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## SCHOOL of nature: ligand-independent immunomodulatory peptides

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Groundbreaking studies in protein biophysics have identified the mechanisms of transmembrane signaling at the level of druggable protein–protein interactions (PPIs). This resulted in the development of the signaling chain homooligomerization (SCHOOL) strategy to modulate cell responses using receptor-specific peptides. Inspired by nature, these short peptides use ligand-independent mechanisms of receptor inhibition and demonstrate potent efficacy *in vitro* and *in vivo*. The SCHOOL strategy is especially important when receptor ligands are unknown. An example is the triggering receptor expressed on myeloid cells-1 (TREM-1) receptor, an emerging therapeutic target involved in the pathogenesis of most inflammatory diseases. Here, I discuss advances in the field with a focus on TREM-1 inhibitory SCHOOL peptides that offer new hope for a ‘magic bullet’ cure for cancer, arthritis, sepsis, retinopathy, and other medical challenges.

### Introduction

Cell surface receptors use a complex fundamental process called transmembrane signal transduction to translate extracellular information into intracellular signaling sequences and further into physiological cell response. This process has a crucial role in health and disease, which makes its therapeutic control of both fundamental and clinical importance. Until recently, progress in this field was impeded by a lack of understanding of transmembrane signaling at the PPI level.

Here, I describe how novel insights at the interface of protein biophysics, immunology, virology, and molecular evolution resulted in our deep understanding of receptor triggering and opened new opportunities in innovative drug

discovery and development. These included the development of receptor-specific immunomodulatory peptides that use a novel, ligand-independent mechanism of action (the so-called ‘SCHOOL peptides’). Ligand-independent modulation is especially important for those receptors the ligands of which are still unknown (e.g., TREM-1). For this reason, the main focus is on ligand-independent TREM-1 inhibitory peptides and their use in multiple cell and animal model systems.

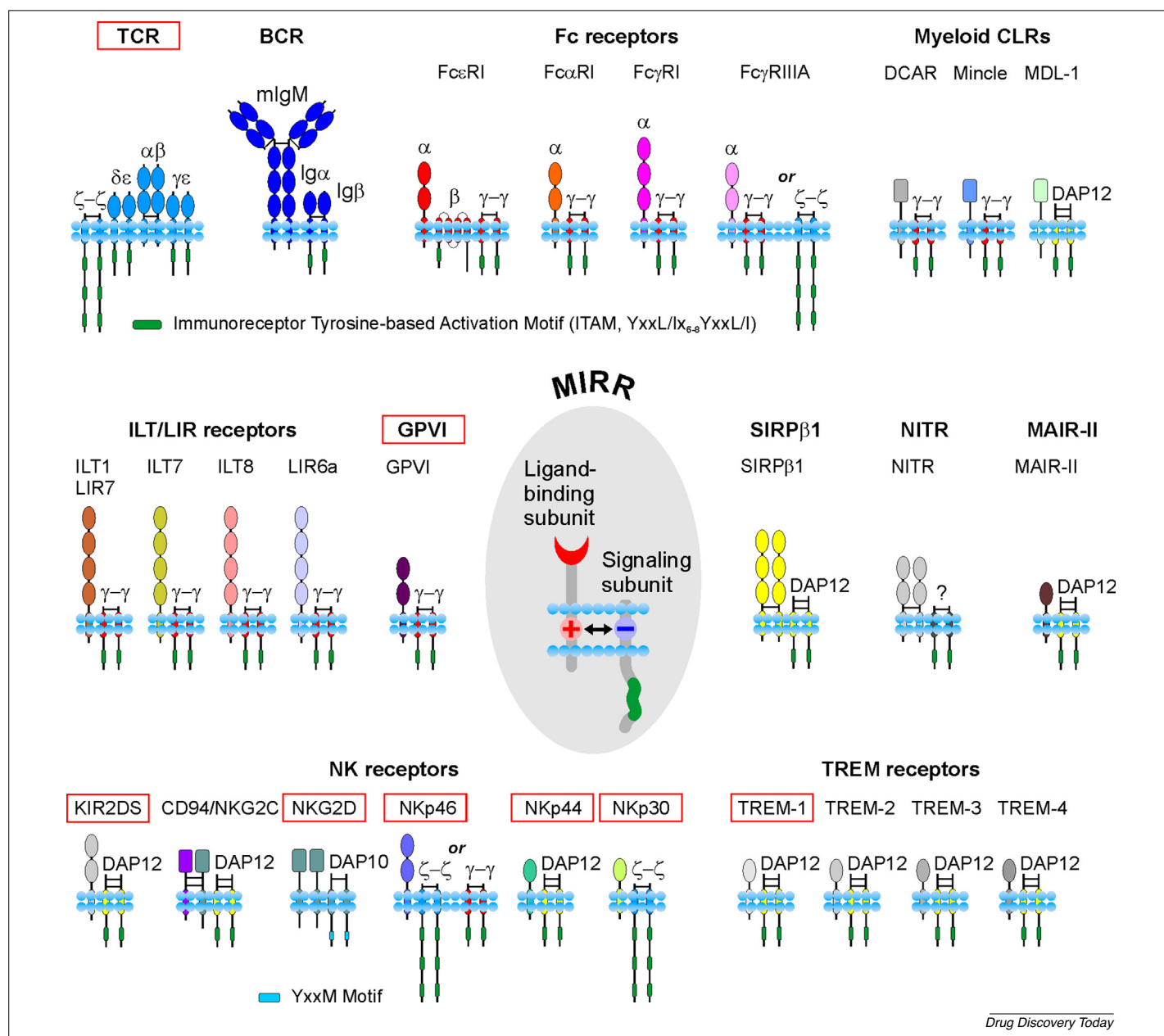
### Multichain immune recognition receptors: different players, same SCHOOL game

Cell receptors the extracellular ligand-binding domains and intracellular signaling domains of which are located on separate subunits are often referred to as multichain immune recognition receptors (MIRRs) (Fig. 1) (reviewed in [1]; see

also [2]). These receptors all have one or more copies of the immunoreceptor tyrosine-based activation motif (ITAM) or the YxxM motif in their cytoplasmic signaling domains [1]. Upon receptor triggering, the ITAM/YxxM tyrosine residues are phosphorylated in an early and obligatory event in the signaling cascade.

One of the main challenges in this field that affects drug discovery is that researchers still cannot come to an agreement on how MIRRs signal. As a result, we continue discussing numerous, seemingly unrelated and mostly descriptive but not predictive, models of MIRR triggering (reviewed in [1]). The most evident example is the T cell receptor (TCR) (Fig. 1). Multiple models of TCR triggering include kinetic proofreading, serial triggering, serial encounter, conformational, permissive geometry, clustering, segregation, and mechanosensor

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**FIGURE 1**

Examples of immune receptors with the extracellular ligand-binding domains and intracellular signaling domains located on separate subunits (multichain immune recognition receptors, MIRR). The inset depicts a schematic representation of the MIRR structure with noncovalent, electrostatically driven interactions between the ligand binding and signaling subunits in the cell membrane (black double-headed solid arrow). Curved lines depict the intrinsic disorder of the cytoplasmic domains of MIRR signaling subunits. ITAMs/YxxM signaling domains are shown in green. Red rectangles depict the receptors for which ligand-independent inhibitory peptides were reported to date (Table 1). Abbreviations: BCR, B cell receptor; CLR, C-type lectin receptor; DAP-10 and DAP-12, DNAX adapter proteins of 10 and 12 kD, respectively; DCAR, dendritic cell immunoactivating receptor; GPVI, glycoprotein VI; ILT, Ig-like transcript; KIR, killer cell Ig-like receptor; LIR, leukocyte Ig-like receptor; MAIR-II, myeloid-associated Ig-like receptor; MDL-1, myeloid DAP12-associating lectin 1; NITR, novel immune-type receptor; NK, natural killer cells; SIRP, signal regulatory protein; TCR, T cell receptor; TREM receptors, triggering receptors expressed on myeloid cells.

models (reviewed in [1]; see also [3]). However, none of these models explain the mechanisms of TCR triggering at the PPI level, and most are seemingly contradictory. For example, whereas the clustering model cannot explain the existence of TCR oligomers on resting T cells, the conformational model cannot explain why multi- but not monovalent ligands trigger TCR. The failure of the existing models of MIRR

triggering to describe and explain the data accumulated to date as well as to predict accurately can be probably explained by the 'Blind men and an elephant' paradigm limitations, when each model considers a different but only one part of the process.

Another challenge is the continuing lack of recognition of how important the translation of the structural similarity of MIRRs to the similarity

of MIRR triggering mechanisms is. An example is the important role of ligand-induced oligomerization of MIRRs in their triggering, with dimerization of MIRRs being the most frequent (reviewed in [1]). Although for TCR this has been known at least since 1995 [4], despite extensive studies since TREM-1 was discovered in 2000 [5], the importance of multimerization for TREM-1 triggering was found only recently [6].

### SCHOOL model of transmembrane signaling

First reported in 2004 [7], the unique and previously unreported phenomenon in the field of protein biophysics, namely the existence of specific homotypic interactions between intrinsically disordered proteins (IDP), which is strongly distinct from the nonspecific aggregation of IDPs, remains a matter of debate in the field [8]. Nevertheless, researchers considered the observed homooligomerization of the cytoplasmic domains of MIRR signaling subunits [7] as a missing piece of the longstanding puzzle of MIRR triggering and developed a novel, all-inclusive platform of MIRR triggering, the

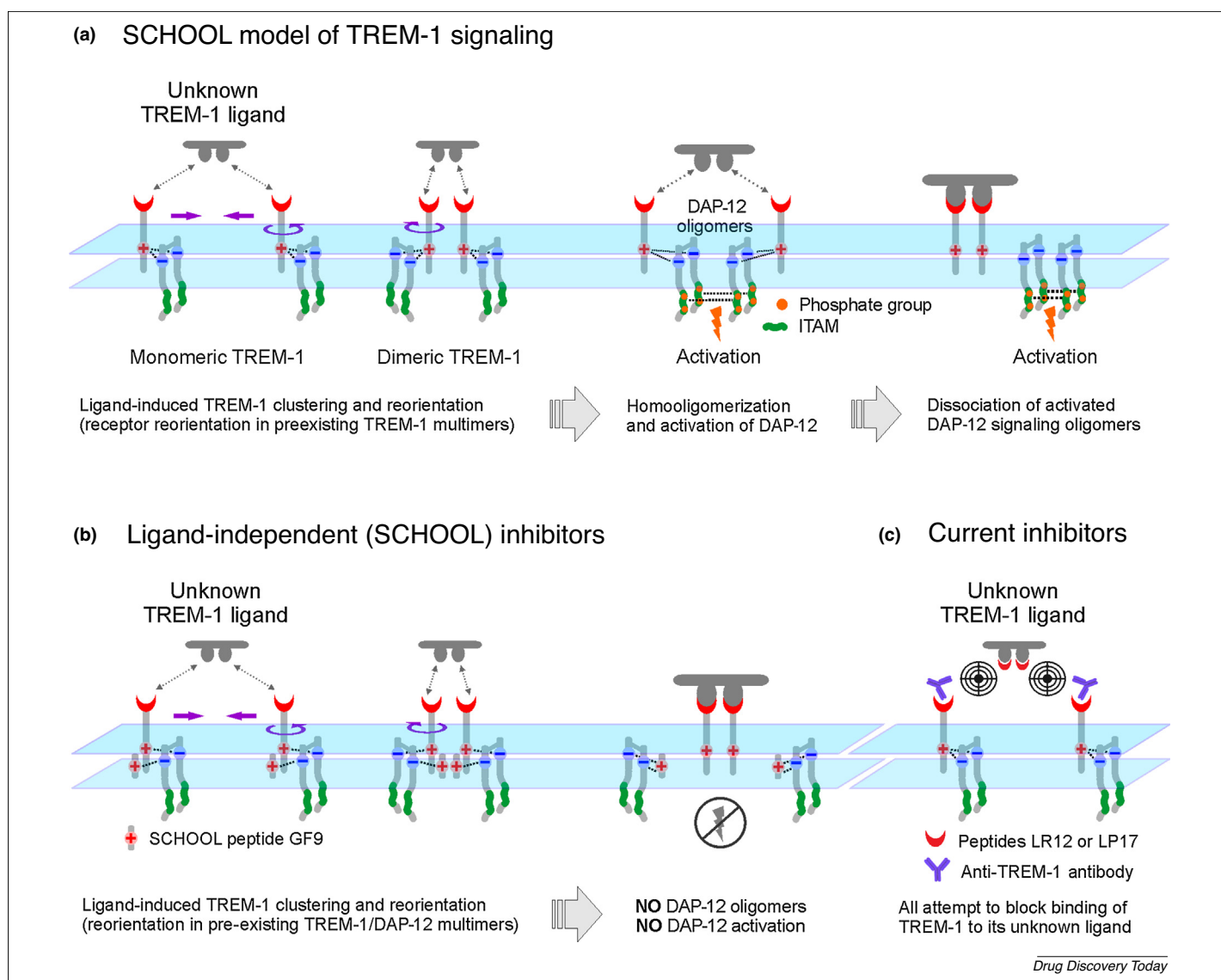
SCHOOL model [9]. The main SCHOOL concept is that the similar structural architecture of MIRRs (Fig. 1) dictates similar molecular mechanisms of their triggering.

In Fig. 2a, the SCHOOL platform is exemplary illustrated for TREM-1 triggering. Multivalent ligand binding outside the cell induces (or tunes) receptor oligomerization (clustering), which is then translated across the membrane into formation of competent DAP-12 signaling homooligomers in the cytoplasmic milieu, a force that drives transmembrane signaling and is necessary and sufficient to trigger TREM-1 and activate the cell (Fig. 2a). As mentioned earlier, the importance of ligand-induced oligomerization for TREM-1

triggering (Fig. 2a) predicted by the SCHOOL platform [9,10] was recently experimentally confirmed [6], illustrating the predictive power of this platform.

### SCHOOL immunomodulation strategy

Within the SCHOOL drug discovery platform, the similarity of the MIRR-triggering mechanisms provides the similarity of the drug targets revealed at the level of specific PPIs, that is, biochemical processes that can be influenced and controlled for therapeutic purposes [10,11]. The intramembrane PPIs between MIRR ligand-binding and signaling subunits represent one of these targets (Fig. 1).



**FIGURE 2**

Triggering receptor expressed on myeloid cells-1 (TREM-1) signaling and therapeutic inhibition. (a) Signaling chain homooligomerization (SCHOOL) model of TREM-1 signaling: formation of competent DNAX adapter protein 12 (DAP-12) signaling oligomers in the cytoplasmic milieu is the necessary and sufficient event to trigger TREM-1 and induce cell activation. (b) Ligand-independent TREM-1 blockade: SCHOOL inhibitors interrupt the intramembrane interactions between TREM-1 and DAP-12 and prevent formation of DAP-12 signaling oligomers upon binding to the multivalent TREM-1 ligand. Example shown for TREM-1 inhibitory SCHOOL peptide GF9. (c) Ligand-dependent TREM-1 blockade: conventional inhibitors attempt to block binding of TREM-1 to its still uncertain ligand(s). Example shown for anti-TREM-1 blocking antibodies and TREM-1 inhibitory peptides LR12 and LP17.

As illustrated for TREM-1, disruption of the intramembrane PPIs between TREM-1 and DAP-12 by short synthetic peptide sequences derived from the transmembrane domains (TMDs) of TREM-1 or DAP-12 (not shown) allows a novel, ligand-independent ('freedom to bind not to signal') approach to TREM-1 inhibition (Fig. 2b).

#### *Discovered by man, invented by nature*

The potential for the development and evolution of the immune system that nature provided by separation of recognition and signaling functions in the MIRR machinery (Fig. 1) [12] came at the cost of risk-taking. As revealed recently [13,14], several different viruses that are pathogenic for humans can uniformly target MIRRs (e.g., TCR). Although we cannot avoid these risks, we can benefit from learning from nature and use this billion years-old immunomodulation strategy to target the immune system for therapeutic purposes (reviewed in [15,16]).

#### **Ligand-independent (SCHOOL) immunomodulatory peptides**

Examples of use of the SCHOOL peptides that target cell receptors other than TREM-1 are discussed in this section.

#### *Science*

Peptides capable of inhibiting TCR-mediated cell activation in a ligand-independent manner have been known since 1997 [17] (Table 1). However, despite extensive studies (reviewed in [1]), the mechanism of their action remained enigmatic until the SCHOOL model was first introduced and applied to this field in 2004 [9]. Since then, the SCHOOL mechanism remains the only one that is consistent with all data reported so far for these peptides (reviewed in [1]). For example, no other model of TCR triggering can explain why the peptide GLRILLKLV derived from the TCR- $\alpha$  TMD (Table 1) inhibits antigen- but not antibody-mediated T cell activation [18] (see [1] for more details). Another striking example is that the SCHOOL model is the only one that explains mechanistically why the apparent discrepancy between *in vitro* and *in vivo* T cell inhibition results observed for the peptides derived from the TMDs of CD3 $\epsilon$ , CD3 $\delta$ , or CD3 $\gamma$  signaling subunits ([19], Table 1) is not a 'discrepancy' (see [1] for more details). Collectively, these and other numerous examples (reviewed in [1]) suggest that rationally designed SCHOOL peptides can be successfully used to study multiple aspects of MIRR-mediated cell activation. This is especially important for the studies of those MIRRs that signal through more than one signaling subunit

(e.g., TCR and B cell receptor, BCR) because it provides means for modulating specific signals and, as a result, specific cell responses.

#### *Medicine*

The peptide GLRILLKLV has been reported to inhibit T cell-mediated diseases, such as arthritis, neuritis, and diabetes, in relevant animal models [17,18,20–23] (Table 1). In humans, topical treatment with this peptide resulted in a marked improvement or cure of psoriasis, atopic eczema, lichen planus, or contact dermatitis, indicating that this therapy might be a proper treatment for human T cell-mediated dermatoses substituting for corticosteroids [24] (Table 1). The peptides derived from the CD3 $\alpha$ , CD3 $\delta$ , or CD3 $\gamma$  TMDs effectively inhibit an immune response and reduce signs of inflammation in the adjuvant arthritis rat model [19]. Although not tested *in vivo*, the peptides derived from the TMDs of different natural killer (NK) cell receptors have been reported to inhibit NK activity *in vitro* [25]. The treatment of whole-blood human samples with the glycoprotein VI (GPVI) inhibitory peptide GNLVRICLGAV designed using the SCHOOL drug discovery platform resulted in significant and specific reduction in both the percentage of P-selectin-positive platelets and the expression of the platelet activation markers, P-selectin and PAC-1 [26], suggesting a potential use of this inhibitor to inhibit thrombosis without affecting hemostatic plug formation.

#### **TREM-1 receptor: magic bullet in medicine** *Infectious and non-infectious diseases*

TREM-1, an inflammation amplifying receptor, was initially shown to have a role in sepsis [27]. Currently, the crucial pathophysiological role of TREM-1 is defined not only in infectious diseases [28], but also in both acute and chronic forms of aseptic inflammation (reviewed in [29,30]) as well as in cancer (reviewed in [31]). Examples are ischemia-reperfusion, hemorrhagic shock, pancreatitis, brain and spinal cord injuries, inflammatory bowel diseases, autoimmune and cardiovascular diseases, retinopathy, liver diseases, psoriasis, cystic fibrosis, Parkinson's disease, as well as lung, pancreatic, liver, brain, breast, and colon cancers.

Furthermore, at the time of writing, the WHO had reported 2 544 792 worldwide confirmed cases of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), including 175 694 deaths ([www.who.int/docs/default-source/coronaviruse/situation-reports](http://www.who.int/docs/default-source/coronaviruse/situation-reports)). Acute respiratory distress syndrome (ARDS), sepsis, and septic shock are life-threatening complications of

SARS-CoV-2 and common causes of death related to COVID-19 in intensive care units (ICU) [32]. Although most experimental COVID-19 therapeutics and vaccines currently aim to block the spread of the viral infection and treat early stages of the disease, therapeutics that slow down the progression from pneumonia to sepsis and ARDS in patients with COVID-19 could reduce the mortality rate in infected risk groups. In patients with cancer, who occupy up to 15% of all ICU beds, the incidence of ARDS is 11.9% of patients with cancer in the ICU, and 17.8% among those on mechanical ventilation [33]. Mortality caused by ARDS in patients with cancer is greater than that of other ICU populations [34]. In experimental ARDS, TREM-1 blockade has been shown to significantly reduce the lung inflammatory response and extend survival [35], highlighting the potential of TREM-1 as an emerging target for neutrophilic lung inflammation and ARDS in patients with COVID-19 and/or cancer.

Importantly, TREM-1 blockade blunts excessive inflammation, while preserving the capacity for microbial control, in contrast to currently widely used anti-single-cytokine therapies [e.g., anti-tumor necrosis factor (TNF)- $\alpha$  $\beta$ , anti-interleukin (IL)-1 $\beta$ , anti-IL-6, or anti-IL-6 receptor blockers] [36]. Recently, TREM-1 blockade was demonstrated to be safe and well tolerated in patients with septic shock [37]. Collectively, this implicates TREM-1 blockade as a new, highly promising therapeutic approach, a potential 'magic bullet' cure for a plethora of inflammation-associated diseases, disorders and conditions.

#### *Uncertainty of TREM-1 ligand(s)*

Conventional TREM-1 blockers, such as TREM-1 inhibitory peptides LP17 and LR12, first reported in 2006 [38] and 2013 [39], respectively, as well as an anti-TREM-1 blocking antibody first reported in 2016 [40], all attempt to block the receptor binding to its ligand(s) (Fig. 2c).

However, since the discovery of TREM-1 in 2000 [5], the actual nature of the TREM-1 ligand(s) remains uncertain (reviewed in [29]). In addition, different TREM-1 ligands are possibly involved in the pathogenesis of different inflammatory disorders [29]. This emphasizes the hurdles that need to be overcome before TREM-1-targeted therapy can become a clinical reality.

In this regard, ligand-independent TREM-1 inhibitory peptides developed using the SCHOOL drug discovery platform can represent a clinically advantageous avenue to modulate TREM-1 function for therapeutic purposes. Examples of the successful use of these inhibitors *in vitro* and *in vivo* are discussed in the following sections.

TABLE 1

**Ligand-independent inhibitory SCHOOL peptides/peptide sequences reported to date and their *in vitro* and *in vivo* activities<sup>a</sup>**

Receptor	Sequence <sup>b</sup>	Origin	Mechanism of action in cell membrane	<i>In vitro</i> activity	<i>In vivo</i> activity	Disease	Refs
TCR	GLRILLKLV	mTCR <sub>116–124</sub>	Disrupts TCRα–CD3δ $\epsilon$ , and TCRα– $\zeta\zeta$ interactions	Inhibits T cell activation <sup>c</sup>	Inhibits diseases in mice and humans	Arthritis, neuritis, diabetes, dermatoses	[17,18,20–24]
	MWKTPTLKYFG	SARS CoV <sub>770–780</sub> (SARS CoV FP)	Disrupts TCRα–CD3δ $\epsilon$ , and TCRα– $\zeta\zeta$ interactions	Not tested	Inhibits disease in mice	Arthritis	[14]
	IVIVDICT	hCD3 $\epsilon$ <sub>133–141</sub>	Disrupts TCRα–CD3δ $\epsilon$ and TCRβ–CD3 $\gamma\epsilon$ interactions	Not tested	Inhibits disease in mice	Arthritis	[19]
	IIVTDVIATL	hCD3 $\delta$ <sub>110–116</sub>	Disrupts TCRα–CD3δ $\epsilon$ interactions	Does not inhibit T cell activation	Inhibits disease in rats	Arthritis	[19]
	FLFAEIVSI	hCD3 $\gamma$ <sub>118–126</sub>	Disrupts TCRβ–CD3 $\gamma\epsilon$ interactions	Does not inhibit T cell activation	Inhibits disease in rats	Arthritis	[19]
NKp46	LLRMGLAFLV	hNKp46 <sub>256–265</sub>	Disrupts NKp46–FcR $\gamma$ ( $\zeta\zeta$ ) interactions	Inhibits NK cytotoxicity	Not tested	–	[25]
NKp44	GLLVAKSLVLSA	hNKp44 <sub>199–210</sub>	Disrupts NKp44–DAP12 interactions	Inhibits NK cytotoxicity <sup>d</sup>	Not tested	–	[25]
NKp30	GTVLLLRAGFYA	hNKp30 <sub>137–148</sub>	Disrupts NKp30– $\zeta\zeta$ interactions	Inhibits NK cytotoxicity	Not tested	–	[25]
NKG2D	AMGIRFIIMVA	hNKG2D <sub>62–72</sub>	Disrupts NKG2D–DAP10 interactions	Inhibits NK cytotoxicity	Not tested	–	[25]
NKG2C	MATVLKTIVLI	hNKG2C <sub>84–94</sub>	Disrupts NKG2C–DAP12 interactions	NS	Not tested	–	[25]
KIR2DS	VLIGTSVVKIPFTILL	hKIR2DS <sub>246–261</sub>	Disrupts KIR2DS–DAP12 interactions	Inhibits NK cytotoxicity	Not tested	–	[25]
GPVI	GNLVRICLGAV	hGPVI <sub>268–278</sub>	Disrupts GPVI–FcR $\gamma$ interactions	Reduces platelet activation	Not tested	Thrombosis	[26]
TREM-1	GLLSKSLVF	mTREM-1 <sub>210–218</sub>	Disrupts TREM1–DAP12 interactions	Inhibits LPS-induced cell activation	Extends survival in mice	Sepsis, NSCLC	[41]
	GFLSKSLVF	hTREM-1 <sub>213–221</sub>	Disrupts TREM1–DAP12 interactions	–	Inhibits disease in mice	Arthritis	[42]
	GFLSKSLVF	hTREM-1 <sub>213–221</sub>	Disrupts TREM1–DAP12 interactions	–	Synergizes with chemotherapies; extends survival in mice	Pancreatic cancer	[48]
	GFLSKSLVF	hTREM-1 <sub>213–221</sub>	Disrupts TREM1–DAP12 interactions	–	Reduces TGF- $\beta$ ; sensitizes tumor to radiation; extends survival in mice	Glioma	[51]
	GFLSKSLVF	hTREM-1 <sub>213–221</sub>	Disrupts TREM1–DAP12 interactions	–	Prevents retinal neovascularization in mice	Retinopathy	[43]
	GFLSKSLVF	hTREM-1 <sub>213–221</sub>	Disrupts TREM1–DAP12 interactions	–	Reduces fibrosis and inflammation; inhibits disease in mice	ALD	[44]
	GFLSKSLVF	hTREM-1 <sub>213–221</sub>	Disrupts TREM1–DAP12 interactions	–	Overcomes anti-PD-L1 resistance; extends survival in mice	Liver cancer	[45]
	LSKSLVF	hTREM-1 <sub>215–221</sub>	Disrupts TREM1–DAP12 interactions	Inhibits LPS-induced cell activation	Extends survival in mice	Sepsis	[49]

<sup>a</sup>Abbreviations: FP, fusion peptide; h, human; m, mouse.<sup>b</sup>Positively charged amino acid residues are indicated by red, negatively charged residues are indicated by blue.<sup>c</sup>Stimulated by antigen but not by anti-CD3 or anti-TCR $\beta$  antibodies.<sup>d</sup>Only when five N-terminal Lys residues are added to the peptide sequence: KKKKKGLLVAKSLVLSA.

### Ligand-independent therapeutic inhibition of TREM-1 *in vitro* and *in vivo*

#### Working from outside and inside the cell

The unique feature of ligand-independent immunomodulatory SCHOOL peptides to reach their site of action in the cell membrane from

both outside and inside the cell enables their use in free form or incorporated into cell-targeted delivery vehicles.

As shown for a 9-amino acid TREM-1 inhibitory SCHOOL peptide (GF9), this peptide, when administered in free form, reaches its

intramembrane site of action from outside the cell by self-inserting into the cell membrane and colocalizing with the TREM-1/DAP-12 receptor complex (Fig. 3, route 1). TREM-1 inhibitory SCHOOL peptides mimic natural transmembrane protein sequences that are highly



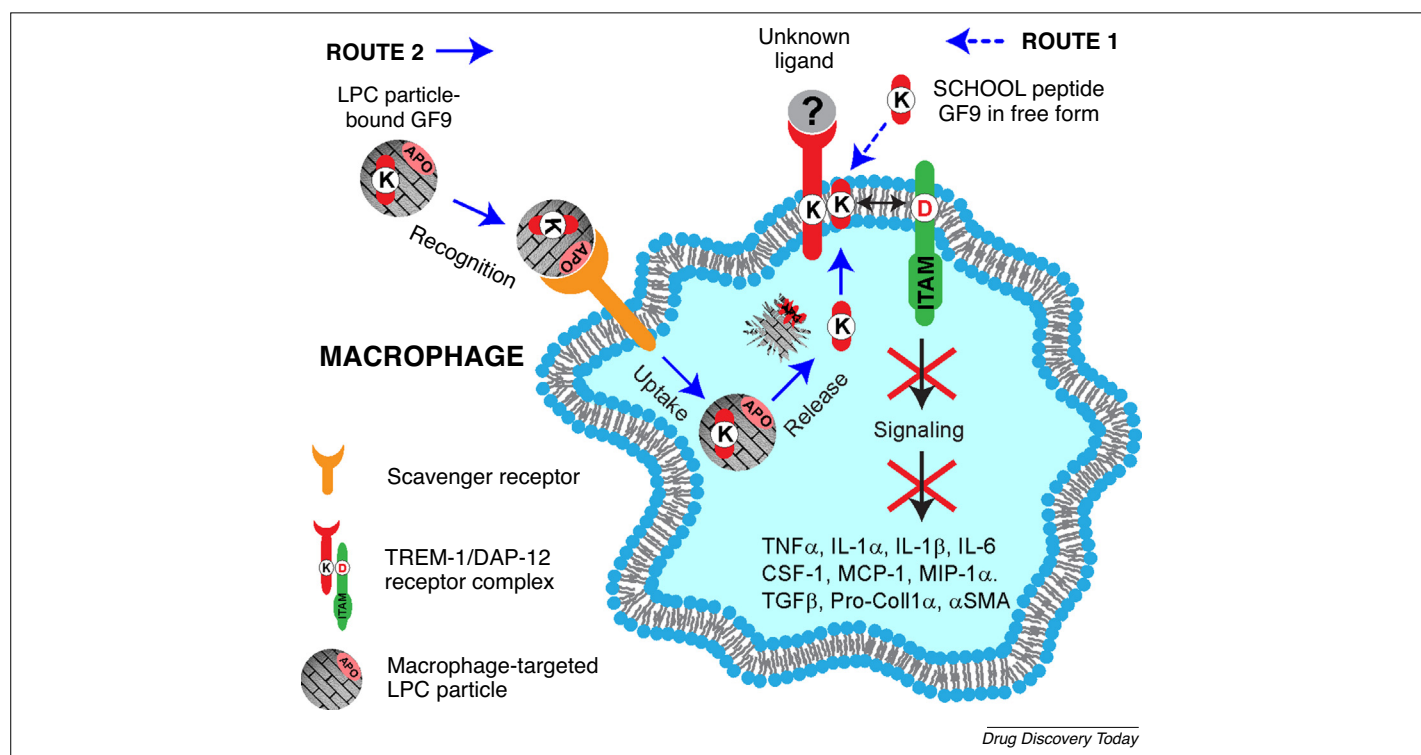


FIGURE 3

Two delivery routes of signaling chain homooligomerization (SCHOOL) inhibitors to their intramembrane site of action. SCHOOL inhibitors used in free form or formulated into cell-specific delivery vehicles can reach their site of action from either outside (route 1) or inside the cell (route 2), respectively. Example shown of TREM-1-expressing macrophages and TREM-1 inhibitory SCHOOL peptide GF9 in free form and formulated into macrophage-targeted lipopeptide complexes (LPC).

conserved between species. Thus, good tolerability and low toxicity of these short synthetic peptides can be anticipated. Indeed, when administered short- or long-term at doses up to at least 300 mg/kg, free GF9 was well tolerated by healthy and diseased mice with no toxicity signs and symptoms observed [41–45].

Alternatively, cell-specific delivery systems and/or constructs can be used to deliver these peptides to TREM-1-expressing cells, such as macrophages. In this scenario, the intracellularly released peptides (peptide constructs) self-insert into the membrane from inside the cell and colocalize with the TREM-1/DAP-12 receptor complex (Fig. 3, route 2). Examples include the use of nature-inspired lipoprotein or lipopeptide complexes (LPC) that mimic human high-density lipoproteins (HDL). In contrast to native HDL, which is not normally endocytosed by macrophages, these complexes contain the synthetic 22-mer fragment(s) of the apolipoprotein (apo) A-I molecule, the main protein constituent of HDL, rationally designed and modified to provide targeted delivery of imaging agents and/or drugs, such as TREM-1 inhibitory SCHOOL peptides (e.g., GF9) to macrophages, enabling the detection and/or treatment of inflammation [41–44,46–48].

In other studies, a 9-amino acid sequence of GF9 was combined with that of the apo A-I 22-mers to generate so-called 'trifunctional' peptides (GA31 and GE31). These peptides were able to assist in the self-assembly of LPC, targeting these complexes to macrophages and inhibiting TREM-1 *in vitro* and *in vivo* [42–44,48]. Recently, other researchers used a similar strategy and embedded a 7-amino acid sequence LSKSLVF (GF9 sequence with two truncated N-terminal residues, Table 1) into a construct capable of inhibiting TREM-1 in the endothelium [49].

Confocal microscopy revealed that free GF9 as well as intracellularly delivered GF9 or GE31 colocalize with TREM-1 in the macrophage membrane [43,48]. This further confirms the SCHOOL mechanism of their action as well as two routes of reaching their intramembrane site of action (Fig. 3). Later, colocalization of LSKSLVF-containing construct with TREM-1 in the endothelial cell membrane was reported [49], expanding findings in macrophages [43,48] to other TREM-1-expressing cells.

Cell-specific delivery of systemically administered TREM-1 SCHOOL peptides could have several advantages: (i) striking the target cell population; (ii) sparing other cells that have no

(or only marginal) effects on the TREM-1-involved disease or condition; (iii) minimizing off-target effects; and (iv) reducing the therapeutic dose. Although some *in vivo* data generated to date and discussed below support this view, further studies are needed to elucidate whether the potential pharmacological advantages in specific applications outweigh more complex manufacturing and regulatory requirements and challenges for the cell-targeted products.

All *in vitro* and *in vivo* studies of ligand-independent TREM-1 inhibitory peptides reported to date are summarized in Table 1.

### Sepsis

The first successful use of a TREM-1 inhibitory SCHOOL peptide (GF9) in sepsis was reported in 2014 [41] (Table 1). Systemically administered free GF9 at 25 mg/kg and macrophage-targeted LPC-formulated GF9 (GF9-LPC) at 5 mg GF9/kg both suppressed TREM-1-mediated production of proinflammatory cytokines  $\text{TNF}\alpha$ ,  $\text{IL-1}\beta$ , and  $\text{IL-6}$  *in vitro* [lipopolysaccharide (LPS)-stimulated J774 macrophages] and *in vivo* (mice with LPS-induced endotoxemia). Both formulations significantly extended the survival of mice with sepsis. The effect was concentration dependent and specific: neither free GF9 at 5 mg/kg nor

free control peptide GLLSGSLVF with a single amino acid substitution of functionally important lysine (highlighted in red in Table 1) for glycine (underlined) at 25 mg/kg were effective [10,41].

Later, the efficacy of TREM-1 ligand-independent inhibition in sepsis was confirmed in another mouse model, the cecal ligation and puncture (CLP) polymicrobial sepsis model [49]. The authors used a construct containing the E-selectin targeting domain and the translocation domain of *Pseudomonas aeruginosa* exotoxin A to deliver the TREM-1 inhibitory SCHOOL sequence LSKSLVF (Table 1) to endothelial cells. The sequence was demonstrated to reduce LPS-induced endothelial cell activation *in vivo* and to confer protection during experimental peritonitis in mice [49].

### Cancer

Ho *et al.* [50] observed that patients with non-small cell lung cancer (NSCLC) with low TREM-1 expression on tumor-associated macrophages (TAMs) had more than a three times higher chance of surviving the first 4 years after diagnosis compared with those with high TREM-1 expression. To establish whether a cause–effect relationship underlies these observations, GF9 therapy was tested in experimental NSCLC [41] (Table 1). The data generated in two NSCLC xenograft models in nude mice demonstrated for the first time a therapeutic efficacy of ligand-independent inhibition of TREM-1 in the treatment of cancer. Whereas 5 mg/kg GF9 did not exhibit any therapeutic activity, 25 mg/kg GF9 and GF9-LPC at 5 mg GF9/kg suppressed tumor growth as effectively as 20 mg/kg paclitaxel (PTX) used as a positive control [41]. In addition, this study also demonstrated the utility of xenograft models in nude mice (with intact macrophage function) for studying macrophage-targeted agents.

By linking inflammation, immunity, and cancer, TAMs have a crucial role in fostering tumor growth and progression in multiple types of tumor. Successful use of GF9 therapy in experimental pancreatic cancer [48] further confirmed the pan-cancer nature of the TREM-1 target. All TREM-1 SCHOOL inhibitory formulations used in this study strongly suppressed the tumor growth and extended animal survival in several xenograft models [48]. The long-lasting antitumor response to GF9 therapy persisted after treatment and correlated significantly with reduced TAM infiltration [48]. As anticipated, the noncytotoxic TREM-1 inhibitory SCHOOL peptides were well tolerated during long-term administration when deployed either in free form

or formulated into macrophage-targeted LPC for peptide half-life extension and targeted delivery. The study revealed for the first time that, in mice with cancer, TREM-1 blockade using TREM-1 inhibitory SCHOOL peptides significantly reduced serum levels of IL-1 $\alpha$ , IL-6, and macrophage colony-stimulating factor (M-CSF or CSF-1), but not vascular endothelial growth factor (VEGF), suggesting a possible contribution of CSF-1-dependent antitumor mechanisms to the observed therapeutic activity [48]. Furthermore, in xenograft models, GF9 therapy standalone was as effective as a first-line standard chemotreatment (gemcitabine + nanoparticle albumin-bound PTX, Abraxane; GEM + ABX), whereas, in combination with GEM + ABX, it synergistically suppressed pancreatic tumor growth and increased survival more than threefold over chemotherapy alone (Alexander Sigalov, unpublished observations, 2018). Future studies need to elucidate whether TREM-1 inhibitory SCHOOL peptides synergize with standard cancer immunotherapy (e.g., anti PD-1/PD-L1), which is largely ineffective in patients with pancreatic cancer.

By mimicking native HDL, LPC are able to cross the blood–brain barrier (BBB). In the mouse glioma 261 tumor model, macrophage-specific LPC-formulated GF9 sensitized glioblastoma tumors to radiation and significantly (up to sixfold) lowered TGF- $\beta$  levels in brain TAMs, which is consistent with data from *in vitro* studies of TREM-1 [51].

Recent independent studies in experimental hepatocellular carcinoma (HCC) [45] (Table 1) revealed that TREM-1 blockade using GF9 at 25 mg/kg significantly attenuated CD8 + T cell dysfunction and abrogated the resistance to PD-L1 blockade. This suggests TREM-1 inhibitory SCHOOL peptides as novel mechanism-based drug candidates to improve anti-PD-L1 therapeutic efficacy in HCC and other resistant cancers (e.g., pancreatic and triple-negative breast cancers).

Collectively, these data encourage further studies of TREM-1 inhibitory SCHOOL peptides as safe and noncytotoxic effective therapies to be used stand alone or in combination with current first-line chemo- and immunotherapies for the treatment of multiple types of cancer.

### Arthritis

In mice with collagen-induced arthritis, free GF9 (but not control peptide GFLSGSLVF at the same dose) and macrophage-targeted LPC containing either GF9 or trifunctional peptides GA31 and GE31 significantly suppressed release of plasma TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and CSF-1, decreased

inflammation and strongly protected against bone and cartilage destruction [42]. This expands the range of potential therapeutic applications for TREM-1 inhibitory SCHOOL peptides to autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, atopic dermatitis, colitis, and inflammatory bowel disease, where TREM-1 blockade is of clinical importance.

### Retinopathy

In a mouse model of oxygen-induced retinopathy (OIR), systemically administered macrophage-targeted LPC formulations of GF9 (alone or as a part of trifunctional peptides GA31 and GE31) were well tolerated and significantly (up to 95%) reduced pathological retinal neovascularization [43]. This indicates that ligand-independent TREM-1 inhibition can be used in the prevention and treatment of retinal neovascular diseases. The study also demonstrated the ability of the TREM-1 inhibitory formulations used to not only cross the BBB, but also to penetrate the blood–retina barrier (BRB). Importantly, TREM-1 blockade using GF9 therapy substantially downregulated retinal levels of TREM-1 and CSF-1 but not VEGF [43], suggesting that the mechanisms of TREM-1-dependent suppression of pathological but not physiological angiogenesis involve CSF-1. Thus, GF9 therapy can represent a safe and effective alternative to the current VEGF-targeted therapy that is complicated by damage of healthy vessels, potential adverse effects on neurons, rapid vascular regrowth upon interrupting VEGF blockade, and limited effectiveness in some patients.

### Liver disease

Therapeutic activity of macrophage-targeted LPC formulations of GF9 (alone or as a part of trifunctional peptides GA31 and GE31) recently demonstrated in an alcoholic liver disease (ALD) mouse model [44] further confirmed the potential of ligand-independent TREM-1 inhibition to create therapeutic opportunities for multiple inflammation-associated diseases. The TREM-1 inhibitory SCHOOL formulations used in this study all ameliorated early phases of inflammation and neutrophil and macrophage recruitment and activation in the liver, and attenuated hepatocyte damage and liver steatosis [44]. Given that ALD and nonalcoholic steatohepatitis (NASH) are similar in terms of pathological observations and pathogenesis, these findings suggest that GF9 therapy represents a promising therapeutic approach for the treatment of both liver diseases.

## Concluding remarks

By uncovering the molecular mechanisms of transmembrane signal transduction and revealing key points of its therapeutic control at the level of druggable PPIs, the SCHOOL drug discovery platform significantly contributes to the development of novel pharmacological approaches.

Importantly, the platform suggests that the common structural architecture of functionally unrelated cell receptors dictates the same molecular mechanisms of their triggering. This not only overcomes the limitations of the decades-long 'Blind men and an elephant' paradigm in our molecular understanding of transmembrane signaling, but also suggests for the first time the similarity of therapeutic targets in seemingly unrelated diseases. This, in turn, makes possible the development of global pharmacological approaches by transferring and exchanging our scientific and clinical knowledge, experience, and therapeutic strategies between these diseases.

One of the most interesting and important features of the SCHOOL platform-revealed immunomodulatory strategies is their independence on ligand binding. This is especially important in therapeutic targeting of TREM-1 receptor, the ligand(s) of which remain uncertain.

Successful applications of the SCHOOL strategy *in vitro* and *in vivo* to therapeutically target the intramembrane PPIs involved in triggering of unrelated receptors that are expressed on different cells (e.g., TCR, GPVI, NK receptors, and TREM-1) provided compelling evidence to support the utility of the SCHOOL platform in the drug discovery and development. Furthermore, the use of the SCHOOL mechanisms by different viruses to disarm the immune system and escape the host immune response provides a unique example of how, by learning from nature, we can elucidate the billions years-old strategies that nature uses in organismal evolution and function and utilize them for therapeutic purposes.

## Conflict of interests

A.B.S. is employed by SignaBlok, Inc., a company developing ligand-independent TREM-1 inhibitors.

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## References

- Sigalov, A.B. (2010) The SCHOOL of nature: I. Transmembrane signaling. *Self Nonself*. 1, 4–39
- Keegan, A.D. and Paul, W.E. (1992) Multichain immune recognition receptors: similarities in structure and signaling pathways. *Immunol. Today* 13, 63–68
- Xu, X. *et al.* (2020) Structural understanding of T cell receptor triggering. *Cell. Mol. Immunol.* 17, 193–202
- Abastado, J.P. *et al.* (1995) Dimerization of soluble major histocompatibility complex-peptide complexes is sufficient for activation of T cell hybridoma and induction of unresponsiveness. *J. Exp. Med.* 182, 439–447
- Bouchon, A. *et al.* (2000) Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol.* 164, 4991–4995
- Carrasco, K. *et al.* (2019) TREM-1 multimerization is essential for its activation on monocytes and neutrophils. *Cell. Mol. Immunol.* 16, 460–472
- Sigalov, A. *et al.* (2004) Homooligomerization of the cytoplasmic domain of the T cell receptor  $\zeta$  chain and of other proteins containing the immunoreceptor tyrosine-based activation motif. *Biochemistry* 43, 2049–2061
- Nourse, A. and Mittag, T. (2014) The cytoplasmic domain of the T-cell receptor zeta subunit does not form disordered dimers. *J. Mol. Biol.* 426, 62–70
- Sigalov, A.B. (2004) Multichain immune recognition receptor signaling: different players, same game? *Trends Immunol.* 25, 583–589
- Sigalov, A.B. (2006) Immune cell signaling: a novel mechanistic model reveals new therapeutic targets. *Trends Pharmacol. Sci.* 27, 518–524
- Sigalov, A.B. (2010) The SCHOOL of nature: III. From mechanistic understanding to novel therapies. *Self Nonself*. 1, 192–224
- Sigalov, A.B. (2012) Evolution of immunity: no development without risk. *Immunol. Res.* 52, 176–181
- Sigalov, A.B. (2009) Novel mechanistic insights into viral modulation of immune receptor signaling. *PLoS Pathog.* 5, e1000404
- Shen, Z.T. and Sigalov, A.B. (2016) SARS coronavirus fusion peptide-derived sequence suppresses collagen-induced arthritis in DBA/1J mice. *Sci. Rep.* 6, 28672
- Sigalov, A.B. (2010) The SCHOOL of nature: IV. Learning from viruses. *Self Nonself*. 1, 282–298
- Sigalov, A.B. (2018) Targeting intramembrane protein-protein interactions: novel therapeutic strategy of millions years old. *Adv. Protein Chem. Struct. Biol.* 111, 61–99
- Manolios, N. *et al.* (1997) T-cell antigen receptor transmembrane peptides modulate T-cell function and T cell-mediated disease. *Nat. Med.* 3, 84–88
- Wang, X.M. *et al.* (2002) T-cell antigen receptor peptides inhibit signal transduction within the membrane bilayer. *Clin. Immunol.* 105, 199–207
- Collier, S. *et al.* (2006) Discrepancy in CD3-transmembrane peptide activity between *in vitro* and *in vivo* T-cell inhibition. *Scand. J. Immunol.* 64, 388–391
- Wang, X.M. *et al.* (2002) T cell antigen receptor (TCR) transmembrane peptides colocalize with TCR, not lipid rafts, in surface membranes. *Cell. Immunol.* 215, 12–19
- Wong, M.S. *et al.* (2014) Novel T-cell inhibiting peptides delay the onset of Type 1 diabetes in non-obese diabetic mice. *Diabetes Metab.* 40, 229–234
- Vanniasinghe, A.S. *et al.* (2014) Targeting fibroblast-like synovial cells at sites of inflammation with peptide targeted liposomes results in inhibition of experimental arthritis. *Clin. Immunol.* 151, 43–54
- Amon, M.A. *et al.* (2006) Lipidation and glycosylation of a T cell antigen receptor (TCR) transmembrane hydrophobic peptide dramatically enhances *in vitro* and *in vivo* function. *Biochim. Biophys. Acta* 1763, 879–888
- Gollner, G.P. *et al.* (2000) Therapeutic application of T cell receptor mimic peptides or cDNA in the treatment of T cell-mediated skin diseases. *Gene Ther.* 7, 1000–1004
- Vandebona, H. *et al.* (2006) Immunoreceptor transmembrane peptides and their effect on natural killer (NK) cell cytotoxicity. *Protein Pept. Lett.* 13, 1017–1024
- Sigalov, A.B. (2007) More on: glycoprotein VI oligomerization: a novel concept of platelet inhibition. *J. Thromb. Haemost.* 5, 2310–2312
- Bouchon, A. *et al.* (2001) TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature* 410, 1103–1107
- Roe, K. *et al.* (2014) Triggering receptor expressed on myeloid cells-1 (TREM-1): a new player in antiviral immunity? *Front. Microbiol.* 5, 627
- Tammaro, A. *et al.* (2017) TREM-1 and its potential ligands in non-infectious diseases: from biology to clinical perspectives. *Pharmacol. Ther.* 177, 81–95
- Pelham, C.J. and Agrawal, D.K. (2014) Emerging roles for triggering receptor expressed on myeloid cells receptor family signaling in inflammatory diseases. *Expert Rev. Clin. Immunol.* 10, 243–256
- Nguyen, A.H. *et al.* (2015) Chronic inflammation and cancer: emerging roles of triggering receptors expressed on myeloid cells. *Expert Rev. Clin. Immunol.* 11, 849–857
- Wujtewicz, M. *et al.* (2020) COVID-19 - what should anaesthesiologists and intensivists know about it? *Anaesthesiol. Intensive Ther.* 52, 34–41
- Cardoso, E.M. *et al.* (2018) Acute respiratory distress syndrome in cancer patients: epidemiology, risk factors and outcomes. *Eur. J. Oncol.* 23, 30–40
- Azoulay, E. *et al.* (2014) Acute respiratory distress syndrome in patients with malignancies. *Intensive Care Med.* 40, 1106–1114
- Yuan, Z. *et al.* (2016) TREM-1-accentuated lung injury via miR-155 is inhibited by LP17 nanomedicine. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 310, L426–L438
- Weber, B. *et al.* (2014) TREM-1 deficiency can attenuate disease severity without affecting pathogen clearance. *PLoS Pathog.* 10, e1003900
- Francois, B. *et al.* (2018) P1 Safety and pharmacodynamic activity of a novel TREM-1 pathway inhibitory peptide in septic shock patients: phase IIa clinical trial results. *Intensive Care Med Exp.* 6 (Suppl. 1), 1–33
- Gibot, S. *et al.* (2006) Modulation of the triggering receptor expressed on myeloid cells-1 pathway during pneumonia in rats. *J. Infect. Dis.* 194, 975–983
- Derive, M. *et al.* (2013) Effects of a TREM-like transcript 1-derived peptide during hypodynamic septic shock in pigs. *Shock* 39, 176–182
- Brynjolfsson, S.F. *et al.* (2016) An antibody against triggering receptor expressed on myeloid cells 1 (TREM-1) dampens proinflammatory cytokine secretion by lamina propria cells from patients with IBD. *Inflamm. Bowel Dis.* 22, 1803–1811
- Sigalov, A.B. (2014) A novel ligand-independent peptide inhibitor of TREM-1 suppresses tumor growth in human lung cancer xenografts and prolongs survival of mice with lipopolysaccharide-induced septic shock. *Int. Immunopharmacol.* 21, 208–219



- 42 Shen, Z.T. and Sigalov, A.B. (2017) Rationally designed ligand-independent peptide inhibitors of TREM-1 ameliorate collagen-induced arthritis. *J. Cell. Mol. Med.* 21, 2524–2534
- 43 Rojas, M.A. *et al.* (2018) Blockade of TREM-1 prevents vitreoretinal neovascularization in mice with oxygen-induced retinopathy. *Biochim. Biophys. Acta* 1864, 2761–2768
- 44 Tornai, D. *et al.* (2019) Inhibition of triggering receptor expressed on myeloid cells 1 ameliorates inflammation and macrophage and neutrophil activation in alcoholic liver disease in mice. *Hepatol. Commun.* 3, 99–115
- 45 Wu, Q. *et al.* (2019) Blocking triggering receptor expressed on myeloid cells-1-positive tumor-associated macrophages induced by hypoxia reverses immunosuppression and anti-programmed cell death ligand 1 resistance in liver cancer. *Hepatology* 70, 198–214
- 46 Sigalov, A.B. (2014) Nature-inspired nanoformulations for contrast-enhanced *in vivo* MR imaging of macrophages. *Contrast Media Mol. Imaging* 9, 372–382
- 47 Shen, Z.T. *et al.* (2015) Diagnostic magnetic resonance imaging of atherosclerosis in apolipoprotein E knockout mouse model using macrophage-targeted gadolinium-containing synthetic lipopeptide nanoparticles. *PLoS ONE* 10, e0143453
- 48 Shen, Z.T. and Sigalov, A.B. (2017) Novel TREM-1 inhibitors attenuate tumor growth and prolong survival in experimental pancreatic cancer. *Mol. Pharm.* 14, 4572–4582
- 49 Gibot, S. *et al.* (2019) Triggering receptor expressed on myeloid cells-1 inhibitor targeted to endothelium decreases cell activation. *Front. Immunol.* 10, 2314
- 50 Ho, C.C. *et al.* (2008) TREM-1 expression in tumor-associated macrophages and clinical outcome in lung cancer. *Am. J. Respir. Crit. Care Med.* 177, 763–770
- 51 Mark, D.J. *et al.* (2019) Targeting tumor-associated macrophages to radiosensitize glioblastoma in a mouse model. *Int. J. Radiation Oncol. Biol. Phys.* 105, E646–E647

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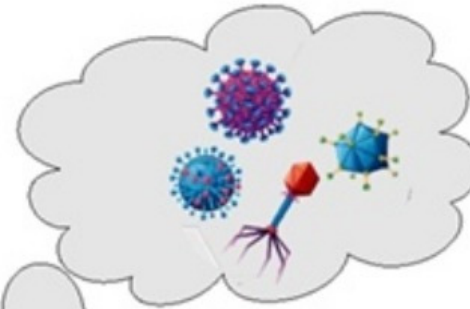
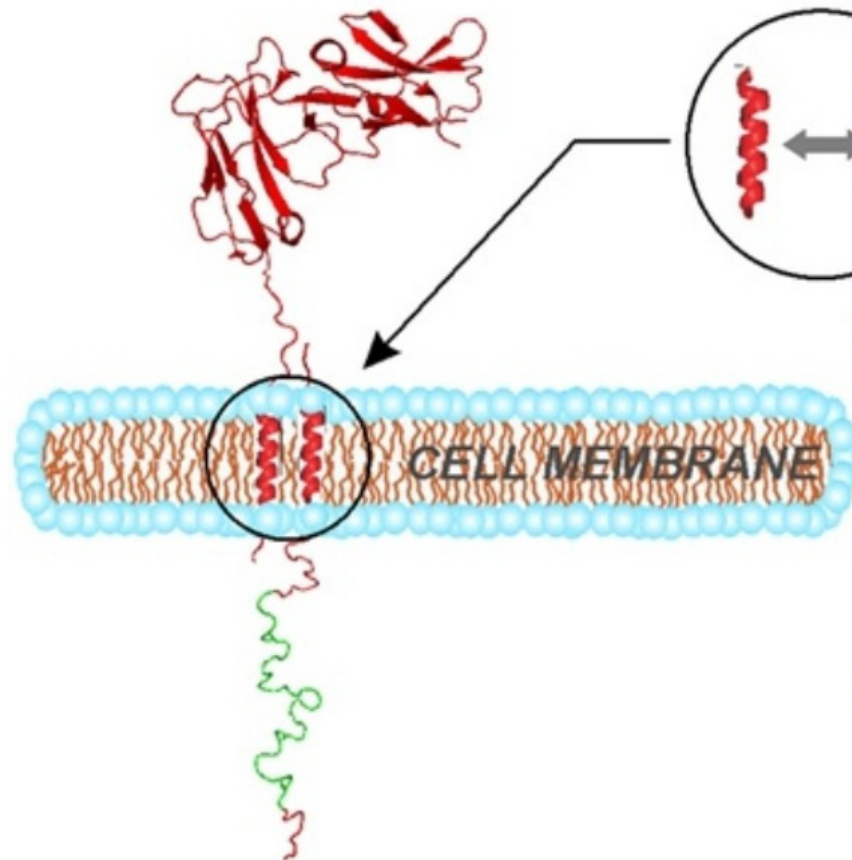
*SignaBlok, Inc., Shrewsbury, MA, USA*

## RISKY POINT

*Point of viral attack  
to disarm the cell*

## Multichain cell receptor

- **Different** protein subunits provide ligand binding outside the cell and signaling inside the cell



**"There are two sides  
to every question"**  
*Protagoras*



## SCHOOL DRUG TARGET

*Novel drug target to treat cancer,  
sepsis, cardiovascular diseases,  
rheumatoid arthritis, and others*