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CHAPTER TWO

Targeting Intramembrane Protein–Protein Interactions: Novel Therapeutic Strategy of Millions Years Old

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Contents

Abstract

Intramembrane protein–protein interactions (PPIs) are involved in transmembrane signal transduction mediated by cell surface receptors and play an important role in health and disease. Recently, receptor-specific modulatory peptides rationally designed using a general platform of transmembrane signaling, the signaling chain homooligomerization (SCHOOL) model, have been proposed to therapeutically target these interactions in a variety of serious diseases with unmet needs including cancer, sepsis, arthritis, retinopathy, and thrombosis. These peptide drug candidates use ligand-independent mechanisms of action (SCHOOL mechanisms) and demonstrate potent efficacy in vitro and in vivo. Recent studies surprisingly revealed that in order to modify and/or escape the host immune response, human viruses use similar mechanisms and

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modulate cell surface receptors by targeting intramembrane PPIs in a ligand-independent manner. Here, I review these intriguing mechanistic similarities and discuss how the viral strategies optimized over a billion years of the coevolution of viruses and their hosts can help to revolutionize drug discovery science and develop new, disruptive therapies. Examples are given.

1. INTRODUCTION

Protein–protein interactions (PPIs) are involved in the vast majority of biological processes (Fletcher & Hamilton, 2006; Fry, 2006; Ryan & Matthews, 2005; Toogood, 2002; Zinzalla & Thurston, 2009). Nearly every important pathway in health and disease includes and is critically influenced by PPIs (Fry, 2006). In this context, the specific and controlled modulation (inhibition) of PPIs provides a promising avenue for rational drug design, as revealed by recent progress in the design of inhibitory antibodies, peptides, and small molecules (Berg, 2008; Che, Brooks, & Marshall, 2006; Fry, 2006; Pagliaro et al., 2004; Robinson, 2009; Sillerud & Larson, 2005; Veselovsky & Archakov, 2007).

Cell surface receptors are integral membrane proteins that translate extracellular information into intracellular signaling sequences and further into physiological cell response in the complex fundamental process called transmembrane signal transduction. This process plays a crucial role in health and disease (Kadmiel & Cidlowski, 2013; Rudd, 2006; Sabroe et al., 2003; Sigalov, 2008a, 2010g), which makes therapeutic control of PPIs involved in this process of both fundamental and clinical importance. However, until recently, the lack of a mechanistic understanding of what PPIs are actually taking place during transmembrane signal transduction has significantly impeded progress not only in fundamental studies in biology and life sciences but also in molecular target-driven drug discovery. The result of this lack of understanding is that the most widely used therapeutic strategies of receptor modulation attempt to prevent binding of receptors to their cognate ligands using clinically relevant antibodies (Hansel, Kropshofer, Singer, Mitchell, & George, 2010) or soluble receptor domains (Bouchon, Facchetti, Weigand, & Colonna, 2001; Molloy, Sewell, & Jakobsen, 2005). However, these large protein entities possess severe disadvantages including a long and costly development process, low activity per mass, manufacturing difficulties, unintended immune response, side effects, low organ and tissue penetration, and a lack of oral bioavailability (Hansel et al., 2010).

As integral membrane proteins, cell surface receptors consist of three domains: (1) extracellular ligand-binding domains that function to detect and bind external stimuli, (2) transmembrane domains that function as anchors to the cell membrane and mediators of signal transduction, and (3) cytoplasmic signaling domains that initiate an intracellular signal transduction cascades. Depending on whether extracellular and intracellular domains are located on the same or separate protein chains, unrelated and functionally diverse cell receptors can be divided into two main structural families: single-chain and multichain receptors (SRs and MRs), respectively (Fig. 1) (Keegan & Paul, 1992; Sigalov, 2004, 2010c). MRs most commonly represent immune receptors and are often referred to as multichain immune recognition receptors (MIRRs) (Keegan & Paul, 1992; Sigalov, 2004). The signature feature of MIRRs is the presence of one or more copies of the immunoreceptor tyrosine-based activation motif (ITAM) regions (Reth, 1989) or the YxxM motif (Wu, Cherwinski, Spies, Phillips, & Lanier, 2000) in their cytoplasmic signaling domains. Upon receptor triggering, tyrosine residues of the ITAM/YxxM regions are phosphorylated in an early and obligatory event in the signaling cascade.

A general platform for receptor-mediated transmembrane signal transduction, the signaling chain homooligomerization (SCHOOL) platform (Sigalov, 2004, 2006, 2008a, 2008b, 2008c, 2010b, 2010c) uncovers for the first time the major mechanisms coupling recognition and signaling functions. Based on the unusual biophysical phenomenon—the ability of the intrinsically disordered cytoplasmic domains of MIRR signaling subunits to form specific homodimers and higher homooligomers (Sigalov, Aivazian, & Stern, 2004; Sigalov, Zhuravleva, & Orekhov, 2007), this platform was originally suggested for MIRRs (Sigalov, 2004) and then—as a general platform for transmembrane signaling mediated by MIRRs and SRs (Sigalov, 2008a, 2010c). Conceptually, the SCHOOL platform suggests that the similar architecture of the receptors dictates similar mechanisms of receptor triggering. Mechanistically, according to the platform, multivalent ligand binding outside the cell induces (or tunes) receptor oligomerization (clustering) which is then translated across the membrane into formation of competent signaling homooligomers in the cytoplasmic milieu—a force that drives transmembrane signaling and is necessary and sufficient to trigger receptor activation (Fig. 1). This provides the similarity of the targets revealed at the level of specific PPIs—biochemical processes that can be influenced and controlled for therapeutic purposes (Sigalov, 2004, 2008a, 2010b, 2010c).

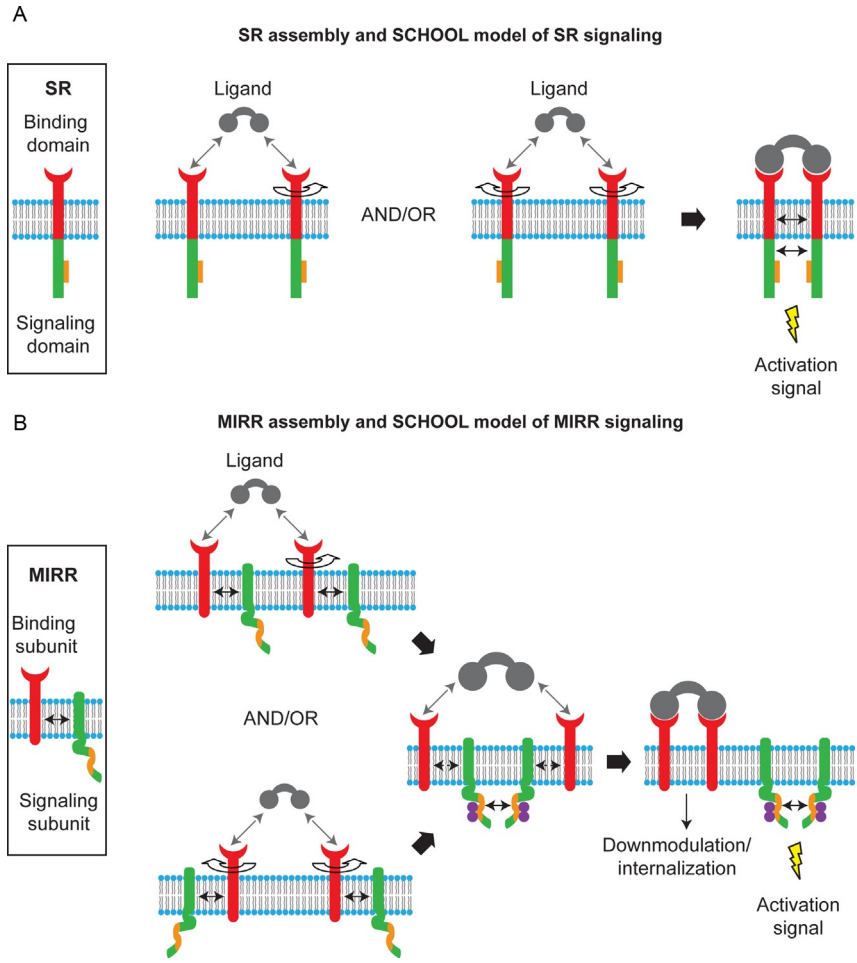

Fig. 1 Single and multichain activating receptor assembly and signaling. (A) In single-chain receptors (SRs), extracellular recognition domain (*red*), and intracellular signaling domain (*green*) with a signaling sequence (*orange rectangles*) are located on the same protein chain. The signaling chain homooligomerization (SCHOOL) model of SR signaling model proposes that receptor homooligomerization in the cytoplasmic milieu plays a central role in triggering SRs. Ligand-induced SR clustering and reorientation (and/or receptor reorientation in preexisting SR clusters) results in SR oligomerization mediated by transmembrane interactions between charged residues. In these oligomers, receptors are in sufficient proximity and adopt a correct interreceptor relative orientation and geometry to promote homointeractions between cytoplasmic domains. Formation of competent signaling oligomers results in generation of the activation signal (for receptor tyrosine kinases, this means transautophosphorylation of Tyr residues in cytoplasmic signaling sequences) and thus triggers downstream signaling pathways. Protein–protein interactions are shown by *solid black arrows*. (B) In multichain immune

2. PPIs IN TRANSMEMBRANE SIGNALING

The SCHOOL platform considers homooligomerization of cytoplasmic signaling domains of cell surface receptors (both SRs and MIRRs) as a driving force of transmembrane signaling and suggests that bringing these domains in the correct orientation and close enough proximity to one other to promote homotypic PPIs between them is an obligatory step to trigger the receptor (Sigalov, 2004, 2010c). This defines the process of ligand-induced receptor triggering and activation as an outcome of the interplay between three major driving forces: extracellular PPIs between receptors and their cognate ligands, inter- (SR) and intrareceptor (MIRR) PPIs in the cell membrane, and interreceptor homotypic PPIs in the cytoplasmic milieu. Interestingly, in transmembrane signaling mediated by receptor tyrosine kinases (RTKs), members of the SR family, a weak dimerization propensity for all RTK transmembrane domains allows for a tight control of the ratio between receptor monomers and dimers (Finger, Escher, & Schneider, 2009; Li & Hristova, 2006). In MIRR-mediated signaling, three major PPIs indicated earlier all are characterized by micromolar affinity and relatively rapid kinetics (Bormann & Engelman, 1992; Davis et al., 1998; Finger et al., 2006; Sigalov et al., 2004). This conjugated and well-balanced system of PPIs involved in receptor triggering explains the molecular mechanisms underlying the ability of MIRRs to transduce the diverse recognition

recognition receptors (MIRRs), the extracellular recognition domains and intracellular ITAM-containing signaling domains are located on separate subunits bound together by noncovalent intramembrane interactions (*solid arrow*). ITAMs are colored *orange*. The signaling chain homooligomerization (SCHOOL) model of MIRR signaling. The model proposes that the homooligomerization of signaling subunits in the cytoplasmic milieu plays a key role in triggering MIRRs. Ligand-induced MIRR clustering and reorientation (and/or receptor reorientation in preexisting MIRR clusters) lead to formation of a dimeric/oligomeric intermediate. In this intermediate, receptors are in sufficient proximity and adopt the correct relative orientation and geometry to promote trans-homointeractions between cytoplasmic domains of signaling subunits resulting in formation of competent signaling oligomers. In these oligomers, protein tyrosine kinases phosphorylate the ITAM tyrosine residues, leading to the generation of activation signal(s), dissociation of signaling oligomers, and internalization of the engaged MIRR ligand-binding subunits. *Circular arrows* indicate ligand-induced receptor reorientation. All interchain interactions in a dimeric intermediate are shown by *dotted black arrows* reflecting their transition state. *Curved lines* depict disorder of the cytoplasmic domains of MIRR signaling subunits. Phosphate groups are shown as *purple circles*. Abbreviations: *ITAM*, immunoreceptor tyrosine-based activation motif.

(discrimination) information across the cell membrane and translate it into different signaling cascades, thus triggering different intracellular pathways and providing different cell responses. In the immune defense, this system of PPIs explains mechanistically how immune cells can have high specificity, selectivity, and sensitivity in the recognition and discrimination of different antigens (ligands) and how this recognition (discrimination) results in different functional outcomes.

In contrast to well-established strategies that target interaction of receptors with their ligands in order to modulate receptor signaling, the SCHOOL strategy is to target other PPIs involved in receptor-mediated transmembrane signal transduction—intramembrane and cytoplasmic homotypic PPIs. This "Freedom to Bind not to Signal" strategy allows for effective and selective therapeutic targeting using small molecule inhibitors and modulatory peptides and peptidomimetics.

2.1 Intramembrane and Cytoplasmic Interactions

2.1.1 Single-Chain Receptors

According to the SCHOOL platform (Sigalov, 2004, 2010c), upon binding to multivalent ligand, intramembrane PPIs between SRs mediate dimerization (oligomerization) of SRs to bring the receptors into close proximity and the correct interreceptor geometry to promote cytoplasmic homotypic PPIs and thus triggers the receptor (Fig. 1A). Interestingly, RTKs and some other SRs such as members of the tumor necrosis factor (TNF) receptor superfamily (Chan, 2007; Chan, Siegel, & Lenardo, 2000) can exist as preassembled dimers (oligomers) on the surface of resting cells. In this scenario, the platform suggests that binding to multivalent ligand leads to reorientation of receptors in these dimers (oligomers) to adopt the geometry competent to promote dimerization (oligomerization) of cytoplasmic signaling domains that trigger the receptor (Fig. 1A). Indeed, in RTK-mediated signaling, multivalent ligand binding results in a conformational change in the receptor extracellular domain, leading to the rotation of the whole receptor (Jiang & Hunter, 1999; Moriki, Maruyama, & Maruyama, 2001). Triggering of the Neu RTK occurs only for a specific transmembrane dimer interface, the rotation of which leads to periodic oscillations in kinase activity (Bell et al., 2000), further confirming the necessity of the correct interreceptor orientation in the receptor dimers (oligomers) formed upon binding to multivalent ligand. In addition, the rotation of the RTK domain with respect to its transmembrane domain by inserting residues into the C-terminal transmembrane flanking region restores the RTK activity. Furthermore, upon

binding of the epidermal growth factor receptor (EGFR) to its cognate ligand, the EGFR extracellular domain rotates and this rotation is transmitted to the receptor transmembrane domain (Moriki et al., 2001). Inter-receptor orientation in receptor dimers plays a critical role in erythropoietin receptor-mediated transmembrane signaling (Livnah et al., 1998; Syed et al., 1998), suggesting a tight coupling of the extracellular domain orientation to the cytoplasmic signaling events. Furthermore, signal transduction via interleukin (IL)-6 requires not only gp130 homodimerization but also the correct relative orientation of the gp130 cytoplasmic domains in a ligand-specific receptor dimer, suggesting that subtle changes in the orientation of the receptor chains relative to each other might result in very different responses (Greiser, Stross, Heinrich, Behrmann, & Hermanns, 2002). Dimerization of the cytokine receptors by monoclonal antibodies is in most cases not enough to induce signal transduction (Autissier et al., 1998). Formation of homooligomers of Fas cytoplasmic tails plays an important role in receptor triggering (Siegel et al., 2004). Similarly, cytoplasmic domain-mediated dimerization of toll-like receptor-4 (TLR-4) is necessary for TLR-4 triggering and to activate signaling cascades (Lee, Dunzendorfer, & Tobias, 2004).

Thus, these and other studies of SRs strongly support the general SCHOOL platform concept and demonstrate that ligand-induced receptor dimerization is translated into protein dimerization in the cell membrane milieu and that the receptor dimer intramembrane interface contains the critical structural information that positions the receptor cytoplasmic signaling domains in a way competent for oligomerization and receptor triggering (Fig. 1A).

2.1.2 Multichain Receptors

Transmembrane signaling mediated by MIRRs can be roughly divided into four stages: (1) recognition and binding of the receptor to its multivalent cognate ligand outside the cell, (2) transmembrane transduction of this information to the cytoplasmic milieu, (3) phosphorylation of the ITAM or YxxM tyrosine residues by protein tyrosine kinases followed by activation of specific intracellular signaling cascades, and (4) activation of genes in the nucleus. While the molecular mechanisms underlying the stages 1, 3, and 4 are understood in significant detail, the mechanisms by which the MIRRs transduce recognition (discrimination) information via receptor transmembrane and juxtamembrane regions into intracellular biochemical events (stage 2) have been a long-standing mystery. It was also not clear how this

putative mechanism could explain the intriguing ability of immune cells to discern and differentially respond to slightly different ligands in the immune defense. Ultimately, the specific PPIs involved in this process have remained largely unknown preventing the development of novel therapeutic approaches.

By uncovering a crucial physiological role of homotypic interactions between intrinsically disordered cytoplasmic domains of MIRR signaling subunits (Sigalov, 2011c; Sigalov et al., 2004, 2007), the biophysically unique phenomenon that still remains a matter of debate whether it exists or not (Nourse & Mittag, 2014; Sigalov, 2016; Uversky & Dunker, 2013), the SCHOOL model suggests that formation of competent MIRR signaling subunit oligomers is necessary and sufficient to trigger the receptor and induce downstream signaling cascades (Fig. 1B) (Sigalov, 2004, 2005, 2006, 2008c, 2010b). This is consistent with the structural hypothesis of cross-phosphorylation (Ortega, 1995; Pribluda, Pribluda, & Metzger, 1994) that assumes that the kinase(s) responsible for catalyzing ITAM Tyr residue phosphorylation exist associated with the receptors, and that for steric reasons, these kinases cannot phosphorylate tyrosine residues on ITAM-containing signaling chains of the same receptor complex. In contrast, in MIRR dimers (oligomers) formed upon binding to their multivalent cognate ligands, the kinases phosphorylate the tyrosines of a distinct receptor complex (cross-phosphorylation or transphosphorylation), thus triggering the receptor (Ortega, 1995).

In unstimulated (resting) cells, intramembrane PPIs between transmembrane helices of recognition and signaling MIRR subunits maintain receptor integrity and determine the relative positions of these subunits in the receptor complex (angles, distances, etc.), thus dictating the overall geometry and topology of MIRRs (Biassoni et al., 2000; Call, Pyrdol, Wiedmann, & Wucherpfennig, 2002; Daeron, 1997; DeFranco et al., 1994; Kim et al., 2003; Klesney-Tait, Turnbull, & Colonna, 2006; Moroi & Jung, 2004; Sigalov, 2004, 2005, 2008c). The SCHOOL platform suggests that MIRR engagement by multivalent ligand or anti-MIRR antibodies (e.g., antibodies against CD3ε and T cell receptor β (TCRβ) chains of TCR or anti-Igβ antibodies for B cell receptor) leads to receptor dimerization (oligomerization) coupled with a multistep structural reorganization aiming to promote homotypic PPIs between MIRR signaling subunits in the cytoplasmic milieu that trigger the receptor and initiate downstream signaling cascades (Fig. 1B). According to the SCHOOL model, sufficiently close interreceptor proximity and correct relative orientation in MIRR dimers

(oligomers) as well as long enough duration of the MIRR–ligand interaction and sufficient lifetime of an individual receptor in these MIRR clusters all play important roles for productive MIRR triggering as strongly supported by a growing body of evidence (Carreno, Gonzalez, & Kalergis, 2006; Holler, Lim, Cho, Rund, & Kranz, 2001; Holowka, Sil, Torigoe, & Baird, 2007; Kiessling, Gestwicki, & Strong, 2006; Klemm, Schreiber, & Crabtree, 1998; Metzger, 2004; Miura, Takahashi, Jung, & Moroi, 2002; Ortega, Schweitzer-Stenner, & Pecht, 1988; Posner et al., 2002; Puffer, Pontrello, Hollenbeck, Kink, & Kiessling, 2007; Radaev & Sun, 2003; Reth & Wienands, 1997; Schweitzer-Stenner, Tamir, & Pecht, 1997; Tamir & Cambier, 1998; Yamasaki, Ishikawa, Kohno, & Saito, 2004).

Some MIRRs such as TCR and major platelet collagen receptor, glycoprotein VI (GPVI), can exist as preassembled oligomers on the surface of resting cells (Berlanga et al., 2007; Jung, Tsuji, & Moroi, 2009; Schamel et al., 2005). In this scenario, similarly to that of SRs, the SCHOOL model suggests reorientation of MIRRs in these dimers (oligomers) to adopt the geometry competent to promote homotypic PPIs between cytoplasmic signaling domains that trigger the receptor.

In the immune defense, the SCHOOL mechanisms suggest that the diversity of the immune cell response is largely provided by the combinatorial nature of MIRR-mediated signaling. More specifically, signal diversification may be achieved through different patterns of MIRR signaling subunit oligomerization (Sigalov, 2004, 2005, 2006) in combination with distinct activation signals provided by different MIRR signaling modules (Jensen et al., 1997; Pike, Baig, & Ratcliffe, 2004; Pitcher & van Oers, 2003; Sanchez-Mejorada & Rosales, 1998) and/or different ITAMs located on the same signaling module (e.g., TCRζ chain) (Chae et al., 2004). Thus, according to the model, the diversity of cell functional outcomes in response to stimulation by different ligands is increasing with the number of different signaling subunits that the MIRR complex has.

2.2 Transmembrane Signaling and Viral Evasion Strategies: Evolutionary and Molecular Aspects

Structural consideration of transmembrane signaling mediated by a variety of functionally unrelated receptors expressed on various cells leads us to the important observation that recognition and signaling functions can be combined on one protein chain (SRs) or separated between different protein chains (MIRRs) (Fig. 1). Another observation is that while extracellular (ligand recognition), transmembrane and cytoplasmic (signaling) domains

of SRs are all well structured (Sigalov, 2010c, 2011a, 2012b), cytoplasmic domains of MIRR signaling subunits are all intrinsically disordered (i.e., these domains lack a well-defined ordered structure under physiological conditions in vitro) in contrast to the well-structured MIRR extracellular (ligand recognition) and transmembrane domains (Sigalov, 2011a, 2011c, 2012b; Sigalov et al., 2004, 2007; Sigalov, Aivazian, Uversky, & Stern, 2006). Ultimately, these observations raise two intriguing questions (Sigalov, 2011a, 2012a). First, why did nature separate recognition and signaling functions for MIRRs, thereby increasing the risk of malfunction and potential attack by pathogens, and second, why did nature select protein disorder for MIRRs to translate recognition of distinct ligands into appropriate activation signals that would induce distinct specific functional outcomes (Fig. 2)? Answering the first question, one can suggest that the modular assembly of MIRRs brings multiple benefits the most important of which is the capability to diversify and vary signal transduction (Sigalov, 2010c, 2010d, 2011b, 2012a). Ultimately, this provides the mechanistic basis for

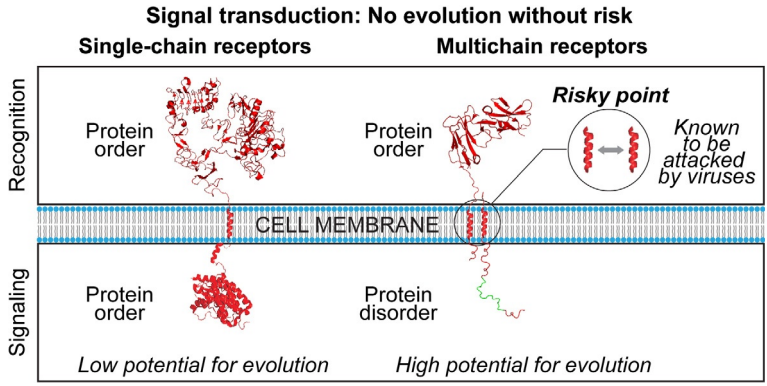

Fig. 2 Evolutionary and molecular aspects of transmembrane signal transduction mediated by single- and multichain cell surface receptors. Images were created using PyMol (www.pymol.org) from Protein Data Bank entries 1NQL and 3GOP for the EGFR extra- and intracellular (juxtamembrane and kinase) domains, respectively (shown as an example of structure of a single-chain receptor), and entry 1UCT for the FcαRI extracellular domain (shown as an example of structure of a multichain receptor recognition subunit). For illustrative purposes, the cytoplasmic domain of a multichain receptor-associated signaling subunit is shown as a monomer and using arbitrary idealized structural elements to represent the ensemble of unfolded conformations of an IDR. The immunoreceptor tyrosine-based activation motif (ITAM) of multichain receptors is depicted in *green*. Intramembrane interactions between recognition and signaling subunits of multichain receptor are shown by a *gray arrow*. Abbreviations: *EGFR*, epidermal growth factor receptor; *FcαRI*, Fc receptor I for IgA.

the diversity and variability of the immune response. According to Charles Darwin, diversity and variability are at the very core of evolutionary processes (Darwin, 1861). One can conclude that nature takes the risks associated with separation of functions in MIRRs in exchange for high potential for evolution of signal transduction (Figs. 2 and 3). In the context of immune signaling, this is of great importance in the development and evolution of the immune defense. Protein disorder contributes to the diversity and variability of signal transduction without compromising efficiency and specificity of signaling (Sigalov, 2010c, 2010d, 2011b, 2012a, 2012b). Thus, evolutionary and developmental benefits of immune signaling-related protein disorder outweigh attendant disadvantages which are largely compensated for by increasing the host defense.

There exist two types of immune receptors: the innate receptors, the germ line-encoded receptors that detect a limited set of conserved antigens; and the adaptive receptors, the somatically generated antigen receptors of the T and B cells (TCR and BCR, respectively) (Table 1). From the evolutionary standpoint, the adaptive immune response evolved long after the innate mechanisms of self-defense and provided significant added value in

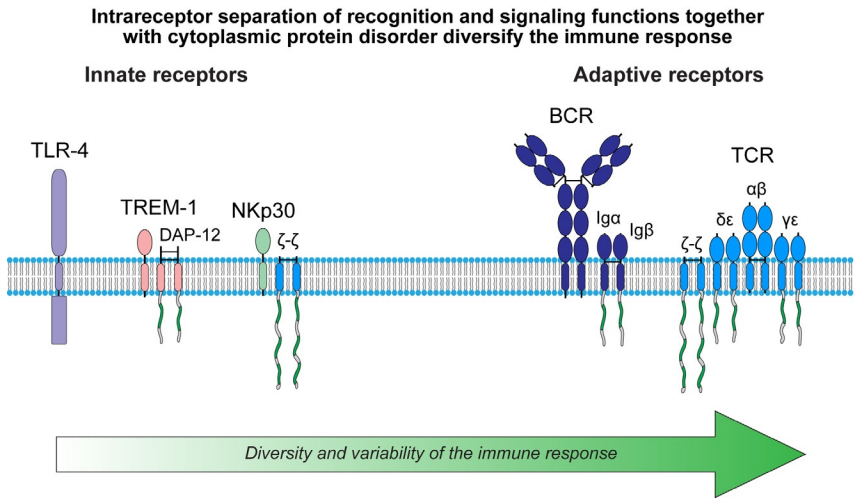

Fig. 3 Diversity and variability of the immune response. The immunoreceptor tyrosine-based activation motif (ITAM) is shown in *green*. *Curved lines* depict intrinsic disorder of the cytoplasmic domains of MIRR signaling subunits. Abbreviations: *BCR*, B cell receptor; *DAP-12*, DNAX adapter protein of 12 kDa; *Ig*, immunoglobulin; *MIRR*, multichain immune recognition receptor; *NK*, natural killer cell; *TCR*, T cell receptor; *TLR-4*, toll-like receptor 4; *TREM-1*, triggering receptor expressed on myeloid cells 1.

Table 1 The Characteristics of the Innate and Adaptive Receptors

Innate Receptors	Adaptive Receptors
Germline encoded	Encoded in multiple gene segments
Nonclonal distribution	Clonal distribution
Do not require gene rearrangement	Require gene rearrangement
Trigger immediate response	Trigger delayed response
Broad specificity: recognize pathogen-associated molecular patterns	Narrow specificity: recognize a particular epitope

promoting survival (Cooper & Alder, 2006; Kimbrell & Beutler, 2001; Litman & Cooper, 2007). Structurally, the adaptive receptors belong to the MIRR family with two (BCR) and four (TCR) signaling chains (Fig. 3). In contrast, the innate receptors belong to the SR family (e.g., TLRs) or represent MIRRs that contain only one signaling chain (e.g., triggering receptors expressed on myeloid cells, TREMs, or natural killer cell receptors, NK receptors) (Fig. 3). Importantly, functions of the ITAM modules, including those located on different (Jensen et al., 1997; Pike et al., 2004; Pitcher & van Oers, 2003; Sanchez-Mejorada & Rosales, 1998; Sigalov, 2004, 2006, 2010c, 2011a, 2012b) or the same (e.g., three different ITAMs on the ζ chain) (Chae et al., 2004) signaling chains and those located on the same chain are rather distinct instead of redundant. This provides a molecular explanation for significantly higher diversity and variability of the adaptive response as compared to the innate response. Interestingly, following this logic, one can conclude that the extent of diversity and variability of NKp30-mediated signaling should be an intermediate between the innate (e.g., TLRs) and adaptive receptors (Fig. 3). This is indeed consistent with the idea that NK cells represent an "evolutionary bridge" between innate and adaptive immunity (Sun & Lanier, 2009).

As biochemical processes that can be influenced and controlled (Archakov et al., 2003; Arkin & Wells, 2004; Berg, 2003; Fletcher & Hamilton, 2007; Pagliaro et al., 2004), intramembrane PPIs between MIRR recognition and signaling subunits as well as homotypic interactions between cytoplasmic domains of MIRR signaling subunits represent potential points of attack by pathogens in order to modulate the MIRR-mediated signaling and thus to modulate the immune response (Sigalov, 2009, 2010f, 2012a).

Examples are viruses that in the billion-years-long struggle for their existence, in order to establish a successful infection, replicate, and persist in the host, have evolved numerous strategies to counter and evade host antiviral immune responses as well as to exploit them for productive viral replication. Several different viruses such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), severe acute respiratory syndrome coronavirus (SARS-CoV), and human herpesvirus 6, that are pathogenic for humans uniformly target members of the MIRR family, including innate and adaptive receptors. Intriguingly, these viruses use either a modular assembly of MIRRs to disrupt receptor-mediated signaling or cytoplasmic protein disorder of MIRRs to surprisingly augment cell activation as required for self-preservation (Cohen, Cohen, Antonovsky, Cohen, & Shai, 2010; Shen & Sigalov, 2016; Sigalov, 2007a, 2008a, 2009, 2010f, 2012a). The example of the coevolution of viruses and their hosts confirms that there is no evolution and development without risks. Although we cannot avoid these risks, we can turn this lemon into lemonade and learn from nature how to efficiently target the immune system for therapeutic purposes (Kim & Sigalov, 2008; Shen & Sigalov, 2016; Sigalov, 2010a, 2010f). This will be further illustrated in the next section on the example of intramembrane PPIs as a therapeutic target.

3. INTRAMEMBRANE PPIs AS A THERAPEUTIC TARGET

Although there is a growing line of experimental evidence indicating that targeting intramembrane PPIs to inhibit (modulate) SR-mediated cell activation might represent a promising therapeutic approach (Bennasroune et al., 2004, 2005; Hebert et al., 1996; Sigalov, 2010a, 2010e; Smith et al., 2002; Tarasova, Rice, & Michejda, 1999), in this work, I will focus on therapeutic intramembrane PPIs using short synthetic peptides (SCHOOL peptides) in order to inhibit MIRR-mediated transmembrane signaling for therapeutic purposes.

Importantly, the receptor-specific SCHOOL peptides employ the molecular mechanisms of receptor inhibition that are not dependent upon binding of the receptor to its cognate ligand. This is especially important in those situations when ligands are still unknown. Example is TREM-1 receptor. Despite some recent evidence that peptidoglycan (PGN) recognition protein 1 (PGLYRP1) may potentially act as a ligand for TREM-1 (Read et al., 2015), the actual nature of the TREM-1 ligand(s) is still not yet well understood, impeding the development of clinically relevant

inhibitors of TREM-1. This receptor functions as a potent amplifier of inflammation and mediates production of proinflammatory cytokines such as TNFα, IL-1β, and IL-6 (Bouchon et al., 2001; Murakami et al., 2009; Schenk, Bouchon, Seibold, & Mueller, 2007). Importantly, TREM-1 activation preferentially induces expression of functional macrophage colony-stimulating factor (M-CSF, also known as CSF-1) (Dower, Ellis, Saraf, Jelinsky, & Lin, 2008) and in contrast to cytokine blockers, blockade of TREM-1 can blunt excessive inflammation while preserving the capacity for microbial control (Weber et al., 2014). Collectively, these findings implicate TREM-1 as a promising therapeutic target in a variety of inflammation-associated diseases (Bosco, Raggi, & Varesio, 2016; Ford & McVicar, 2009; Tammaro et al., 2017).

3.1 Cancer

3.1.1 Nonsmall Cell Lung Cancer

Lung cancer is the leading cause of cancer deaths worldwide. There are two types of this cancer—nonsmall cell lung cancer (NSCLC) and small cell lung cancer. Despite advances made in chemotherapy (Felip, Santarpia, & Rosell, 2007), NSCLC kills over 1.1 million people annually worldwide, and the 5-year survival rate for patients with NSCLC is only 15% (Jemal et al., 2011). Similar to most solid tumors, the infiltrate of NSCLC, contains tumor-associated macrophages (TAMs) (Solinas, Germano, Mantovani, & Allavena, 2009). By secreting a variety of growth factors, cytokines, chemokines, and enzymes, TAMs regulate tumor growth, angiogenesis, invasion, and metastasis (Shih, Yuan, Chen, & Yang, 2006). High TAM content correlates with the promotion of tumor growth and metastasis (Solinas et al., 2009) and is associated with poor prognosis in NSCLC (Welsh et al., 2005). TAM recruitment, activation, growth, and differentiation are regulated by M-CSF (Elgert, Alleva, & Mullins, 1998). Increased pretreatment serum M-CSF level is a significant independent predictor of poor survival in patients with NSCLC (Kaminska et al., 2006).

In patients with NSCLC, TREM-1 expression on TAMs is associated with cancer recurrence and poor survival: patients with low TREM-1 expression have a 4-year survival rate of over 60%, compared with less than 20% for patients with high TREM-1 expression (Ho et al., 2008), which suggests inhibition of the TREM-1 pathway may be a promising target for the development of a targeted anticancer therapy.

Considering the unknown nature of TREM-1 ligand(s), in our recent study (Sigalov, 2014), we used the SCHOOL platform to design a novel,

ligand-independent TREM-1 inhibitory peptide sequence GF9, and demonstrated that the GF9 peptide specifically blocks TREM-1-mediated transmembrane signaling in vitro and in vivo. Utilizing two human NSCLC xenograft nude mouse models (H292 and A549), we demonstrated for the first time that blockade of TREM-1 function using GF9 substantially decreases cytokine production in vitro and significantly delays tumor growth in vivo. We also showed that the SCHOOL peptide inhibitor of TREM-1 GF9 can be formulated into self-assembling macrophage-specific lipopeptide and lipoprotein complexes that mimic human high density lipoproteins (HDLs) for peptide half-life extension and targeted delivery (Sigalov, 2014). This incorporation significantly (up to 10-fold) increases GF9 therapeutic efficacy (Sigalov, 2014).

3.1.2 Pancreatic Cancer

Pancreatic cancer (PC, 85% of which are pancreatic ductal adenocarcinomas) is the fourth leading cause of cancer-related mortality across the world with very poor clinical outcome (Hariharan, Saied, & Kocher, 2008). Current treatments of PC all only marginally prolong survival or relieve symptoms in patients with PC (Schneider, Siveke, Eckel, & Schmid, 2005). There has been no significant progress in the field of targeted therapy for PC (Walker & Ko, 2014) and despite tremendous efforts, the 5-year survival rate remains less than 5%.

As lung cancer and many other solid tumors, PC is characterized by a marked infiltration of macrophages into the stromal compartment (Shih et al., 2006; Solinas et al., 2009). In patients with PC, macrophage infiltration begins during the preinvasive stage of the disease and increases progressively (Clark et al., 2007). The number of TAMs is significantly higher in patients with metastases (Gardian, Janczewska, Olszewski, & Durlik, 2012). Presence of TAMs in the PC stroma correlates with increased angiogenesis (Esposito et al., 2004), a known predictor of poor prognosis (Kuwahara et al., 2003). Similar to NSCLC, high pretreatment serum M-CSF is a strong independent predictor of poor survival in PC patients (Groblewska et al., 2007). Interestingly, M-CSF blockade not only suppresses tumor angiogenesis and lymphangiogenesis (Kubota et al., 2009) but also upregulates T cell checkpoint molecules, including programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen-4 (CTLA-4), thereby overcoming limitations of PD-1 and CTLA-4 antagonists to achieve regression in even well-established tumors (Zhu et al., 2014). Importantly, continuous M-CSF inhibition affects only pathological

angiogenesis but not healthy vascular and lymphatic systems outside tumors (Kubota et al., 2009). In contrast to blockade of vascular endothelial growth factor (VEGF), interruption of M-CSF inhibition does not promote rapid vascular regrowth (Kubota et al., 2009). TREM-1 activation is known to enhance release of most cytokines that are increased in patients with PC (Tjomsland et al., 2011; Yako, Kruger, Smith, & Brand, 2016) and play a vital role in creating and sustaining inflammation in the tumor favorable microenvironment, thus affecting patient survival. These include monocyte chemoattractant protein-1 (MCP-1), TNFα, IL-1α, IL-1β, IL-6, and M-CSF (Lagler et al., 2009; Schenk et al., 2007; Sigalov, 2014). Collectively, these findings suggest that a therapy that targets TREM-1-mediated intratumoral inflammation, angiogenesis, and vascularization can represent a promising strategy for treating PC.

Recently, we tested the therapeutic potential of TREM-1 inhibitory GF9 peptide sequences in three human PC xenograft mouse models (AsPC-1, BxPC-3, and Capan-1) (Zu T. Shen and Alexander B. Sigalov, unpublished). Administration of these SCHOOL peptides resulted in a strong antitumor effect achieving an optimal-treated/control (T/C) value of 19% depending on the xenograft and formulation used and persisting even after treatment was halted. The effect correlated significantly with increased survival, reduced angiogenesis, and suppressed TAM infiltration. The peptides were well tolerated when deployed in either free form or formulated into HDL-mimicking macrophage-targeted lipopeptide complexes. Further, blockade of TREM-1 significantly reduced serum levels of IL-1α, IL-6, and M-CSF, but not VEGF, suggesting an M-CSF-dependent inhibitory effect on angiogenesis.

Taken together with our previous study (Sigalov, 2014), these data suggest that SCHOOL TREM-1-specific peptide inhibitors have a cancer type independent, therapeutically beneficial antitumor activity and can be potentially used as a stand-alone therapy or as a component of combinational therapy for PC, NSCLC, and other solid tumors.

3.2 Autoimmune Diseases

Autoimmune diseases are a fascinating but poorly understood group of diseases that are characterized by a specific adaptive immune response that is mounted against self-antigens (Davidson & Diamond, 2001). As a result, the effector pathways of immunity cause chronic inflammatory injury to tissues, which may often prove lethal. Examples are rheumatoid arthritis (RA),

systemic lupus erythematosus, inflammatory bowel disease, multiple sclerosis, atopic dermatitis (AD), and other disorders. T cells and macrophages, two main subtypes of immune cells, are known to be implicated in mediating many aspects of autoimmune inflammation.

RA is a chronic, systemic inflammatory disorder that causes chronic inflammation of the joints and affects about 1% of the world's population, with women affected three times more often than men. The economic burden created by RA is enormous with annual direct and indirect costs per patient of $2500–14,500 and $1500–45,000, respectively, explaining the high demand for new therapies (Kukar, Petryna, & Efthimiou, 2009). Typically, new approaches to RA involve the use of monoclonal antibodies, peptides, antigen mimetics, T cell vaccinations, infusion/injection of antiinflammatory cytokines, or cytokine inhibitors (Kukar et al., 2009; Kurosaka, Ali, Byth, & Manolios, 2007). However, current RA treatments including TNF blockers such as Humira, all have multiple shortcomings including a high level of serious side effects and insufficient efficacy, thus highlighting the need for new and improved treatments. The pathogenesis of RA suggests a key role for aberrant pathways of T cell activation in the initiation and/or perpetuation of disease (Cope, Schulze-Koops, & Aringer, 2007; Sakaguchi, Benham, Cope, & Thomas, 2012). This makes inhibition of pathways involved in T cell activation including TCR-mediated cell activation a promising therapeutic strategy for novel RA therapies (Lorenz, 2003; Won & Lee, 2008).

AD is an inflammatory skin autoimmune disease characterized by impaired epidermal barrier function and cutaneous inflammation. The prevalence of AD has steadily increased during the past few decades. AD affects 1%–3% of adults and up to 20% of children, in 85% of them the disease onset is before the age of 5 years (Novak, 2009). Novel therapeutic approaches are required, as most of the treatments of AD are limited to symptomatic therapies. Activation and skin-selective homing of peripheral blood T cells, and effector functions in the skin, are involved in the pathogenesis of AD (Akdis, Akdis, Trautmann, & Blaser, 2000). Numerous studies have pointed to the role of activated CD4+ T cells in AD (Nomura & Kabashima, 2016). Topical immunomodulators that affect T cells and block TCR-mediated cell activation may therefore have a role in the treatment of AD (Nomura & Kabashima, 2016).

Furthermore, macrophages are central to the pathogenesis of autoimmune diseases including RA and AD (Kasraie & Werfel, 2013; Kinne, Brauer, Stuhlmuller, Palombo-Kinne, & Burmester, 2000). The abundance

and activation of macrophages in the inflamed synovial membrane significantly correlates with the severity of RA (Kinne et al., 2000; Mulherin, Fitzgerald, & Bresnihan, 1996). In order to be clinically effective, RA therapies should reduce the number of synovial sublining macrophages (Bresnihan et al., 2007). Major macrophage-related targets in RA and AD include TNFα, IL-1, IL-6, and M-CSF (Li, Hsu, & Mountz, 2012). M-CSF-dependent cells are known to be essential for collagen-induced arthritis (CIA) development (Campbell, Rich, Bischof, & Hamilton, 2000). These and other findings (Colonna & Facchetti, 2003; Genua, Rutella, Correale, & Danese, 2014; Kasraie & Werfel, 2013; Kuai et al., 2009) suggest targeting macrophage activation including TREM-1-mediated cell activation as another promising strategy to treat RA and AD.

3.2.1 Targeting T Cell Receptor

Structurally, TCR is a member of the MIRR family and has α and β antigen-binding subunits (TCRα and TCRβ, respectively) that are bound by electrostatic intramembrane PPIs with three signaling homo- and heterodimers: ζζ, CD3εδ, and CD3εγ (Fig. 3). Short synthetic peptides capable of inhibiting TCR-mediated cell activation in a ligand-independent manner are known since 1997 (Manolios et al., 1997) when TCR-targeted inhibitory activity was first reported for a synthetic peptide corresponding to the sequence of the TCRα transmembrane domain (the so-called core peptide, CP). In the TCR complex, this domain is known to interact with the transmembrane domains of CD3εδ and ζ (Call et al., 2002; Manolios, Bonifacino, & Klausner, 1990). Later, the TCRα CP has been shown to reduce inflammation and inhibits the progression of experimental arthritis (Amon et al., 2006; Kurosaka, Ali, et al., 2007). In patients with AD, psoriasis, or lichen planus, a daily topical application of this peptide over three consecutive days has been demonstrated to inhibit inflammation and ameliorate the disease equally as well as betamethasone (Enk & Knop, 2000; Gollner, Muller, Alt, Knop, & Enk, 2000).

Interestingly, similar ligand-independent TCR inhibitory activity was later reported for HIV fusion peptide (FP) found in the N terminus of the HIV envelope glycoprotein 41 (gp41) (Bloch et al., 2007; Quintana, Gerber, Kent, Cohen, & Shai, 2005). The patterns of inhibition of TCR-mediated cell activation exhibited by TCR CP and HIV gp41 FP were intriguingly similar: both peptides inhibit antigen—but not anti-CD3-stimulated T cell activation (Quintana et al., 2005; Wang, Djordjevic, Kurosaka, Schibeci, Lee, Williamson, et al., 2002). Similar to

TCRα CP, HIV gp41 FP was shown to reduce inflammation and ameliorate T cell-mediated autoimmune arthritis in animal models (Quintana et al., 2005).

However, despite extensive studies (Ali et al., 2006; Amon et al., 2006; Bloch et al., 2007; Collier, Bolte, & Manolios, 2006; Kurosaka, Bolte, Ali, & Manolios, 2007; Manolios et al., 1997; Quintana et al., 2005; Wang, Djordjevic, Bender, & Manolios, 2002), the mode of action of these clinically relevant peptides was enigmatic until the SCHOOL model was first introduced and applied to this field (Sigalov, 2004, 2006, 2007a). According to the model (Sigalov, 2004, 2005, 2006, 2010c, 2010e), TCRα CP and HIV gp41 both compete with the TCRα chain for binding to CD3δε and ζζ and disrupt the corresponding intramembrane PPIs. This results in disconnection/predissociation of the affected signaling subunits from the remaining receptor complex and upon stimulation by multivalent antigen, leads to inhibition of antigen—but not antibody-mediated TCR triggering and cell activation. Importantly, TCR assembly and cell surface expression are not affected by treatment with TCRα CP (Kurosaka, Bolte, et al., 2007). This finding as well as colocalization of TCR CP with TCR in the membrane of resting cells (Wang, Djordjevic, Bender, et al., 2002) directly prove the hypothesis about "pre-" rather than full dissociation state of the unstimulated TCR complex in the presence of TCRα CP, whereas upon stimulation, the affected signaling subunits, ζζ and CD3εδ, become physically disconnected from the remaining receptor complex. It should be noted that the proposed SCHOOL mechanism is the only mechanism consistent with all experimental and clinical data reported up to date for transmembrane peptides of TCR and other MIRRs as well as for lipid and/or sugar conjugates of these peptides (Ali, Amon, Bender, & Manolios, 2005; Ali, De Planque, Huynh, Manolios, & Separovic, 2002; Amon et al., 2006, 2008; Bender, Ali, Amon, Diefenbach, & Manolios, 2004; Collier et al., 2006; Enk & Knop, 2000; Gerber, Quintana, Bloch, Cohen, & Shai, 2005; Gollner et al., 2000; Huynh, Ffrench, Boadle, & Manolios, 2003; Kurosaka, Bolte, et al., 2007; Manolios, Huynh, & Collier, 2002; Sigalov, 2007b, 2008a; Wang, Djordjevic, Bender, et al., 2002; Wang, Djordjevic, Kurosaka, et al., 2002).

Later, the use of the SCHOOL model and comparative primary sequence analysis of proven and predicted immunomodulatory sequences of viral fusion protein regions allowed not only to suggest the specific molecular mechanisms of inhibition of TCR-mediated cell activation by TCRα CP and HIV gp41 FP (Sigalov, 2007a, 2008b, 2009) but also to predict

similar immunomodulatory activity for other viral FPs such as SARS-CoV FP (Sigalov, 2009). Our recent study (Shen & Sigalov, 2016) provided compelling experimental in vivo evidence in support of this hypothesis. As demonstrated, a synthetic 11 amino acid-long peptide-derived from SARS CoV FP predicted using the SCHOOL model to disrupt intramembrane PPIs in the TCR complex was able to reduce inflammation in DBA/1J mice with CIA and protect mice against bone and cartilage damage (Shen & Sigalov, 2016). Formulation of the peptide into self-assembling macrophage-targeted lipopeptide nanoparticles that mimic native human HDLs significantly increased peptide dosage efficacy (Shen & Sigalov, 2016). Collectively, these findings further confirm that viral immune evasion strategies evolved during the host–virus coevolution can be transferred to therapeutic strategies that require similar functionalities (e.g., in the treatment of autoimmune diseases).

3.2.2 Targeting TREM-1

TREM-1 expressed on macrophages is a promising target for the development of new rational therapies for autoimmune diseases including RA (Kuai et al., 2009). In addition, targeted delivery of antirheumatic drugs to macrophages is another highly desirable strategy for the treatment of RA because it would not only strike the cells that mediate or amplify most of the permanent tissue destruction but also spare other cells that do not affect joint damage (Garrood & Pitzalis, 2006; Kinne, Stuhlmuller, & Burmester, 2007).

Recently, we demonstrated that a TREM-1-specific inhibitory nonapeptide GF9 designed using the SCHOOL model and capable of inhibiting TREM-1-mediated macrophage activation in a ligand-independent manner, suppresses release of proinflammatory cytokines and M-CSF, decreases inflammation and protects against bone and cartilage destruction in experimental arthritis (Shen & Sigalov, 2017). Administration of GF9 reduced the plasma levels of M-CSF, TNFα, IL-1, and IL-6 (Shen & Sigalov, 2017). Interestingly, 31-mer peptides with sequences from GF9 and helices 4 (GE31) and 6 (GA31) of the major HDL protein, apolipoprotein A–I, were able to perform three functions: assist in the self-assembly of GA/E31-HDL, target these particles to macrophages, and block TREM-1 signaling (Shen & Sigalov, 2017). Similar to our cancer studies (Sigalov, 2014), formulation of GF9 alone or as a part of GE31 and GA31 peptides into HDL significantly increased its therapeutic efficacy. Colocalization of GF9 with TREM-1 in the macrophage membrane observed using confocal microscopy indicates that GF9 self-inserts into the cell membrane and disrupts intramembrane

PPIs between TREM-1 and its signaling partner, DAP-12, further confirming the SCHOOL mechanisms of action of this peptide (Shen & Sigalov, 2017). Collectively, these findings suggest that TREM-1 inhibitory SCHOOL sequences may be promising alternatives for the treatment of RA and possibly, other TREM-1-mediated autoimmune diseases.

3.3 Sepsis

Septic shock is a complex clinical syndrome that results from the systemic response to infection and is characterized by overwhelming production of proinflammatory cytokines that leads not only to tissue damage, but also to hemodynamic changes, multiple organ failure, and ultimately death (Cohen, 2002). Despite the use of potent antibiotics and advanced resuscitative equipment costing $17 billion annually, sepsis still kills about 375,000 Americans each year and mortality rates for patients with septic shock are nearly 50% (Martin, Mannino, Eaton, & Moss, 2003). The only approved sepsis drug, Xigris by Ely Lilly and Company, has been withdrawn from all markets in 2011 and currently, no approved drugs are available. In addition, over 30 drug candidates have failed late-stage clinical trials. Together, this highlights an urgent unmet medical need for new sepsis treatments.

Macrophages are known to secrete high levels of TNFα, IL-6, and IL-1 in septic mice (Ayala & Chaudry, 1996). Further, in patients with sepsis, M-CSF is overproduced (Oren, Duman, Abacioglu, Ozkan, & Irken, 2001). Furthermore, elevated levels of TNFα along with other proinflammatory cytokines are closely linked with poor patient outcome (Gogos, Drosou, Bassaris, & Skoutelis, 2000). Importantly, in patients with sepsis, TREM-1 expression on macrophages is markedly increased (Gibot, 2005; van Bremen et al., 2013). Initial findings established TREM-1 as an amplifier of the systemic inflammatory response syndrome associated with sepsis (Bouchon, Dietrich, & Colonna, 2000; Bouchon et al., 2001). Blockade of TREM-1 in septic mice lowers expression levels of TNF-α and IL-6 and increases survival from 5% to 10% in control animals to 70%–80% in treated animals (Bouchon et al., 2001; Gibot et al., 2007; Wang et al., 2012). Taken together, this suggests TREM-1-mediated macrophage activation as a promising therapeutic target for this syndrome.

However, as discussed earlier, the development of conventional inhibitors of TREM-1 that attempt to block binding of TREM-1 to its ligand (Bouchon et al., 2001; Gibot et al., 2007; Wang et al., 2012) is significantly complicated by the unknown nature of TREM-1 ligand(s) and hence the

increased risk of failure of these approaches in clinical development. In this context, TREM-1-specific inhibitory SCHOOL peptide sequences that employ ligand-independent mechanism of receptor inhibition represent a promising alternative to conventional approaches. Ligand-independent blockade of the TREM-1 signaling pathway in mice with experimental lipopolysaccharide (LPS)-induced septic shock using TREM-1 inhibitory SCHOOL peptide GF9 was shown to substantially suppress proinflammatory cytokine production and prolong survival of septic mice (Sigalov, 2014). Targeted delivery of GF9 to macrophages using self-assembling lipopeptide complexes significantly increased peptide half-life and therapeutic efficacy (Sigalov, 2014). These data strongly support the hypothesis that ligand-independent modulation of TREM-1 function using small synthetic peptides might be a suitable treatment for sepsis.

3.4 Thrombosis

Damage to the integrity of the vessel triggers platelet adhesion and aggregation resulting in the formation of a thrombus, which prevents blood loss at sites of injury or leads to occlusion and irreversible tissue damage or infarction in diseased vessels (Leslie, 2010; Nieswandt et al., 2001). Despite intensive research and recent advances in antithrombotic drug discovery and development (Angiolillo, Bhatt, Gurbel, & Jennings, 2009), uncontrolled hemorrhage still remains the most common side effect associated with antithrombotic drugs that are currently in use on the about $11 billion market.

Engagement and clustering of GPVI, the major collagen receptor on platelets, play a crucial role in platelet adhesion, aggregation, and activation induced by collagen (Jung & Moroi, 2008). The selective inhibition of GPVI is believed to inhibit thrombosis without affecting hemostatic plug formation, thus providing new therapeutic strategies to fight platelet-mediated diseases (Li et al., 2007; Lockyer et al., 2006). Thus, in contrast to current drugs, GPVI-specific inhibitors may represent an ideal class of clinically suitable antithrombotics.

Despite intensive studies (Farndale, 2006; Gawaz, 2004; Gibbins, Okuma, Farndale, Barnes, & Watson, 1997; Moroi & Jung, 2004), the molecular mechanisms underlying GPVI triggering and platelet activation were not known until recently when the SCHOOL model was introduced and applied to GPVI triggering and transmembrane signal transduction (Sigalov, 2004, 2005, 2006, 2007b, 2007c, 2008a). This resulted in the development of a novel concept of platelet inhibition and the invention

of new platelet inhibitors that employ the ligand-independent SCHOOL mechanisms of GPVI inhibition (Sigalov, 2007a, 2007b, 2008a).

Structurally, GPVI is a member of the MIRR family and signals through its signaling partner, the FcRγ chain (Moroi & Jung, 2004). Uncovering the molecular mechanisms of collagen-stimulated triggering of the GPVI-mediated signal cascade, the SCHOOL model reveals GPVI-FcRγ intramembrane PPIs as a novel therapeutic target for the prevention and treatment of platelet-mediated thrombotic events (Sigalov, 2006, 2007b, 2007c, 2008a, 2010a). Specific blockade or disruption of these PPIs causes a physical and functional disconnection of the GPVI and FcRγ subunits. Experimental data obtained using the GPVI-specific inhibitory SCHOOL peptide GV11 (Sigalov, 2007a, 2007b, 2008a) provided support for this novel concept of platelet inhibition and demonstrated that depending on the donor, incubation of whole blood samples with GV11 prior to addition of collagen (10 and 20 μg/mL) or convulxin (10 ng/mL) leads to a 30%–60% reduction in both the percentage of P-selectin-positive platelets and the expression of the platelet activation markers, P-selectin and PAC-1. This effect is specific: platelet activation via ADP (20 μM) is not affected by the peptide. Collectively, these findings suggest that targeting intramembrane PPIs using the SCHOOL technology opens exciting new avenues in innovative antithrombotic drug discovery and development.

3.5 Retinopathy

Pathologic retinal neovascularization (RNV) causes an angiogenesis-related vision impairment in retinopathy of prematurity (ROP), diabetic retinopathy (DR), and retinal vein occlusion (RVO), which are the most common causes of vision loss and blindness in each age group (Jo & Kim, 2010; Laouri, Chen, Looman, & Gallagher, 2011; Mutlu & Sarici, 2013). In premature infants, normal retinal vascular development is interrupted resulting in retinal ischemia and invasion of the vitreous by abnormal neovessels. In addition, vitreoretinal neovascularization can promote traction retinal detachment, leading to blindness (Al-Shabrawey et al., 2013). In the United States, 14,000–16,000 premature infants are affected by ROP annually and about 4.1 million adults over 40 years have DR (Bashinsky, 2017; Hartnett, 2017). Complications of conventional therapeutic options including laser ablation (corneal edema, anterior chamber reaction, intraocular hemorrhage, cataract formation, and intraocular pressure changes) (Mutlu & Sarici, 2013) and anti-VEGF therapy (damage of healthy vessels, potential

side effects on neurons, rapid vascular regrowth upon interrupting the VEGF blockade, and limited effectiveness in some patients) (Maharaj et al., 2008; Mancuso et al., 2006; Pieramici & Rabena, 2008; Verheul & Pinedo, 2007) highlight an unmet need for new targeted therapies that can better address the pathogenesis of neovascular retinal diseases and improve their treatment. In addition, drug delivery to the retina via systemic administration is ideal but it is still a challenge due to the blood–retinal barrier (BRB) (Gaudana, Ananthula, Parenky, & Mitra, 2010).

Retinal microglia and blood-derived macrophages (BDM) that regulate angiogenesis are critically involved in the pathogenesis of RNV diseases (Checchin, Sennlaub, Levavasseur, Leduc, & Chemtob, 2006; Espinosa-Heidmann et al., 2003; Kubota et al., 2009). In ROP, the retina is infiltrated by activated leukocytes and macrophages (Davies, Eubanks, & Powers, 2006). While retinal microglia can become activated in response to retinal pathologies (Penfold, Madigan, Gillies, & Provis, 2001), infiltration and activation of BDM may provide a major contribution in retinal degeneration (Caicedo, Espinosa-Heidmann, Pina, Hernandez, & Cousins, 2005). In mice with oxygen-reduced retinopathy (OIR), macrophages promote the development of pathological RNV (Gao et al., 2016). Macrophages induce angiogenesis by secreting multiple proangiogenic factors, including VEGF (Liu et al., 2013), MCP-1 (Niu, Azfer, Zhelyabovska, Fatma, & Kolattukudy, 2008) and proinflammatory cytokines, such as TNFα and IL-1 (Nagai et al., 2001). Inhibition of MCP-1 suppresses RNV (Kim, Byeon, Hong, Han, & Jeong, 2005; Yoshida, Yoshida, Ishibashi, Elner, & Elner, 2003). M-CSF that acts as an "angiogenic switch" (Lin et al., 2006) is involved in the differentiation of tissue macrophages and microglia during postnatal development (Cecchini et al., 1994). In contrast to VEGF blockade, interruption of M-CSF inhibition does not promote rapid vascular regrowth (Kubota et al., 2009). In the OIR mice, M-CSF is required for pathological RNV but not for the recovery of normal vasculature (Kubota et al., 2009). In the retinas of diabetic rats and in the vitreous of patients with proliferative DR, M-CSF levels are significantly higher compared to control subjects (Liu, Xu, Jiang, & Da, 2009; Yoshida et al., 2015). This suggests targeting M-CSF either directly or via the specific inflammatory signaling pathway as a highly promising strategy for treating ocular neovascular diseases including ROP.

TREM-1 is upregulated under a variety of inflammatory conditions (Pelham & Agrawal, 2014). Upon activation, TREM-1 enhances the production of multiple cytokines and growth factors including MCP-1, TNFα,

IL-1α, IL-1β, IL-6, and M-CSF (Dower et al., 2008; Lagler et al., 2009; Schenk et al., 2007; Shen & Sigalov, 2017; Sigalov, 2014). However, despite the recent identification of TREM-1 as a novel hypoxic marker in vivo and in vitro (Bosco et al., 2011), the role of TREM-1 in RNV diseases including ROP remained unclear. Recently, we found that TREM-1 is highly overexpressed in the retina of the OIR mice compared to controls (Modesto A. Rojas, Zu T. Shen, Ruth B. Caldwell, and Alexander B. Sigalov; unpublished data). Blockade of TREM-1 using the TREM-1 inhibitory SCHOOL GF9 peptide sequences substantially suppressed retinal protein levels of TREM-1 and M-CSF, suggesting an M-CSF-dependent inhibitory effect on angiogenesis, and significantly (up to 90%) reduced the area of vitreoretinal neovascularization (Modesto A. Rojas, Zu T. Shen, Ruth B. Caldwell, and Alexander B. Sigalov, unpublished data). Collectively, these promising data suggest that targeting intramembrane PPIs in the TREM-1/DAP-12 receptor complex using TREM-1-specific SCHOOL peptide inhibitors represent a novel strategy to treat RNV diseases including ROP.

4. CONCLUSIONS

There is a growing interest in therapeutic targeting specific PPIs involved in receptor-mediated transmembrane signal transduction and our improved understanding of the molecular mechanisms underlying this process can significantly contribute into the development of novel pharmacological approaches to a variety of diseases.

Example is the SCHOOL platform that by uncovering the molecular mechanisms of transmembrane signaling mediated by unrelated and functionally diversed surface receptors expressed on various cells, revealed the specific PPIs involved in receptor triggering as key points of therapeutic control. Importantly, the SCHOOL platform suggests that within the SR and MIRR families, the similar structural architecture of the receptors dictates similar mechanisms of receptor triggering. This, in turn, suggests similarity of therapeutic targets in seemingly unrelated diseases, which makes possible the development of global pharmacological approaches as well as the transfer of our clinical knowledge, experience, and therapeutic strategies between these diseases. Successful application of the SCHOOL strategy to target in vivo intramembrane PPIs involved in triggering of such unrelated receptors as TCR, TREM-1, and GPVI expressed on T cells, macrophages, and platelets, respectively, provided compelling evidence to support this

hypothesis and resulted in the discovery of new drug candidates for a variety of diseases including cancer, sepsis, RA, AD, and retinopathy.

Further, the SCHOOL platform significantly improved our understanding of the immunomodulatory activity of many human viruses. It appears that different viruses use their fusogenic peptides not only to fuse their membranes to their target host cells but also to disarm the immune system using the SCHOOL mechanisms of receptor inhibition and to escape the host immune response. This gives an interesting example of how our advances in mechanistic understanding of the fundamental natural processes and their interactions can elucidate the billions years old strategies nature uses for combinatorial control and optimization at different scales, from evolution to organismal function.

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