different strands of the protofilament (Figure 2b). Interestingly, all receptor-derived S3 peptides selectively bound adapter TIR domains, i.e. TIRAP or MyD88. Thus, peptides from S3 of TLR2 and TLR4 strongly bound TIRAP, but not the TIR of corresponding receptors.[40,44,47] Similarly, the S3 CPDPs from the TLR1 or TLR6 libraries differentially bound adapters MyD88 or TIRAP, respectively, but not the TLR2 TIR.[43] The S3 peptide from TIRAP, TR9, however, demonstrated a measurable binding to TLR2 TIR.[43] The S3 site in the TIRAP complex interacted with the surface jointly formed by α-helices B and C (Figures 1a and 2b).[59] This site, defined as Site 2 (S2),[42] corresponded to two peptides in TIR libraries, Region 5 (α-helix B) and Region 6 (mostly α-helix C) peptides. Peptides from S2 were inhibitory in all adapter libraries tested (Table 1).[45–47] In the TIRAP library, CPDPs from both helices that form S2, αB, and αC, inhibited the TLR signaling,[45] whereas in TRIF and TRAM libraries, only one of two S2 peptides, i.e. TF5-ΔC and TM6, respectively, inhibited (Table 1). The receptor-derived S2 CPDPs were only partly active in the TLR4 and TLR9 libraries.[40,42] The adapter-derived S2 inhibitory peptides bound TIRs of TLRs and TLR adapters (Table 1; Figure 2d to g).[43,44,47]

The similar topology of TIR segments that have produced inhibitory CPDPs suggests a common mechanism of the initiation of intracellular signaling by different TLRs. The first step of this mechanism is dimerization of TIR domains of two TLR molecules through the asymmetric S1–S4 interaction (Figure 2d). The formation of TLR TIR dimer creates a composite binding site for adapter recruitment through two S2–S3 interactions. Importantly, the geometry of TIR interfaces is such that the recruited adapter interacts with both TIRs of the dimer simultaneously through S2–S3 and reciprocal S3–S2 interactions (Figure 2d). Such a trimeric interaction is a perfect example of a cooperative interaction predicted to stabilize TIR oligomers.[40] The recruitment of the first adapter TIR domain creates two additional bipartite sites for recruitment of additional adapters through simultaneous S1-S4 adapter-adapter and S2-S3 receptor-adapter interactions (Figure 2e). This stage creates two strands of the initial complex, each of which can elongate further through intrastrand S1–S4 interactions that occur simultaneously with the stabilizing S2-S3 interstrand interaction (Figure 2e). Because peptides from S1 and S4 sites of TLR9 and TLR4 demonstrated multi-specificity and

bound receptor and adapter TIRs, we proposed that TLR9 and TLR4 signalosomes can elongate bidirectionally (Figure 2f).[42] The bidirectional elongation of the initial TLR4 signaling complex is supported by a recent study of Latty et al.[65] Using the single-molecule imaging, this study detected a conditional formation of "super" MyDDosome complexes, which contained twice as many MyD88 molecules per TLR4 as the "regular" MyDDosomes.[65]

Interestingly, the S1 peptide from TLR9 library, 9R34-ΔN, bound the TLR9 and TIRAP TIRs, but not Myd88 TIR.[42] This observation led us to propose a mechanistic explanation for the non-obligatory, facilitating role that TIRAP plays in TLR9 signaling.[42] The observation that, even though TLR9 can signal in the TIRAP absence, the signaling is significantly augmented by TIRAP, was originally made by Bonham et al.[66] and later supported by our finding that TLR9 is sensitive to a TIRAP-targeting CPDP.[44] The finding that the S1 peptide from TLR9 library bound TIRAP, but not MyD88 suggested that TIRAP is required for elongation of the initial complex in the S1 direction (Figure 2f).[42] Remarkably, the S4 CPDP from TLR9 library, 9R11, bound TIRs of both TIRAP and MyD88 equally, thus explaining the ability of TLR9 to signal in the TIRAP-deficient models (Figure 2g). Based on the limited inhibitory ability of S1 CPDPs from TLR2 co-receptors,[43] we proposed that the TLR2 signalosome elongates unidirectionally, similarly to the TLR9 signalosome in the TIRAP-deficient models.[42]

Conclusions and perspectives

Studies of the last decade have discovered a number of TIR-derived CPDPs and led to considerable progress in the understanding of mechanisms of their action. Available data suggest that inhibitory peptides generally correspond to four TIR sites that mediate the assembly of primary TLR signaling complexes, the architecture of which is similar to the architecture of filamentous complexes self-assembled from the TIRAP TIR domains. Two of these sites are located near β-strands that form two opposite edges of the β-sheet; whereas the other two are formed by three helices located on the convex surface of the β-sheet.

Direct binding of TIR-derived peptides to the TIR domains have been visualized in cell-based assays by FLIM and confirmed in functional and immunoprecipitation studies. The inhibitory TIR-derived CPDPs often demonstrate the multispecific binding, as some peptides are capable of binding several TIRs with comparable affinities. On the other hand, there are several examples of selective CPDP-TIR interactions. Different binding specificities of inhibitory CPDPs

manifest in the selective TLR inhibition by some CPDPs and may result in varying inhibition potency in the cells of different backgrounds.

The *in vivo* potency has been firmly established for many TIR-derived CPDPs. The systemic TLR inhibition is achievable in small animal models through peptide administration via i.p. or i.v. route. For several CPDPs, a single dose of 10 nmol/g was sufficient for systemic TLR inhibition and prevention of TLR-induced lethality. CPDPs were also used in the treatment of chronic conditions as a several days' course.[44] Multispecificity, i.e. the ability to target several TIR domains, apparently contributes to the *in vivo* potency of some CPDPs and is important for the understanding of their biological effects.

Although the size of inhibitory CPDP collection is quite large, the molecular determinants of CPDP inhibitory activity are not well understood at this time. The key factors that account for this lack of understanding are (i) the divergence of inhibitory sequences, (ii) divergence of TIR sites to which the multispecific inhibitory CPDPs bind, and (iii) the absence of resolved structures of TIR–peptide heterocomplexes. Resolution of fine atomic structures of TIR–peptide complexes should significantly advance the molecular understanding of TIR–TIR and TIR–peptide recognition mechanisms and lay a foundation for optimization of inhibitory sequences. The second area of potential progress is the optimization of cell-permeable vectors for the targeted delivery of decoy peptides to the intended intracellular targets. Another avenue for potential progress is in the use of non-peptidic backbones to develop CPDP peptidomimetic analogs with improved biological stability, analogously to the recent study of Trifonov et al.[67]

Declaration of conflicting interests

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ORCID iD

Vladimir Y Toshchakov https://orcid.org/0000-0002-7942-2294

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