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Commentary

Soluble Immune Response Suppressor (SIRS): Reassessing the immunosuppressant potential of an elusive peptide

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ABSTRACT

A previously studied immunosuppressive cytokine, Soluble Immune Response Suppressor (SIRS), may have relevance to current studies of immune suppression in a variety of human disease states. Despite extensive efforts using experimental models, mainly in mice, much remains to be discovered as to how autoimmune cells in mice and humans escape normal regulation and, conversely, how tumor cells evade evoking an immune response. It is the contention of this commentary that the literature pre-2000 contain results that might inform current studies. The broadly immunosuppressive protein, SIRS, was studied extensively from the 1970s to 1990s and culminated in the determination of the n-terminal 21mer sequence of this 15 kDa protein which had high homology to the short neurotoxins from sea snakes, that are canonical members of the three finger neurotoxin superfamily (3FTx). It was not until 2007 that the prophylactic administration of the synthetic N-terminal peptide of the SIRS 21mer, identical to the published sequence, was reported to inhibit or delay the development of two autoimmune diseases in mice: experimental allergic encephalomyelitis (EAE) and type I diabetes (T1D). These findings were consistent with other studies of the 3FTx superfamily as important probes in the study of mammalian pharmacology. It is the perspective of this commentary that SIRS, SIRS peptide and the anti-peptide mAb, represent useful, pharmacologically-active probes for the study of the immune response as well as in the potential treatment of autoimmune, inflammatory diseases and cancer.

1. Introduction

The role of soluble substances (first called 'factors,' later lymphokines and finally cytokines) in the generation and regulation of immune responses was first proposed in the 1960s (for additional details see [1]) along with the two-signal hypothesis of lymphocyte activation [2,3]. In the early 1970s, Gershon and colleagues suggested that a unique class of T-cells, termed suppressor cells, could negatively regulate immune responses [4]. This coincided with the explosive growth of studies around soluble factors (lymphokines) regulating immunity as illustrated in a series of workshops [5–8]. The simultaneous improvement in analytical tools in molecular biology (cloning and DNA sequencing) and protein chemistry (HPLC and improved protein sequencing) in the 1970s and early 1980s foreshadowed the areas genomics and proteomics. These new tools allowed the purification of factors and the determination that many, but certainly not all, were protein in nature.

Molecular cloning techniques developed in the late 1970s–early1980s allowed investigators to confirm that many of these factors came from genes expressed during immune responses and led to their sequence identity being determined [5–8]. During this time, limited progress was made in the unambiguous determination of the biochemical nature of suppressor factors. The author worked diligently on this problem along with many colleagues from 1975 to 1990 [9]. By the late 1980s with a few exceptions, little clarity had been reached either in terms of the precise biochemical composition of these suppressor factors or their genetic origins. Complicating this work was the question of whether such factors showed antigen specificity (i.e. were antigen specific suppressor factors). It was clear that the suppressor T cells could show apparent antigen specificity, but all protein purification and cloning approaches failed to confirm that soluble suppressor factors could account for this [9]. In the meantime the nature of the antigen receptor on T-cells was discovered and characterized both at the gene and protein level. A precise link between the T-cell antigen receptor and the antigen-specific suppressor factors has yet to be unambiguously demonstrated [10–14].

This failure to identify the genetic source of antigen-specific suppressor factors led to a profound skepticism about this research topic [15]. This was further supported by the successful identification of cytokines such as TGF-β, interferons and IL-10 as suppressive cytokines that lacked antigen specificity [16–19]. A

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further complication came from observations that products of the eicosanoid pathway, e.g., prostaglandins and leukotrienes, as well as other small molecule ligands acting via G-protein coupled receptors could also function as non-specific regulators of immunity [20,21]. Thus it was clear that there were a plethora of ways in which immune cells could be regulated that did not require antigen specificity.

2. Soluble Immune Response Suppressor (SIRS)

SIRS had originally been identified as a non-antigen specific suppressor factor in the early 1970s [22]. It was produced by Ly 2+ (now called CD8) T-cells in response to stimulation by the mitogen concanavalin A (Con A) or interferon beta (IFNβ), (interferon-α (IFN-α) also seems to work) or by infectious agents [23–28] A large body of work, principally by Aune and Pierce and their collaborators, further characterized SIRS from Con A stimulated spleen cell cultures as requiring oxidation by macrophages or H_2O_2 in order to be biologically active [24,25]. Critically for the discussion below, it was shown that the oxidized form of SIRS, SIRSox disrupted the cytoplasmic array of microtubules in cells which could explain its capacity to inhibit cell proliferation in both lymphocytes and neoplastic cells [26,27]. SIRS was also produced and purified from a T cell hybridoma (393D2.6) and characterized as having a molecular weight of ~15 kDa by SDS–PAGE [28] with other researchers also reporting the identification of suppressor factors with properties similar to SIRS [29–32]. Of great importance were observations suggesting that a similar, if not identical factor to SIRS was produced in humans, particularly patients with nephrotic syndrome [33–36]. Around this time, a rat monoclonal anti-SIRS antibody was developed that could detect partially purified SIRS in both mouse and human material but not the material in crude cell supernatants [31,36].

2.1. Sequencing SIRS

In 1987, Webb et al. obtained the N-terminal, 21 amino acid sequence of a SIRS protein isolated from the T cell hybridoma, 393D2.6 (MTEENQQSSQPKTTINNAGNS). A rabbit anti-peptide antiserum was generated that was specific to that sequence [37,38]. A surprise came when a blast search of the sequence, (BLAST, www.ncbi.nim.nih.gov) revealed a very high homology to a family of short neurotoxins, part of the superfamily termed 3FTxs, isolated from sea snakes (see Tables 1 and 2 for an updated BLAST search). As discussed below in more detail, these initial BLAST results did not show any evidence of significant homology to any mammalian proteins. To confirm that this sequence was indeed related to SIRS, it was shown that the rabbit anti-SIRS peptide antiserum blocked SIRS activity in an in vitro antibody-forming cell response. It should be noted in passing that, at that time the anti-peptide antiserum was not checked to see whether it could neutralize snake neurotoxin activity. In addition, using a cDNA probe based on the peptide sequence, the selection of poly A+ RNA that produced SIRS activity was confirmed in a rabbit reticulocyte assay using TGF-β1 as a control [38].

All these data suggest that the 21mer SIRS peptide sequence was related to SIRS. However, despite detecting a poly A+ RNA that could code for a SIRS-like molecule using the cDNA sequence probe, several attempts to clone the SIRS cDNA using techniques available in the early 1990 s failed (Freire-Moar & Webb, unpublished data). Of particular note was the observation that several recombinant interferons (rat IFN-γ and human rIFN α-A/D) generated inhibitory activity (measured in vitro in cytotoxic T lymphocyte (CTL) assays) that could be blocked by the rabbit-anti-SIRS peptide antibody, strongly implying that IFNs suppressive activity was due, in part, to SIRS [38] an observation germane to the discussion below. As one of the controls in these experiments, the SIRS peptide was added to CTL cultures and showed evidence of suppression of the in vitro CTL assay. However, at the time, this observation was not followed up (see below).

2.2. Suppressor cell studies move on

The publication of these results coincided with a period of transition for the field of suppressor cell studies. Preliminary reports by

Table 1
Significant alignments produced by the SIRS peptide with sequences shown. The data were obtained using BLAST, (www.ncbi.nim.nih.gov).

RecName: Full = Short neurotoxin 1				
Sequence ID: sp|P68412.1|3S11_HYDST				
Length: 60 Number of Matches: 1				
Alignment statistics for match #1				
Score	Expect	Identities	Positives	Gaps
43.1 bits(94)	0.002	15/17(88%)	15/17(88%)	0/17(0%)
Query 5 NQQSSQPKTTINNAGNS 21				
NQQSSQPKTT N AGNS				
Sbjct 5 NQQSSQPKTTTNCAGNS 21				
RecName: Full = Short neurotoxin 2				
Sequence ID: sp|Q5UFR7.1|3S12_HYDPR				
Length: 81 Number of Matches: 1				
Alignment statistics for match #1				
Score	Expect	Identities	Positives	Gaps
43.1 bits(94)	0.003	15/17(88%)	15/17(88%)	0/17(0%)
Query 5 NQQSSQPKTTINNAGNS 21				
NQQSSQPKTT N AGNS				
Sbjct 26 NQQSSQPKTTTNCAGNS 42				
RecName: Full = Short neurotoxin 1 neurotoxin precursor				
Sequence ID: sp|Q5UFR8.1|3S11_HYDPR				
Length: 81 Number of Matches: 1				
Alignment statistics for match #1				
Score	Expect	Identities	Positives	Gaps
43.1 bits(94)	0.003	15/17(88%)	15/17(88%)	0/17(0%)
Query 5 NQQSSQPKTTINNAGNS 21				
NQQSSQPKTT N AGNS				
Sbjct 26 NQQSSQPKTTTNCAGNS 42				

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Table 2
The SIRS peptide was searched against the non-redundant protein sequence database (nr) using Blastp (protein–protein BLAST). Search parameters were specified as PAM-30 scoring matrix, word size 2, and gap costs of existence 9 and extension 1. For this reason, despite high identity scores (e.g. 90%) the gap costs will result in a lower total score as shown for example with short neurotoxin 2 precursor.

Description	Max score	Total score	Query cover (%)	*E* Value	Ident. (%)	Accession
RecName: Full = Short Neurotoxin 1 AltName: Full: Toxin A	43.1	43.1	80	0.002	88	P68412.1
RecName: Full = short Neurotoxin 2 Flags: Precursor	43.1	43.1	80	0.003	88	QSUFR7.1
RecName: Full = Short neurotoxin 1 AltName: Full = Major/minor ne Neurotoxin Flags: precursor	43.1	43.1	80	0.003	88	QSUFR8.1
RecName: Full = Short neurotoxin 1 AltName: Full = toxin 4	36.7	36.7	57	0.30	92	P68415.1
RecName: Full = Pelamitoxin a	36.7	36.7	57	0.30	92	P62388.1
RecName: Short neurotoxin C	36.7	36.7	80	0.30	76	P19958.1
RecName: Short neurotoxin A	36.7	36.7	80	0.30	76	P32879.1
RecName: Full = Short neurotoxin 1 AltName: Full = Hydrophitoxin a	36.7	36.7	57	0.30	92	P25494.1
RecName: Full = Short neurotoxin 1; AltName: Full = Neurotoxin A	36.7	36.7	57	0.30	92	P01437.1
RecName: short chain neurotoxin 2 precursor [*Deinagkistrodon acutus*]	36.7	36.7	57	0.32	92	AC148330.1
RecName: short chain neurotoxin 2 precursor [*Hydrophis cyanocinctus*]	36.7	36.7	57	0.32	92	AC148329.1
RecName: Full = Short neurotoxin 2; AltName: Full = Hydrophitoxin b; Flags: Precursor	36.7	36.7	57	0.34	92	P62376.1
RecName: Full = Short neurotoxin B: Flags: Precursor	36.7	36.7	80	0.35	76	P19959.1
RecName: Full = Short neurotoxin 1; AltName: Full = SM12' AltName: Full = Toxin 4; Flags; Precursor	36.7	36.7	57	0.35	92	P68416.2
RecName: Short chain neurotoxin isoformPrecursor [*Hydrophis hardwickii*]	36.7	36.7	57	0.35	92	ABN54805.1
RecName: Full = Short neurotoxin 2; AltName: Full = SN36; Flags: Precursor	36.7	36.7	57	0.35	92	Q8UW26.1
RecName: Full = Short neurotoxin SN160; Flags: Precursor	36.7	36.7	57	0.35	92	Q8UW27.1
RecName: hypothetical protein [*Acinetobacter jonsonii*]	35.8	35.8	95	0.83	62	WP0049819
RecName: truncated short neurotoxin [*Aipysurus eydouxii*]	34.1	34.1	47	1.9	100	AAT11123.1
RecName: Full = Short neurotoxin 1; AltName: Full = Toxin Aa c	34.1	34.1	47	2.2	100	P01434.1
RecName: Short-chain neurotoxin isoform 9 [*Parasuta nigriceps*]	34.1	34.1	47	2.4	100	ACY68694.1
RecName: Full = Short neurotoxin D; Flags: Precursor	34.1	34.1	47	2.4	100	P19960.1
RecName: Short-chain neurotoxin isoform 8 [*Parasuta nigriceps*]	34.1	34.1	47	2.4	100	ACY68693.1
RecName: Short-chain neurotoxin isoform 6 [*Parasuta nigriceps*]	34.1	34.1	47	2.4	100	ACY68691.1
RecName: Short-chain neurotoxin isoform 5 [*Parasuta nigriceps*]	34.1	34.1	47	2.4	100	ACY68690.1

Friere-Moar, et al. [39], Terajima et al. [40] and Fukuse et al. [41] in our group, presaged later research from Japan and elsewhere in the U.S. and Europe that identified a $CD4^+$ T cell population that was termed regulatory T cells or Tregs to distinguish them from the previously described $CD8^+$ T suppressor cells. It is noteworthy that the difference in nomenclature is semantic since both populations are exclusively suppressive and virtually all T cells regulate immunity either positively or negatively and, on occasion, they do both [42]. In any event, the $CD4^+$ regulatory T cells required CD25, the IL-2R-α chain, for activation. Also a new, readily reproduced biomarker, FOXP3 was discovered that could be used to identify Tregs in both mouse and human [43,44]. Most researchers interested in immune suppression refocused their attention toward these newly identified Treg cells. Studies of the older, $CD8^+$ T-suppressor cells have also evolved and the phenotypic identification of $CD8^+CD122^+$ regulatory T cells has helped to distinguish them from the $CD4^+$ $FOXP3^+$ regulatory population [45,46]. This is in addition to groups seeking to revive the use of the suppressor cell designation to describe antigen specific suppressor cells and factors [11,12,47,48].

Despite the continued interest in $CD8^+$ T cells as regulatory T cells, the importance of $CD4^+$ Treg cells in suppressing all aspects of immune responses in both humans and mice has become a central tenet of immune regulation in various disease states, including autoimmunity, allergy and asthma, transplantation, cardiovascular disease, neurological diseases, and cancer [13,14,49–51]. As we have become more knowledgeable about immune regulation, it has become abundantly clear that there are a plethora of mechanisms involving cytokines, antigen presenting cells, and other cell types, all of which can contribute to the regulation of both innate and acquired immunity [52]. While it is not the intention of this commentary to deeply review this literature, it is important to understand that with the literal explosion of research in this area, it was relatively easy for older observations to be forgotten. This is quite similar to the situation described by Swanson [53] in the neurosciences where researchers discovered that a potentially useful treatment for migraine had been reported on years earlier and ignored until it was discovered again using newer and more powerful search algorithms [51].

2.3. The absence of follow on studies

A pertinent question is why there was no additional follow up in the research community around SIRS; particularly since it appears to be present in human immune cells and in at least one disease, steroid-responsive nephrotic syndrome, in human [33–35]. Certainly one factor could be lack of a more detailed phenotypic description of $CD8^+$, SIRS producing cells [54]. It is also the case that the original work on SIRS was spearheaded by a group that published mainly in the 1980s and early 1990s with many outside collaborators [23–26,28,31,33,35,36,38–40]. Nonetheless, these studies did establish that SIRS is an important regulator of T-cells, macrophages and B-cells likely due in part to its effect on microtubule formation (as SIRSox) [26] while its production in response to mitogens, antigens, allogeneic stimulus, interferons and infectious organisms, marks it as an important element in the regulation of immune cells. Sadly, the work largely stopped in the early 1990s due to the group leading the research disbanding and moving on to other projects. At around the same time IL-10

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was discovered and TGF-β was also found to be immune suppressive [17–19]. Lack of further characterization and cloning of SIRS may well have contributed to the shift in focus to these more defined cytokines.

Certainly, there are examples in the literature that might have led other researchers to SIRS. Feng et al. [13], used a protocol involving allogeneic stimulation analogous to that used earlier by Devens et al. [38] that leads to the production of SIRS either directly or via IFN-γ, stimulation. This work focused solely on the up-regulation of $CD4^+$ Tregs, and did not make a connection to the earlier $CD8^+$ T suppressor cell literature [14]. Even publications that identified $CD8^+$ regulatory T cells, missed the opportunity to connect to the earlier suppressor T cell literature except that related to IL-10 [45,46,54,55].

2.4. SIRS 21 mer peptide as a therapeutic

In 2007–2008, research on SIRS was briefly revived by two papers by Brod and Hood [56,57] who were studying models of autoimmunity in mice; experimental autoimmune encephalomyelitis (EAE) and type I diabetes (T1D), and who were clearly aware of the earlier papers on SIRS [31,37,38].

In the 1990s Brod and Burns [58] had shown that type I interferon (IFN-α; which also stimulates SIRS production [31]) administered prophylactically (po), could delay or inhibit the development of the autoimmune disease, experimental autoimmune encephalomyelitis (EAE). It has been speculated that IFN-α given po may act via the oral pharyngeal-associated lymphoid tissue or gut associated lymphoid tissue before it is digested via proteolysis. [59–61]. The Brod and Burns data showed that IFN-α could delay EAE development in mouse via a mechanism that appeared to involve inhibition of Th1 cytokines, IL-2, and IFN-γ. They subsequently showed that ingested IFN-α also could inhibit or delay the development of T1D in a NOD mouse model via a mechanism that appeared somewhat different than that seen in the mouse EAE model. Of special note, they also used an oral protocol for the administration of human recombinant interferon (rHIFN-α) in patients with relapsing, remitting multiple sclerosis (RRMS) [56]. They found no toxicity in normal volunteers over a range of 300–100,000 units. Over an rHIFN-α dose range of 10,000–30,000 units, cells taken from RRMS patients showed decreased proliferation in response to Con A. They also reported a decrease in serum soluble intercellular adhesion molecule-1 (ICAM-1), a surrogate marker for disease activity in MS and a decrease in TH-1 cytokines as well as decreases in TGF-β and IL-10.

Up to that point, the use of the synthetic SIRS peptide by itself as a therapeutic had not been considered despite the earlier observation that the peptide could inhibit CTL induction in vitro [38]. In the Brod and Hood EAE study [56], the SIRS peptide was given simultaneously with antigen. At 10 μg, ip or 10–100 μg po, an attenuation of disease severity was observed. At the highest po doses (10 and 100 μg) SIRS peptide delayed disease onset. The authors also reported a decrease in inflammation with the cytokine profile observed being interpreted as indicative of a possible decrease in Th1 cell migration and, consequently, decreased CNS inflammation. They also reported an increase in IL-13 production that was interpreted as indicating a TH2 response leading to a decrease in the duration, severity and incidence of EAE. An increase in TNF-α was paradoxically also observed with its precise role remaining to be explored further although the authors believed it might be counter balanced by TH2 cytokines produced in the model studies. In the NOD mouse model feeding of SIRS daily (1 μg, po) delayed the onset and decreased the frequency of T1D; interestingly, the 10 mg dose fed daily had no effect on disease onset. This result needs to be explored further to understand why the higher dose had no effect on disease onset. However, bell shaped or U shaped dose response curves are a well established phenomenon in pharmacology [62,63]. SIRS-fed mice also showed decreased pancreatic inflammation that was attributed to decreased pro-inflammatory chemokines (SDF-1, MIP1-g, RANTES and GM-CSF). These data clearly require independent confirmation and extension particularly given the existence of human data using IFN-α administered po [64]. This is also important, since all the original studies on SIRS focused almost exclusively on its antiproliferative effects on lymphocytes and cancer cells.

At the time of the original SIRS studies, many chemokines had still to be discovered and characterized [65] which accounts for lack of studies concerning SIRS effects on chemokines and growth factors. Moreover, it is important to reiterate that while oral administration of peptides is an uncommon route, intranasal intradermal and subcutaneous routes being used more often therapeutically, it has been successfully used (e.g. cyclosporine and desmopressin) [61]. In any case, linking the earlier reports on the mechanism of action of SIRS to the Broad and Hood studies remains a critical step in linking the relationship of SIRS and SIRS peptide to their earlier work on IFN-α. Nevertheless, Brod and Hood may be credited with demonstrating that orally administered SIRS peptide can substitute for IFNs and delay the development of both rat EAE and T1D in NOD mice. It also suggested a new direction for work on SIRS; specifically, focusing on the therapeutic utility of the SIRS peptide. The SIRS peptide bears a strong homology to the snake neurotoxins from a wide variety of species all of which are members of the 3FTx superfamily of toxins. The results from the SIRS peptide studies of Brod and Hood highlighted the fact that there might be a heretofore unsuspected link between the biological activities of SIRS and the pharmacological/therapeutic activities of snake venom derived 3FTx peptides. In the next section this connection is explored further.

3. SIRS peptide and the three finger neurotoxin family

In the late 1980s work on the individual protein components of snake neurotoxins had been underway for many years being primarily focused on mechanisms of their toxicity [66,67]. Thus, what information there was concerning their effects on the immune system was largely related to their toxicity or to the capacity of the immune system to make neutralizing antisera [68,69]. It has now been established that neurotoxins closely related to the SIRS sequence will bind to a number of receptors and ion channels in mammalian cells [70]. For the purposes of this Commentary it is important to point out that one family in particular, the three finger toxin family (3FTx) which is found in many snake species (elapids, hydrophiids, colubrids, viperids and crotalids), have proved important among other things, in enabling the isolation of the nicotinic acetylcholine receptor (nAChR; by α-bungarotoxin) which enhanced understanding of myasthenia gravis, another autoimmune disease. The bungarotoxins have also proved useful in deciphering the molecular mechanisms of long-term potentiation and memory [111] Importantly, modified cobratoxin from the Thailand cobra is being developed to treat adrenomyeloneuropathy and MS [59] a finding that is complimentary to the work of Brod and Hood work using the SIRS peptide. The 3FTx family of toxins also affect α- and β-adrenergic receptor function (β-cardiotoxins [71]); antagonize cell adhesion in a variety of cell types (dendroaspins, [72]); potassium channels (cardiotoxins) and $\alpha_V\beta_3$ integrins [73]. All these pharmacological activities are relevant to the function of immune cells and responses [74–77]. Also, as mentioned earlier, SIRS can affect microtubule assembly and function [26]. Certainly, this does not exclude a possible interaction of SIRS with cell surface receptors or ion channels as this remains to be explored.

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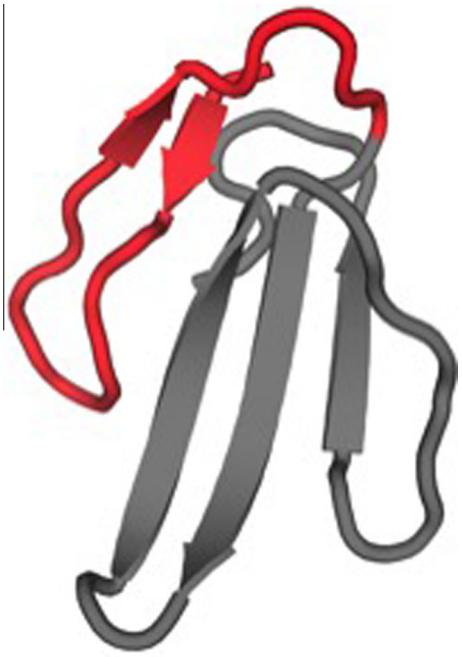

Fig. 1. Proposed location of the SIRS peptide, in red within a 3Ftx protein crystal. The red color indicates where the 21 amino acids of the SIRS peptide sequence might fit into the three dimensional structure of a typical 3FTx family member forming the bulk of the first 'finger' region. The red and gray arrows indicate the position of the β-strands within the fingers.

3.1. SIRS and 3FTx toxin homologies

At the biochemical level, both the 3FTx toxins and SIRS can be sensitive to disulfide active reagents, hydrogen peroxide and reactive oxygen species [24,31,59]. While this a common feature of proteins containing disulfide bonds, lack of Cys residues in the first 21 amino acids of SIRS suggests that the remainder of the protein likely contains at least one disulfide bond. Equally compelling is that the short sequence of SIRS bears a striking similarity of more than 90% to three of the short neurotoxins from sea snakes which are themselves part of the 3FTx toxin superfamily (Tables 1 and 2, Fig. 1). Moreover, as depicted in Table 2, the next 21 highest scoring sequences are all neurotoxins with sequences that are significantly similar to the SIRS peptide. What is remarkable is that after more than 20 years of genomic and proteomic analysis of the genomes of many species, no other mammalian proteins reported to date, appear to have this same degree of similarity. Importantly, there are mammalian examples of orthologs of 3FTxs such as the uPAR/CD87 superfamily that includes CD59/Ly6 [78]. And, at least one of these, Ly6E is also inducible by interferons and is immunosuppressive [78]. Although it is not directly related to the SIRS peptide it is important to keep in mind that viral infections that induce type I IFN cause an increase in expression of a large number genes not all of which are fully characterized [67]. In the case of Ly6E, in the mouse it can limit IL-2 production from activated T cells [79], whereas in monocytes, it acts by down regulating CD14 expression at the transcriptional level [67]. Tsetlin [79] has shown that a member of the Ly6 family, Lynx 1 can act via nAChRs, similarly to another 3FTx member, α-bungarotoxin, thus potentially having a role in treating neurodegenerative diseases, pain and cancer.

3.2. SIRS, immune function and nicotinic receptors

In the immune system, T cells, B cells and macrophages express both nicotinic and muscarinic AChRs [80] with the α7 nAChR being involved in the down regulation of proinflammatory cytokines and modulating antibody production [112]. SIRS also down regulates antibody production [22]. It is reasonable then to suggest that exploring the AChR in connection with SIRS might prove to be useful. Also, CD177, another member of this superfamily is up regulated in murine neutrophils following exposure to endotoxin and plays an important role in neutrophil survival in the bone marrow [81]. Thus there is ample precedence for immune regulatory molecules within this superfamily. Nonetheless, to date, there appears to be no direct biochemical or genetic connection between the uPAR superfamily and SIRS that can be discovered using bioinformatics approaches. Thus while speculative, perhaps SIRS represents a new subset of the 3FTx toxin superfamily?

3.3. Is SIRS part of a larger immunomodulatory protein?

As intriguing as the putative N-terminal SIRS sequence appears to be, until the work of Brod and Hood [56,57] there was little indication that the SIRS peptide might have any therapeutic activity in vivo due, in part, to the assumption that the SIRS peptide was probably part of a larger protein and would likely require the entire protein for full biological activity [37]. In that regard, it is unknown whether the SIRS peptide alone can recapitulate all the pharmacological activity reported to date for SIRS. In particular, examining the effects of SIRS peptide on proliferation and microtubule formation [23–26] would be an important extension of the studies on the mechanism of action. Indeed, both the rabbit anti-SIRS peptide antisera and the rat anti-SIRS monoclonal antibody identified a 30–35 kDa protein by Western blot (possibly a dimer as sizing columns suggested a protein of 14–15 kDa [24,25]). This further underscores the need noted to repeat and extend the work of Brod and Hood to better understand the totality of the data on SIRS and SIRS peptide and how they relate to one another.

In snake, the biological activity of these small peptides is due in part to an exchange of segments and point mutations in exons [70]. The problem of functionally characterizing protein modifications at the level of transcription or post-translationally has been noted in various forms since the pre-genomic sequence era [68–74]. For example, most of current genomic sequences cover areas of the genome that contain gene sequences or sequences that have the characteristics of genes containing regulatory regions e.g. promoters, start codons, exons and introns [68,69]. This leaves substantial room for alternative splice forms [70,72], resulting in small changes in RNA transcription leading to changes in isoform expression of proteins [71]. Post-translational modification, including molecular reassembly is also known to occur [73,74], all of which may need to be explored to some degree in order to understand the genetic origins of SIRS in mice and man.

It is worth noting that within the 3FTxs family, considerable genetic modifications occur [81,82]. For example, the pheromone, Pethodontid Modulating Factor (PMF) is a 7-kDa protein, related to the 3FTxs superfamily. In the male salamander more than 30 unique isoforms of PMF exist with roughly 30% amino acid identity or homology. The existence of so many isoforms may be speculated to be due to powerful, but poorly understood sexual selection pressure [83]. This example is cited to make the point that throughout the vertebrate kingdom, this family of proteins has shown remarkable plasticity in form and function. It may be speculated that the structural features of the 3FTx toxin superfamily lend themselves to genetic modification to suit whatever role they have evolved to perform. Thus the best method to resolve genetic origins of SIRS would be to examine the transcriptome from an appropriately stimulated $CD8^+$ T cell or T-cell line using a RNA-Seq-based approach [84].

At the level of protein structure and function, the 3FTxs toxin family also shows a broad range of biological and pharmacological

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activities noted above, mostly but not exclusively related to their toxic effects. As noted earlier they contain conserved disulfides, something that the SIRS peptide at least, lacks. However, previous studies showed that the SIRS protein is sensitive to sulfhydryl agents making it likely that intra-chain disulfide bonds exist in SIRS that are necessary for the some of the pharmacological properties reported [31]. Also SIRS has no cellular toxicity unlike the 3FTx toxins [9,26,31,56,57]. Thus the SIRS peptide and possibly its precursor protein may have evolved by deleting the invariant Cys residues, at least in the amino terminal peptide, that characterizes all the other members of this superfamily. This may be sufficient to avoid cell toxicity. As suggested earlier, the putative Cys deletion may indicate that SIRS is the first known example of a non-canonical member of the 3FTx family.

3.4. Structural homology

A possible structural relationship between the 3FTx neurotoxins and SIRS is illustrated by the ribbon structure in Fig. 1 based on Gilquin et al. [85]. The SIRS peptide in this figure is superimposed in red indicating the location of the SIRS peptide. Its location and folding within the protein shows that it could comprise one of the three fingers of a typical 3FTx toxin. The red and gray arrows indicate the positions of the β-strands in each of the fingers. The positions of the disulfide bonds are not shown here for simplicity. However, these disulfides are critical not only for the structural integrity of the 3FTx toxins but also for their pharmacological activity. Most of these family members consist of proteins in the 6–8 kDa range [66–70,86]. The size of the entire SIRS protein is estimated as between 15 kDa (gel filtration) and 35 kDa (SDS–PAGE/Western Blotting). It could be that this difference between a typical 3FTx family member, which is a monomer, and SIRS is due to multimeric forms of the SIRS. Alternatively, the proposed relationship to the 3FTx toxin family could be limited to only a single domain. Hence, the critical need to determine the complete sequence of SIRS to more fully understand its domain structure and its relationship to the 3FTx superfamily.

In the absence of an established 3D structure for SIRS, the short sequence length of SIRS, despite the strong similarity to the snake venom toxin clade, complicates any detailed structural analysis. Nevertheless, as Fig. 1 demonstrates, it is possible to fit the SIRS peptide sequence to a typical 3Ftx neurotoxin with little difficulty. Comparisons based on amino acid sequence alone show only the known association with sea-snake short neurotoxins (Tables 1 and 2). Because of the small size of the domain and mutational saturation, it is difficult to approach sequence-based phylogenies in the absence of structural information with any degree of confidence. It is even possible that an as yet undetected relationship exists between SIRS and mammalian orthologs further underscoring the need for additional peptide sequencing either at the genomic or peptide level. Thus, analyses at the level of sequence similarity on one hand, or at the level of tertiary structure homology on the other remain to be explored further once the SIRS gene can be identified.

However, seeking homology at the level of secondary structure may still prove useful. As a complement to large and growing genomic datasets, one can imagine a high throughput pipeline for computational structure prediction where molecular sequences (nucleic acid or protein) can be automatically assigned structures by one or more off-the-shelf secondary structure prediction algorithms [87,88]. This is exemplified by the SBGrid Consortium [89], FATCAT [90] or TOPS++FATCAT [91] that serve as repositories for both protein structures and analysis software.

As outlined by Ye and Godzik [90] and Morin and Sliz [92], the availability of such software can be a powerful tool in understanding the relationships between proteins and peptides that might exist at the structural level that may not be obvious at the sequence level. Unfortunately, the SIRS peptide is too short to be able to carry out reliable structural domain analyses. In that regard, Schultes and his colleagues [93] have been studying smaller peptides in order to make predictions about likely structural motifs [94,95]. His preliminary analysis of the SIRS peptide suggests that by itself, it may assume a structure (not shown) that is similar to that shown in Fig. 1. Clearly, more sequence data will allow a much better prediction of the structure of SIRS to tell whether or not it is truly a member of the 3FTx toxin superfamily. Perhaps as useful from a pharmacological perspective, it is possible to derive 21 mers that have a different amino acid sequence but which show a similar structure to the SIRS peptide. These peptides would make useful congeners for use in a therapeutics discovery program based on the pharmacologic activity of the SIRS peptide.

4. Conclusions

Data on SIRS from the 1970s to 1990s describe a potent immunosuppressive protein induced by antigens, mitogens, interferons and infectious agents that is present in both mice and humans. It has a potential mechanism of action involving the disruption of microtubule formation in a variety of cell types. The SIRS peptide also has pharmacological activity [54,55]. The elusive nature of this peptide and the present mystery surrounding its genetic origin, including its possible link to the 3FTx toxin superfamily, suggests that the time is ripe to revisit this nearly forgotten cytokine by tapping into the exponential growth in genomics data obtained from a wide variety of species since the SIRS peptide was sequenced more than 25 years ago. Thus, it is not unreasonable to expect that a focused reanalysis of human and murine databases might lead to the identification of the genetic source of SIRS in mammals as has been done for virtually every other known protein cytokine [96]. For reasons that remain unclear, this protein seems to be an exception leading to the questions: Why? And how did this sequence emerge from an extract obtained from a murine T cell hybridoma? A deeper genomic analysis using newer tools to probe the genome database has yet to provide a clear answer (Noordewier and Webb, unpublished data).

Linking the sequence to the snake venom 3FTx toxins offers two opportunities. One is to use the apparent similarities between the SIRS peptide and the 3Ftx neurotoxin superfamily to establish a mammalian source of the peptide. A peptide analysis, in the form of a phylogenetic tree suggests the possibility that the source of SIRS could emerge from this direction. The problem with this approach was outlined earlier in the discussion concerning comparative protein/peptide structure. The SIRS peptide at 21 amino acids is just slightly too short to give an unambiguous answer (Noordewier and Webb, unpublished observation). The second is to use the pharmacological information gained from the use of the peptide in vivo to develop the therapeutic potential of this peptide in treating autoimmune diseases, cancer and in organ transplantation. In this regard, a SIRS peptide specific mAb may also have utility as a therapeutic in treating a variety of inflammatory conditions and potentially in cancer treatment as well. The possibility remains that there is indeed an alternative splice form of the SIRS peptide that has yet to be identified in the transcriptome [97,98] or it is the product of post-transcriptional processing [97–105]. The optimal way to explore either of these alternatives is to carry out biochemical studies using a combination of a new anti-SIRS peptide monoclonal antibody and molecular biochemical techniques such as RNA-Seq to identify this peptide.

Given the pharmacological activity of the peptide and that of SIRS, renewed research efforts appear warranted. It is important to independently confirm the SIRS peptide data, analyze more fully the mechanism of action (effect on microtubules?) and extend them

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to other inflammatory conditions as well as tumor models. Snake venom derived peptides have many possible therapeutic uses [70]. It may be argued that the SIRS peptide and its congeners may represent a new direction for anti-inflammatory drug discovery. Indeed, the deletion of the invariant Cys residues in other 3Ftx toxin family members might lead to a new family of anti-inflammatory peptides. The advantage for the SIRS peptide is that unlike the conotoxins [106] for example, it has none of the labile disulfide bonds that limit their effectiveness in treating pain and other neurological conditions.

Finally, the generation of novel mAbs to the SIRS peptide sequence could also be of considerable value in examining whether this molecule plays a significant role in the interferon-based regulation of immune cell function as proposed more than 30 years ago [38–40]. Given the appearance of SIRS in at least one human disease [31–36] it is also possible that such mAbs might themselves be useful as therapeutics in both inflammatory diseases and cancer.

5. Future directions

This commentary and the brief analysis carried out here began with the premise that new, *in silico* based algorithms applied to the ever-growing database of literature and genomics should lead to some answers concerning the seemingly forgotten immunoregulatory protein, SIRS. Using the semantics search engine, EuretosBRAIN it became clear that there was no shortage of possible directions to explore. One of the signal features of the historical SIRS literature was the stimulation of SIRS production by interferons. In addition the Brod and Hood data links interferons and the SIRS peptide in having similar effects on two autoimmune disease models. All this leads the author to posit that the biological effects of type I interferons may be a reasonable place to begin [107]. Since their discovery, a vast literature has arisen and continues to grow, exploring the biology of interferons [108].

Of equal promise are the open source specialized data bases that focus on a more limited set of genes and proteins as exemplified by the Interferome [107]. Thus, the possibility remains that there are perhaps other proteins in the Interferome or outside it that might be important to examine further. An entirely new source of naturally occurring peptides (short open reading frame peptides or SMORFs) has been recently reported to exist in the human genome as well as other species [109] that appear to function mainly intracellularly although they have been identified in plasma. In theory they could be relevant to this discussion, as they exist in sizes consistent with the SIRS peptide. Indeed a long noncoding RNA can code for a 34mer that regulates muscle activity [110].

Clearly there is much still to be learned about the genome and its various modifications to carry out cellular functions. It is to be hoped that the present Commentary will rekindle interest in SIRS as a probe for immune regulation studies as well as a source of potential peptide based drug discovery efforts for the treatment of autoimmune diseases, transplantation rejection and cancer.

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