Molecular Therapy
Review

Noncanonical microprotein regulation of immunity

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The immune system is highly regulated but, when dysregulated, suboptimal protective or overly robust immune responses can lead to immune-mediated disorders. The genetic and molecular mechanisms of immune regulation are incompletely understood, impeding the development of more precise diagnostics and therapeutics for immune-mediated disorders. Recently, thousands of previously unrecognized noncanonical microprotein genes encoded by small open reading frames have been identified. Many of these microproteins perform critical functions, often in a cell- and context-specific manner. Several microproteins are now known to regulate immunity; however, the vast majority are uncharacterized. Therefore, illuminating what is often referred to as the "dark proteome," may present opportunities to tune immune responses more precisely. Here, we review noncanonical microprotein biology, highlight recently discovered examples regulating immunity, and discuss the potential and challenges of modulating dysregulated immune responses by targeting microproteins.

INTRODUCTION

Advances in high-throughput genomics and proteomics have led to the discovery of thousands of unrecognized small open reading frames (sORFs or smORFs).[1] These sORFs can be translated into noncanonical microproteins,[2] also referred to as micropeptides,[3] short ORF-encoded polypeptides (SEPs),[4] or cryptic microproteins.[5] Previously overlooked due to their small size, frequent lack of canonical start codon, and lower sequence conservation relative to annotated protein-coding genes, sORF-derived noncanonical microproteins are now known to be critical for a wide array of cellular functions including metabolism and gene expression.[6–9] Several noncanonical microproteins were recently shown to regulate immunity; however, the vast majority remain unvalidated, particularly for those expressed in immune cells.[10]

The mammalian immune system is a highly regulated host defense mechanism. Its dysregulation underlies inflammatory and autoimmune disorders.[11] However, the molecular and genetic underpinnings of dysregulated immune responses remain incompletely understood, which complicates the development of precise, highly effective immune modulators. Unlocking the role of microproteins in immunity may unveil novel ways of modulating the immune system.

Here, we review noncanonical microprotein-mediated regulation of mouse and human immune cells. We start by defining microproteins and briefly describing their biogenesis, conservation, biochemistry, and function. Next, techniques to identify and characterize microproteins are discussed. We then describe recently discovered microproteins that regulate immunity and highlight how many of these reside on transcripts containing both protein-coding and noncoding functional elements. We conclude by discussing the potential as well as challenges of targeting or using microproteins to predict, diagnose, or modulate pathologic immune responses. With this comprehensive review, we aim to generate interest among immunologists and molecular biologists, while also offering them a framework for characterizing the potentially thousands of microprotein regulators of immunity.

OVERVIEW OF MICROPROTEIN BIOLOGY

Microprotein definition and classification

Noncanonical microproteins are defined as those with less than 100 amino acids that are directly translated from sORFs.[12] sORFs are present in a variety of transcripts including messenger RNAs (mRNAs), long noncoding RNAs (lncRNA), mitochondrially encoded ribosomal RNAs (rRNA), unannotated novel transcripts, pri-microRNAs, pseudogenes, and circular RNAs.[1,7,8,13–16] It is important to note that microproteins discussed here are derived from sORFs and do not include the many biologically active peptides derived from pro-proteins through post-translational processing and proteolytic cleavage.[17–19] However, the biologic importance of these pro-protein-derived peptides (e.g., insulin,[17] adrenal cortical hormone,[18] and neuropeptide Y[19]) underscores the relevance of small proteins in general.

sORFs are located on either noncoding or protein-coding transcripts and are classified based on their spatial relationship to the nearest canonical ORF (Figure 1A).[1] This classification scheme was adopted by a community-led effort to establish a consensus set of human sORFs. Those found in 5′ untranslated regions (UTRs) of mRNAs, termed upstream ORFs (uORFs), are the most prevalent type (42.5%) of sORF in this consensus set.[1] The second most prevalent type of sORF (30.4%) are those in lncRNA transcripts (lncRNA-ORFs). Several other types of sORFs were

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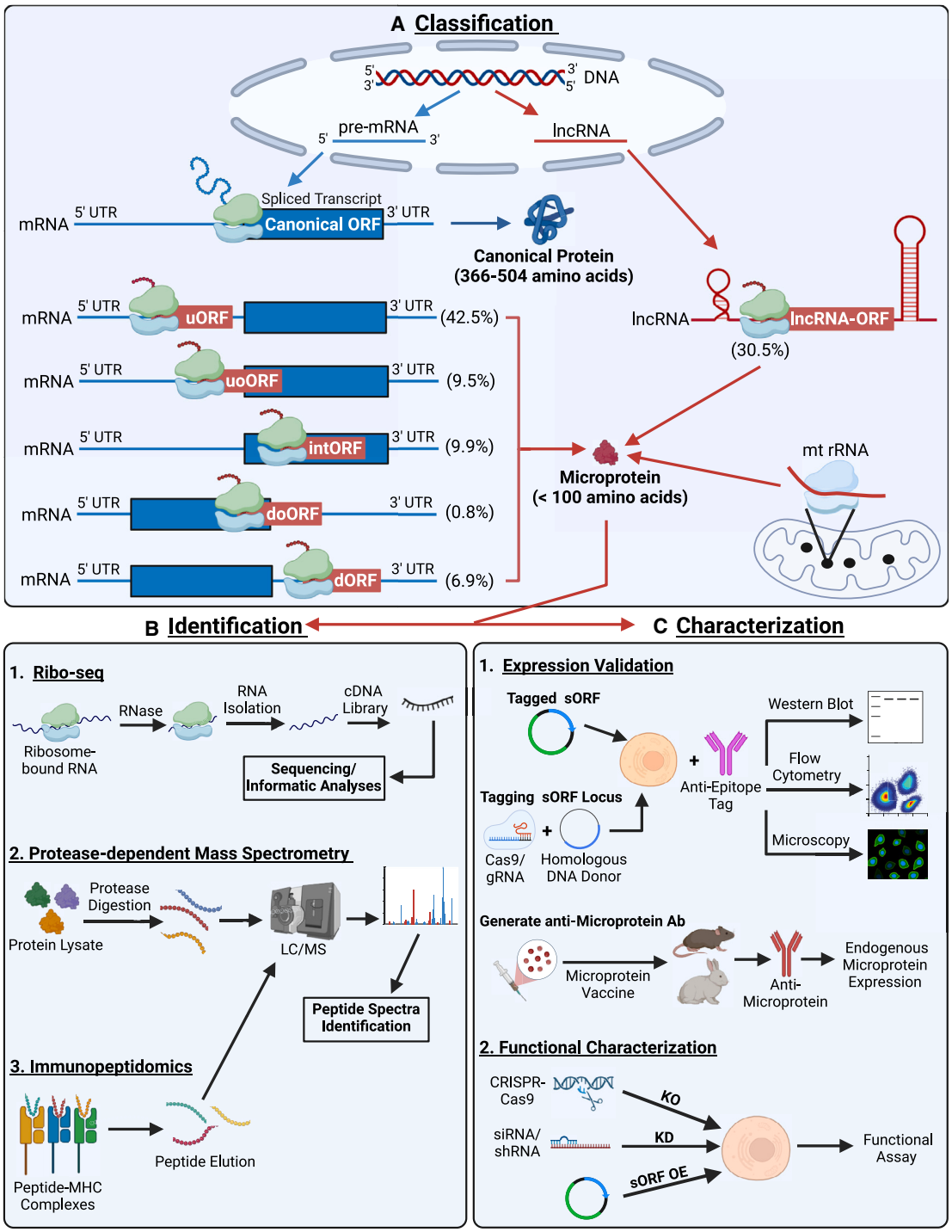


Figure 1. Noncanonical microprotein classification, identification, and characterization

(A) Noncanonical microproteins are encoded by sORFs in mRNAs or noncoding RNAs. sORFs are classified based on their spatial relationship to the nearest canonical ORF. Upstream sORFs (uORFs), upstream overlapping sORFs (uoORF), out-of-frame sORFs (intORFs), downstream overlapping sORFs (doORFs), downstream sORFs (dORFs), or sORFs in lncRNAs (lncRNA-ORFs), are depicted along with their relative abundance in a consensus set of sORFs. (B) Techniques used to identify noncanonical microproteins are depicted. Ribo-seq uses high-throughput sequencing to identify ribosome-bound and thus nuclease-resistant 28- to 30-nucleotide-long RNA fragments. Protease-dependent liquid chromatography-tandem mass spectrometry identifies microproteins by matching peptide spectra to a reference database. Immunopeptidomics

(legend continued on next page)

established by this community-led effort (see Figure 1A) with each making up less than 10% of the consensus set.[1] While this high-confidence group of sORFs is a useful resource, it likely contains false positives (i.e., those sORFs that do not generate stable microproteins[20]) and false negatives (i.e., sORFs that were excluded such as those less than 16 amino acids long,[6] those with a noncanonical start codon,[7,8] those expressed only in specific tissue types not included in this dataset,[15] and those generated from unannotated[14] or circular transcripts[21–23]). Undoubtedly, the true number of sORF genes will be refined as more are identified and characterized in additional tissues and contexts.

Microprotein conservation and potential evolution from noncoding transcripts

Noncanonical microprotein conservation is low relative to canonical proteins.[24] Similar low conservation is observed for lncRNA genes, some of which harbor noncanonical microproteins.[24] By definition, lncRNA transcripts are those with more than 200 nucleotides and no protein coding potential, yet they perform diverse functions using a variety of underlying mechanisms.[25] Consistent with their low conservation, the majority of human sORFs in a consensus set were evolutionarily young and approximately 63% emerged *de novo* from ancestral non-coding transcription.[6] This is consistent with previous reports of *de novo* microproteins emerging from ancestral lncRNAs in primates.[21,26,27] Given that the bulk of transcription is noncoding and that the number of noncoding transcripts increases with organismal complexity,[28,29] *de novo* noncoding transcripts may initially represent aberrant transcription events but ultimately serve as raw material for the evolution of functional noncoding or eventually coding genes.[30]

Microprotein biochemical and molecular characteristics

Noncanonical microprotein biochemical and molecular characteristics differ from canonical proteins at the levels of transcription, translation, and protein biochemistry (see Table 1 for details). At the structural level, signal peptides and hydrophobic transmembrane domains are often present in microproteins and facilitate their membrane localization or secretion.[7,8,23,39] Hydrophobic transmembrane domains may be particularly common among evolutionarily young (i.e., species-specific) microproteins due to their *de novo* emergence from the noncoding genome.[40] The reason for this may be related to enrichment of thymidine and uridine nucleotides in noncoding genomic regions and in codons for hydrophobic amino acids leading to an increased abundance of hydrophobic transmembrane domains in evolutionarily young microproteins.[40]

Noncanonical microproteins also tend to be more disordered and unstable than canonical proteins.[31] An enrichment of carboxy-terminal (C-terminal) hydrophobic tails, which are more common among evolutionarily young microproteins derived from noncoding regions, contributes to their instability.[39] These hydrophobic tails are captured by the ribosome-associated BAG6 membrane protein triage complex for transmembrane insertion, or, if improperly folded, rapid proteasomal degradation.[39] Thus, uncharacterized microproteins with C-terminal hydrophobic tails, especially those derived from genomic regions annotated as noncoding, may be more likely to be rapidly degraded and not functional.

There are also molecular and biochemical characteristics associated with microproteins that are stable and functional. Prensner et al. interrogated a select group of sORFs and found that those stably translated tended to be longer than 50 amino acids, possessed evidence of evolutionary conservation, and had at least one peptide present in previously published mass spectrometry (MS) datasets.[8] At the functional level, sORFs found to impact cellular growth in a CRISPR-Cas9 screen were enriched for those with a Kozak translation initiation motif, lower minimum free energies, greater evolutionary conservation, longer transcript length, and higher translation efficiency.[7] Taken together, uncharacterized noncanonical microproteins that are relatively long (i.e., >50 amino acids), conserved, and lack C-terminal hydrophobic regions may more likely be stably translated and functional. Notably, counterexamples of very small, hydrophobic and poorly conserved microproteins are well documented,[24,41–45] underscoring the need for improved methods to predict microprotein function from amino acid sequence.

METHODS OF MICROPROTEIN IDENTIFICATION

Several technological advances facilitated the rapid increase in the number of identified microproteins over the last 20 years. For a detailed discussion of these methods, we refer readers to several recent excellent reviews.[32,33,42,46,47] Here, we provide an overview of the three most widely used global techniques: ribosome sequencing (Ribo-seq), protease-dependent MS, and immunopeptidomics (Figure 1B).[2]

Ribo-seq is the most sensitive and widely used technology for identifying sORFs.[1] The technique uses high-throughput sequencing to identify ribosome-bound and thus nuclease-resistant 28- to 30-nucleotide-long RNA fragments.[32,34] Notably, Ribo-seq measures global ribosome binding to RNA but does not distinguish between ribosome binding and translation. To identify Ribo-seq sORFs that are likely being translated, several bioinformatic metrics[2,48,49] and modified experimental workflows have been developed. Bioinformatic parameters associated with bona fide translation include an enriched three-nucleotide periodicity of the sequencing reads in the reading frame of the putative microprotein,[21] consistent Ribo-seq read density across the sORF,[21] and a high ribosome release score (ratio of Ribo-seq reads in sORFs relative to their 3′ UTRs).[50] Many of

matches spectra of peptides eluted from purified MHC molecules against a reference database. (C) After being identified, microprotein expression can be validated using various techniques including overexpression of an epitope-tagged microprotein, using CRISPR-Cas9 homology-directed repair to epitope tag an endogenous microprotein locus, and generating a microprotein-specific antibody. Once expression is confirmed, microprotein function can be examined using overexpression (OE), knockdown (KD), or knockout (KO) systems.

Table 1. Comparison of noncanonical microprotein and canonical protein biochemical and molecular characteristics

		Microproteins	Canonical proteins	Reference
Transcription and translation characteristics	exons (avg)	2	11	Chothani et al.[31]
	transcript expression	low relative to canonical proteins for some sORF types	high relative to some sORF types	Vakirlis et al., Schlesinger and Elsässer, Chothani et al., Ingolia et al.[9,32–34]
	alternative start codon usage	∼20%–50% (CUG is most common)	∼20%	Prensner et al., Martinez et al., Chothani et al., Ingolia et al., Ruiz Cuevas et al., Laumont et al.[8,14,31,35–37]
	translation efficiency	lower for dORFs but otherwise similar to mRNAs	similar to microproteins other than those from dORFs	Chen et al., Chothani et al., Ruiz Cuevas et al.[7,31,36]
Protein biochemical characteristics	number of amino acids (median)	all: 33–55; uORF: 17–39 lncRNA-ORF: 42-50	366–504	Martinez et al., Martinez et al., Ji et al., 2022, Ruiz Cuevas et al., Laumont et al., Ouspenskaia et al.[14,15,21,36–38]
	amino acid composition[a]	enriched: A, G, P, R depleted: D, E, I, K, Y		Vakirlis et al., Li et al.[9,10]
	hydrophobicity[a]	increased relative to canonical proteins		Chen et al.[7]

[a]Not replicated in these[3,23] datasets.

these and other parameters are often incorporated into bioinformatic tools used to identify putative sORFs from Ribo-seq data (expertly reviewed elsewhere[2,48,49]). Modified experimental workflows that enrich for translated sORFs from Ribo-seq data include isolating RNAs bound by multiple ribosomes (polysomes)[51] and using ribosomal inhibitors to enrich for reads corresponding to translation initiation while decreasing background reads from non-specific ribosome binding.[35,52,53]

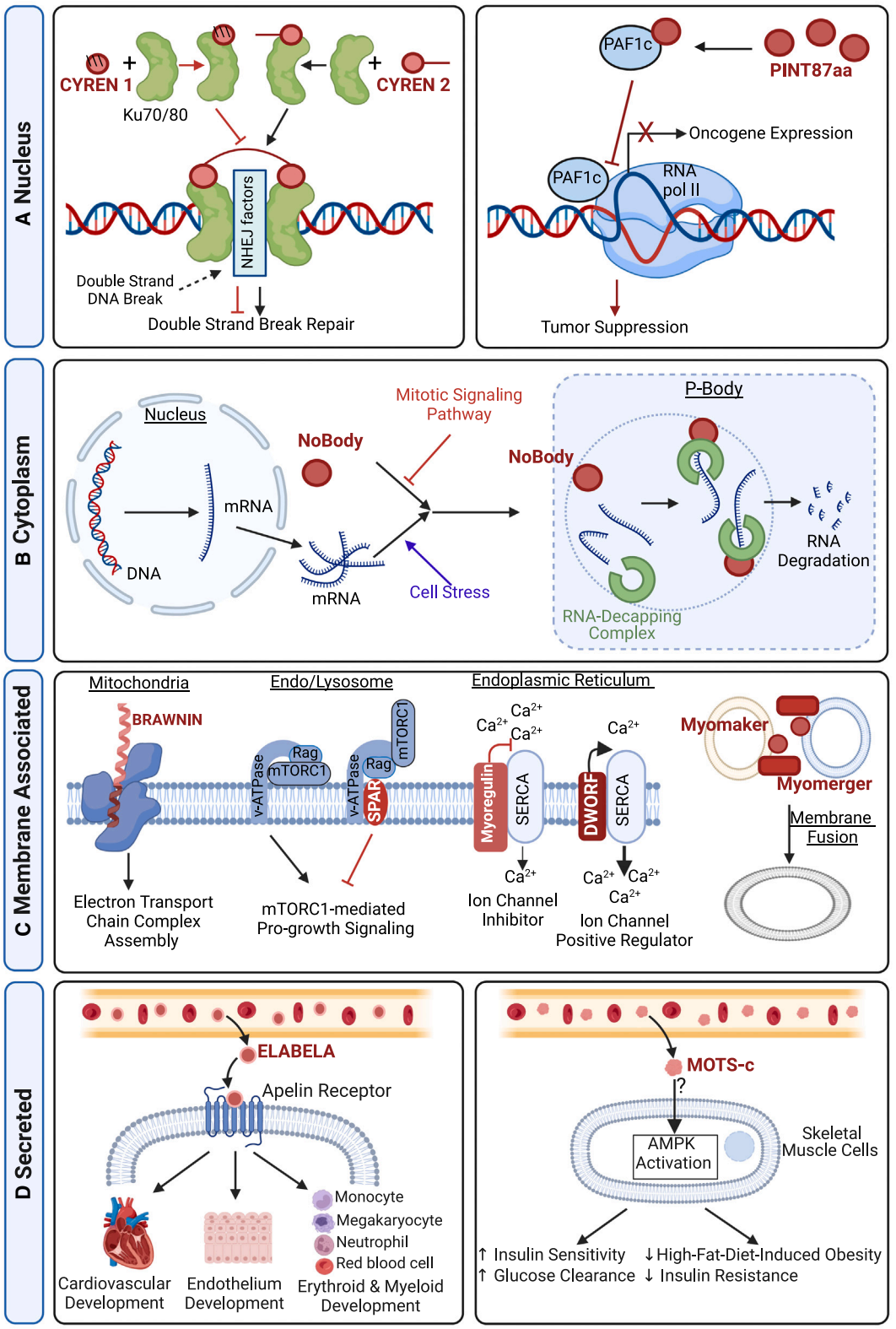
MS is commonly used to validate the expression of microproteins and identify new microproteins on a global scale.[7,15,38] The general MS workflow relies on protease digestion of lysates followed by liquid chromatography-tandem MS (LC-MS/MS). Peptide spectra are then matched against a reference database. This workflow performs well for large canonical proteins but presents several challenges when used to identify microproteins.[36,54] First, microproteins often contain just one, two, or even zero protease recognition sites thereby greatly reducing sensitivity and specificity.[7,15,38] Second, microproteins are generally less abundant, more unstable, and more prone to loss during LC-MS/MS sample preparation.[32] Third, most microproteins are not annotated in reference databases and are subsequently discarded as background noise.[1] Efforts to address these difficulties include using protease-independent methods, using protocols that enrich for small molecular weight proteins to reduce background peptides, utilizing highly sensitive methods that can detect a wide range of proteins such as data-independent MS, and using RNA sequencing and/or Ribo-seq to create databases that more accurately reflect potential microproteins.[15,36,54–56]

Immunopeptidomics is a protease-independent approach that uses MS to identify peptides eluted from major histocompatibility class I (MHC class I) molecules.[7,38] Compared with conventional MS approaches, the MHC class I immunopeptidome prolongs the half-life of bound peptides facilitating the identification of less stable noncanonical microproteins.[36,37] The MHC class I immunopeptidome is more commonly used for microprotein identification compared with the MHC class II immunopeptidome because MHC class I is more broadly expressed and better able to present peptides from proteins with both low and high levels of abundance.[7,36–38,54,57] Limitations of MHC class I immunopeptidomics include that only small peptides (8–12 amino acids) are presented and that each MHC class I allele is restricted to a specific subset of peptides sharing a common motif.[20,21,34] Despite these limitations, immunopeptidomics is a useful technique, particularly for identifying novel microproteins expressed in malignant cells that may be exploited as targets for immunotherapy.[38,58,59]

To overcome the limitations of each high-throughput microprotein identification technique described above, genomic and proteomic techniques are often performed in parallel and integrated using bioinformatic tools.[14,15,31,36–38,54,60–62] Microprotein databases are also available (expertly reviewed elsewhere),[32,46] and collaborative efforts are now combining datasets to establish a standardized annotation of noncanonical microproteins.[1] Furthermore, bioinformatic tools are available to predict subcellular localization[63] and function of uncharacterized microproteins.[63,64] As interest in and understanding of microproteins increases, identification techniques, and algorithms to predict their function will likely steadily improve.

MICROPROTEIN CHARACTERIZATION

After a microprotein is identified, its expression must be validated, and its function determined (Figure 1C). A common low-throughput technique to verify expression is to ectopically overexpress an epitope-tagged version.[7,8] Drawbacks of this method are that it does not provide evidence of endogenous expression and epitope tags may alter microprotein stability.[65] To confirm endogenous



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microprotein expression, CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) coupled with homology directed repair may be used to epitope tag the microprotein within its endogenous locus,[66] but stability problems related to the epitope tag remain possible. Microprotein-specific antibodies can surmount both limitations, but their production and validation can be costly, time consuming, and may not be feasible for extremely small microproteins lacking specific immunogenic sequences.[45,67] If both antibody- and epitope-tagging strategies fail, combining genetic manipulation of a sORF with targeted proteomics using isotopically labeled peptides can also provide evidence for endogenous sORF expression.[48,68] Given the inherent challenges posed by their small size and limited preexisting molecular data, validation of putative microprotein expression frequently requires some degree of trial and error. As the field progresses, validation techniques will likely improve. One exciting possibility is that of direct microprotein sequencing using technology similar to Nanopore long-read DNA and RNA sequencing.[69]

Once expression of a noncanonical microprotein has been verified, the next challenge is to establish its function. A good place to start is to determine its subcellular localization as this is often associated with function.[66,70] Subcellular localization is determined with immunofluorescence, immunohistochemistry, or subcellular fractionation using epitope-tagged versions of the microprotein or microprotein-specific antibodies.[7,8,71–73] Functional predictions based on sequence or folding homology to other characterized proteins may also provide important clues[63,64,74–80] but have limited utility for the majority of evolutionarily young microproteins.[24,31] To test the predicted function of any microprotein, ectopic overexpression systems, shRNA-mediated knockdown, and knockout using CRISPR-Cas9 are used.[7,8,81] CRISPR-Cas9 screens using a library of guide RNAs targeting sORFs can also be used for high-throughput identification of functional, noncanonical microproteins.[7,8] If the microprotein sORF overlaps a potentially functional noncoding sequence, frameshift or start codon missense mutations may be used to ablate translation of the microprotein but maintain transcription of the noncoding RNA thereby enabling the separation of microprotein-dependent from microprotein-independent effects.[66] Complementation can be tested by combining knockdown and overexpression techniques.[66] Interacting proteins may be determined through immunoprecipitation/affinity purification followed by MS.[82] Pathway and ontology enrichment analyses can then be performed on these co-purifying proteins to elucidate potential molecular mechanisms.[71] When employed in the systematic manner described above, it is possible to determine whether any unvalidated noncanonical sORF generates a stable functional microprotein.

MICROPROTEIN MOLECULAR MECHANISM THEMES

While the functions of most microproteins are unknown, those that are known vary widely and include regulating metabolism,[70,83] gene expression,[7,8,84–86] signal transduction,[87,88] DNA repair,[89–91] apoptosis,[16] proliferation,[66] regeneration,[71,92] stress responses,[93] intercellular communication,[94–98] and malignant cell function.[12,42,99] The molecular mechanisms governing these functions are equally varied and depend on their interactions with partner macromolecules, most commonly other proteins. Although there are a wide variety of microprotein mechanisms, themes emerge when microproteins are grouped by subcellular location. In this section, microprotein molecular mechanism themes are briefly reviewed.

Nuclear localization

Interactions of microproteins with multiprotein complexes in the nucleus regulate critical nuclear processes such as DNA repair[89,91] and transcription (Figure 2A).[100] For example, cell-cycle regulator of non-homologous end-joining (CYREN-1) and CYREN-2 are nuclear microproteins that interact with the non-homologous end-joining (NHEJ) DNA repair proteins Ku70 and Ku80 to either inhibit or promote NHEJ, respectively.[89–91] PINT-87aa is a microprotein derived from the circular form of the lncRNA *LINC-PINT*. It inhibits transcription of several oncogenes through its interaction with the polymerase-associated factor 1 complex, a regulator of RNA polymerase II recruitment and elongation.[100]

Cytoplasmic localization

In the cytoplasm, microproteins interact with other proteins or multiprotein complexes to regulate signal transduction,[82,101] translation,[85] mRNA stability,[85] and apoptosis (Figure 2B).[16] For instance,

Figure 2. Microprotein molecular mechanism themes

Molecular mechanism themes of microproteins, grouped by subcellular localization are illustrated. (A) In the nucleus, CYREN-1 blocks, whereas CYREN-2 promotes, double-strand break (DSB) repair by competitively binding to the DSB repair proteins Ku70/80. PINT-87aa inhibits transcription through its interaction with the polymerase-associated factor 1 (PAF1c) complex, a regulator of RNA polymerase II (RNA-pol II) recruitment and elongation. (B) In the cytoplasm, NoBody localizes in P-bodies where it interacts with and enhances the activity of the mRNA decapping complex. NoBody is an example of a microprotein that is intrinsically disordered, and intrinsically disordered proteins or those with intrinsically disordered domains are important for forming non-membranous hydrogel-like structures including P-bodies. Multiple mitotic signaling pathways phosphorylate NoBody leading to P-body dissociation. (C) Transmembrane microproteins localize to a variety of organelles. In mitochondria, BRAWNIN localizes to the inner mitochondrial membrane where it interacts with and is required for respiratory chain complex III assembly and function. The microprotein small regulatory polypeptide of amino acid response, is located in endo/lysosomes and plays an important role in muscle regeneration. It interacts with v-ATPase preventing mammalian target of rapamycin complex 1 (mTORC1) from interacting with Ragulator thereby limiting mTORC1 activation and pro-growth signaling. DWORF (dwarf open reading frame) and myoregulin are ER membrane-associated microproteins that positively and negatively regulate the calcium pump sarco-endoplasmic reticulum Ca^{2+} adenosine triphosphatase (SERCA), respectively. Myomaker and Myomerger are two integral membrane microproteins that positively regulate membrane fusion in myoblasts. (D) Many microproteins are secreted and are important intercellular messengers. ELABELA is one example that signals through the Apelin receptor and controls cardiovascular development, early endothelial development, erythropoiesis, and myeloid development. MOTS-c is a mitochondrial DNA-encoded microprotein hormone that enhances insulin sensitivity and glucose metabolism in muscle cells by enhancing AMPK signaling.

nonannotated P-body dissociating polypeptide (NoBody) localizes to processing bodies (P-bodies), which are non-membranous ribonucleoprotein complexes that regulate mRNA decapping, stability, and translation.[85] P-bodies assemble via a biophysical process called liquid-liquid phase separation (LLPS),[102] which underlies non-membranous organelle formation.[103] LLPS often requires proteins or protein domains that lack a stable structure (i.e., are intrinsically disordered).[102,103] Within P-bodies, NoBody regulates and interacts with protein components of the mRNA decapping complex,[84] and, due to its intrinsic disorder, NoBody may also regulate P-body liquid phase remixing in response to phosphorylation from multiple signal transduction pathways.[85] Notably, many microproteins are intrinsically disordered,[6,90,104,105] suggesting that some intrinsically disordered microproteins may be important regulators of non-membranous organelle formation.

Membrane localization

Signal peptides and transmembrane domains are common among microproteins,[23] allowing many to localize to the plasma membrane and membrane-bound organelles. Mechanistic themes of membrane-localized microproteins include interactions with other transmembrane proteins and multiprotein complexes resulting in regulation of ion pumps,[106] signaling complexes,[71] and membrane fusion (Figure 2C).[64,77,84,104,105] Transmembrane microproteins are particularly common in mitochondria where, through interactions with other transmembrane proteins, they regulate electron transport chain (ETC) assembly and function.[67,86,107–109] Collectively, these examples illustrate the importance of transmembrane microproteins on membrane-dependent cellular functions.

Extracellular localization

Many microproteins are secreted into the extracellular space[15,23,70] where they interact with cell surface or intracellular targets to affect development,[81,83,110] tumor proliferation,[8] appetite,[9,111] glucose metabolism,[83] insulin sensitivity,[83] and inflammation (Figure 2D).[112] Apelin receptor early endogenous ligand (ELEBELA, also known as Toddler) is an example of a secreted hormone-like microprotein that signals through the Apelin receptor.[95,97,110] It is critical for cardiovascular, endothelial, erythroid, and myeloid development, and its loss during pregnancy may lead to preeclampsia.[94–98,110] By contrast, mitochondrial ORF of the 12S rRNA type-C (MOTS-c) is a mitochondrial DNA-encoded microprotein hormone that targets intracellular adenosine monophosphate-activated protein kinase signaling in skeletal muscle thereby regulating glucose metabolism and insulin sensitivity.[83] However, it is unclear which secretory and endocytic pathway(s) are used by MOTS-c and other secreted microproteins.[45,83,111] Altogether, these examples suggest that microprotein hormones are important for intercellular communication.

MICROPROTEINS IN POLYCISTRONIC AND BIFUNCTIONAL EURKARYOTIC TRANSCRIPTS

Prokaryotic transcripts often contain multiple ORFs (i.e., are polycistronic) generating several proteins from the same transcript.[113] By contrast, eukaryotic protein-coding transcripts were historically assumed to be monocistronic.[7] The discovery of functional uORF-derived microproteins on eukaryotic transcripts challenged this assumption and suggested that eukaryotic mRNA, like prokaryotic mRNA, can also be polycistronic.[3,99] Certainly, not all eukaryotic uORFs generate functional microproteins, but instead a portion[23,31] regulate translation of their downstream main ORF through ribosomal sequestration or nonsense-mediated decay.[114–116] Thus, some uORFs function as *cis* regulators of their downstream main ORF while others code for a diverse pool of functional microproteins.[7]

While uORFs challenged the monocistronic assumption of eukaryotic mRNA, lncRNA-encoded microproteins now challenge the coding versus non-coding dichotomy of eukaryotic genes.[82,116–122] Many microproteins encoded by noncoding RNAs demonstrate functions separate from that of their parent lncRNA.[82,117–123] This suggests that bifunctional (coding and noncoding) genes[124] may be enriched among microproteins. In addition, protein- and RNA-dependent functions of bifunctional genes are often not observed in the same context, indicating that subcellular localization, cell type, and context influence coding versus noncoding functions of bifunctional genes.[72,73,124,125]

Among the immunomodulatory microproteins identified thus far (Table 2), the majority (seven of nine) appear to be bifunctional.[45,72,112,127,130–132,144–147] In some cases, the microprotein is present in a single species, either human specific or lower on the evolutionary scale.[112,148] Because most evolutionarily young microproteins arise *de novo* from noncoding transcription,[6] one may speculate that some bifunctional loci represent evolutionary intermediates. Alternatively, bifunctional loci may instead be evolutionarily stable (e.g., MOCCI and miR-147b described below)[61,62,149] and underappreciated, suggesting that even known protein-coding mRNA molecules may have unrecognized noncoding functions.[150] As more microproteins are characterized, the prevalence of bifunctional genes within the immune system and elsewhere will continue to be ascertained.

IMMUNOMODULATORY MICROPROTEINS

Microproteins regulate both innate (Figure 3) and adaptive immunity (Figure 4). Here, we catalog functions and mechanisms of microproteins involved in immune regulation and, for those derived from bifunctional loci, we also detail the function and mechanism of their corresponding noncoding RNA (Table 2).

Microprotein modulation of innate immunity

Mm47/Stmp1, a positive regulator of the NLRP3 inflammasome pathway

Mitochondria are regulatory hubs for innate antiviral and inflammasome pathways.[151] Mitochondrial microproteins (mito-SEPS) are emerging as important regulators of these pathways.[72] The nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (Nlrp3) inflammasome is a multiprotein complex that is highly expressed in innate immune cells and is critical for detecting and mounting rapid immune responses to infection.[152] It becomes

Table 2. Noncanonical microprotein and bifunctional genes that regulate immune cells

	Microprotein					Noncoding element					
Gene name	Name	Cell site of action	Function	Immuno-modulatory effect	Disease state(s) studied	Name	Cell site of action	Function	Immuno-modulatory effect	Disease state(s) studied	Ref.
Innate immunity											
1810058124Rik/ C7orf73	Mm47/Stmp1	macrophage/ microglia	activation of NIrp3 inflammasome/ increased ROS, increased mitochondrial fission	pro-inflammatory	ischemia-reperfusion (glaucoma and diabetic retinopathy)						Zheng et al., Bhatta et al.[3,65]
	STMP1	tumor cells	promotes electron transport chain complex IV activity and mitochondrial fission leading to proliferation and metastasis		cancer						Spencer et al., Papaioannou et al.[122,123]
C15ORF48/NMES-1/MISTRAV	MOCCI/C15ORF48/ MISTRAV	macrophage, monocyte	decreases electron transport chain complex IV activity, reduces ROS, reduces mitochondrial membrane potential, reduces inflammatory cytokine production	anti-inflammatory	rheumatoid arthritis, COVID-19, viral infection	miR-147b	macrophage/ monocyte	decreases complex IV activity, reduces inflammatory cytokine production, promotes antiviral signaling	anti-inflammatory	viral infection	Laumont et al., Erhard et al., Spencer et al.[37,57,122]
Hemotin/Stanin	hemotin/stanin	hemocytes (*Drosophila* macrophages)/ macrophages	regulates endosomal maturation of macrophages	anti-bacterial/promotes phagocytosis	anti-bacterial phagocytosis						Pueyo et al.[126]
LINC00998	MAVI1	macrophages	inhibits MAVS protein aggregation and type 1 interferon signaling	pro-viral	viral infection	*LINC00998*	tumor cells	tumor suppressive in glioma and AML		cancer	Shi et al., Yang et al., Pang et al.[127–129]
	SMIM30	tumor cells	promotes hepatocellular carcinoma growth		cancer						
U90926	U9-ORF	macrophages/ dendritic cells	TLR-induced paracrine secretion of microprotein dampens IL-6-mediated inflammation	pro-inflammatory	septic shock	*U90926*	microglia	stabilizes *Cxcl2* neutrophil chemoattractant mRNA by preventing RNA decay	pro-inflammatory	ischemia-reperfusion/stroke	Sabikunnahar et al., Chen et al.[112,130]

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Table 2. *Continued*

	Microprotein					Noncoding element					
Gene name	Name	Cell site of action	Function	Immuno-modulatory effect	Disease state(s) studied	Name	Cell site of action	Function	Immuno-modulatory effect	Disease state(s) studied	Ref.
Adaptive immunity											
Aw112010	Aw112010	macrophages	increased production of inflammatory cytokines	pro-inflammatory/ anti-bacterial	*Salmonella* infection, colitis	*Aw112010*	$CD4^+$ T cells, macrophages	promotes $CD4^+$ T cell polarization toward Th1 and Th17 subsets; suppresses anti-inflammatory IL-10 cytokine expression; promotes inflammatory cytokine production in macrophages	pro-inflammatory		Yang et al., Jackson et al.[131,132]
Dleu2/DLEU2	Dleu2-17aa/ DLEU2-25aa	Tregs	promotes Treg polarization by enhancing Smad3 recruitment to the *Foxp3* promoter	anti-inflammatory	experimental autoimmune encephalitis	miR-15a/16-1	B and T cells	restricts proliferation, survival, and memory T cell differentiation		chronic viral infection, cancer	Tang et al., 2024, Gagnon et al., 2019, Urena et al., 2022, Klein et al., 2010[44,133–135]
Mir31/MIR31HG	miPEP31	Tregs	promotes Treg polarization by inhibiting transcription of miR-31	anti-inflammatory	experimental autoimmune encephalitis	miR-31	Tregs	inhibits Treg polarization by targeting *FOXP3* and *Gprc5a* transcripts	pro-inflammatory	experimental autoimmune encephalitis	Zhou et al., Zhang et al., Rouas et al.[43,136,137]
MIR155HG	P155/miPEP155	dendritic cells	binds HSC70 and downregulates MHC class II-mediated antigen presentation leading to decreased Th1 and Th17 polarization	anti-inflammatory	psoriasis, experimental autoimmune encephalitis	miR-155	bone marrow-derived hematopoietic cells	promotes $CD4^+$ T cell polarization toward Th1 and Th17 subsets; regulates immunoglobulin class switching	pro-inflammatory	multiple inflammatory disorders	Niu et al., Seddiki et al., Chen et al., Dunand-Sauthier et al., Rodriguez et al., Alivernini et al.[45,138–142]
MT-RNR-1	MOTS-c	T cells	suppresses mTOR signaling, which likely contributes to enhanced Treg and suppressed Th1 responses	anti-inflammatory	type 1 diabetes, obesity						Schlesinger et al., Graham et al., 2023[67,158]
	MOTS-c	skeletal muscle	regulates glucose metabolism and insulin sensitivity through AMPK		type 2 diabetes						

activated in response to pathogen-associated molecular patterns and host-derived danger-associated molecular patterns. Activation of the Nlrp3 inflammasome results in the maturation of inflammatory cytokines (e.g., interleukin-1β [IL-1β]) important for antimicrobial responses and induction of the pyroptosis cell death pathway.[152] The highly conserved mitochondrial micropreptide-47 (Mm47, also called Stmp1), encoded by the lncRNA *1801158124Rik*, contributes to activation of the Nlrp3 inflammasome in murine macrophages (Figure 3A).[65] Lipopolysaccharide (LPS) treatment reduces the expression of the *Mm47* transcript in a TLR4-TRIF (Toll-like receptor 4/TIR domain-containing adaptor inducing interferon-β)-dependent manner. Despite downregulation of its transcript following activation, genetic deletion reduces IL-1β production following Nlrp3 inflammasome activation, and ectopic Mm47 expression rescues IL-1β maturation in Mm47 knockout macrophages. Altogether, these data suggest that Mm47 is required for inflammasome activation while the downregulation of its transcript following macrophage activation may serve to prevent prolonged inflammasome signaling.

Mm47 is also necessary for inflammasome-mediated inflammation in microglia, which are resident macrophage-like cells in the central nervous system. Following activation by oxygen-glucose deprivation and reperfusion, Mm47 augments expression of Nlrp3 inflammasome components, inflammasome activation, and Nlrp3-driven inflammatory cytokine production in primary murine microglia.[3] Furthermore, mitochondrial membrane potential and reactive oxygen species (ROS), positive regulators of inflammasome pathway activity,[149,153] are decreased in *Mm47*-deficient microglia.[3] In a retinal *in vivo* ischemia-reperfusion (IR) model of inflammation, microglia activation, inflammasome activation in retinal tissue, and apoptosis of retinal ganglion cells are reduced in *Mm47* knockout mice. Because IR-mediated retinal inflammation contributes to the pathogenesis of diabetic retinopathy and glaucoma, these data suggest that targeting Mm47 may prove beneficial for these disorders.

Combined, these two studies offer an initial glimpse of how a mitochondrial microprotein can regulate innate immune cells. They also highlight important cell- and context-specific regulation of its function and expression. For example, only in the IR microglial model does Mm47 drive increased expression of Nlrp3 inflammasome pathway genes, enhance pyroptosis, and increase ROS production.[3] Regarding expression, Mm47 rapidly declines in LPS-stimulated bone marrow-derived macrophages[65] whereas it increases in microglia following IR-induced inflammation.[3] Outside of the immune system, Mm47 is also highly expressed in hepatocellular carcinoma (HCC) where it promotes the activity of complex IV of the ETC and mitochondrial fission leading to increased proliferation and metastasis.[154,155] Its enhancement of mitochondrial fission in HCC is consistent with that observed in microglia.[3] By contrast, Mm47 does not alter complex IV activity in microglia.[3] Thus, there is much to learn about the regulation of Mm47 expression, its cell-type-specific molecular mechanisms, and its function in the many other tissues where it is abundantly expressed.[3,65]

MOCCI, a negative regulator of inflammation

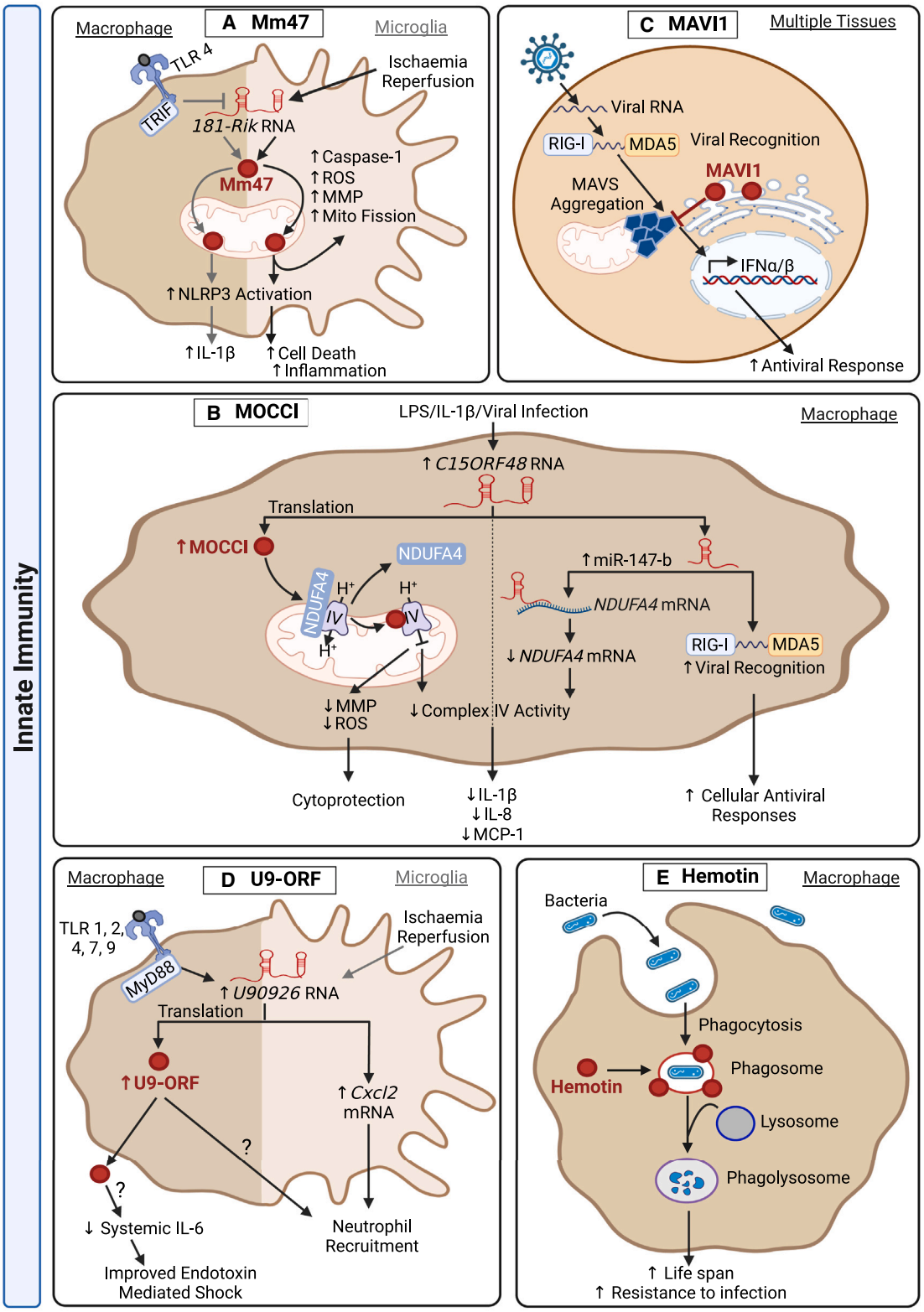
Modulator of cytochrome c oxidase during inflammation (MOCCI, also referred to as C15ORF48, MISTRAV, and NMES-1), encoded by the highly conserved gene *C15ORF48*, is a mitochondrial microprotein that negatively regulates innate immune cell inflammatory responses (Figure 3B).[72,73,125] MOCCI is induced in a variety of cell types including macrophages, endothelial cells, epithelial cells, and fibroblasts by inflammatory stimuli (e.g., LPS, IL-1β, tumor necrosis factor-α, interferon-γ [IFN-γ], viral infection, and chemical hypoxia),[37,57,122] but its induction is complex and varies by cell type, context, and species.[72,73,125] MOCCI dampens inflammatory responses by negatively regulating complex IV of the ETC. Specifically, MOCCI replaces its paralog, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 4 (NDUFA4), a component of complex IV, resulting in lower complex IV activity, lower mitochondrial membrane potential, and reduced ROS production leading to cyto-protection and decreased inflammatory cytokine production.[72,73,125]

The *C15ORF48* gene also encodes a highly conserved micro-RNA in its 3′ UTR, miR-147b.[72,73,125] Complementary to MOCCI's effect on NDUFA4, miR-147b targets and reduces the expression of the *NDUFA4* transcript resulting in decreased complex IV activity and inflammation.[72,73] Unlike MOCCI, miR-147b does not alter membrane potential or ROS production.[72,73] While MOCCI and miR-147b cooperate to dampen complex IV activity and inflammation, their effects on innate antiviral pattern recognition signaling pathways through retinoic acid inducible gene I/melanoma differentiation-associated protein 5 (RIG-I/MDA-5) diverge. MiR-147b potentiates RIG-I/MDA-5/nuclear factor κB activity, enhancing cellular antiviral responses, whereas MOCCI suppresses this response protecting against excessive cell death from hyperinflammation.[72] *C15ORF48* is therefore an example of a bifunctional gene where the coding and noncoding elements have both convergent and divergent functions.

Interestingly, MOCCI and miR-147b may not be co-expressed in some cell types or the ratio of the two may differ by cell type. For instance, monocytes express mainly miR-147b in response to IL-1β.[72] This may aid their critical role in viral defense. On the other hand, endothelial cells produce high levels of MOCCI in response to IL-1β, perhaps reflecting their need to avoid ROS production that could lead to vascular damage.[72] However, the mechanisms governing translation of MOCCI and other microproteins in bifunctional loci are not yet known.

MAVI1, a negative regulator of antiviral pattern recognition receptor pathways

Innate antiviral pattern recognition pathways alert the immune system that a viral infection is taking place and induce a rapid, potent antiviral response.[143,156] Frequently, these pathways are activated first



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in non-immune cells by the cytosolic nucleic acid pattern recognition receptors, RIG-I and MDA-5, that signal through the adaptor, mitochondrial antiviral signaling (MAVS) protein.[122,123] Downstream kinases then activate the transcription factors interferon regulatory factors 3 and 7 thereby inducing antiviral type I interferons.[122,123] The microprotein MAVI1 (microprotein in antiviral immunity 1) is a conserved, endoplasmic reticulum transmembrane microprotein previously annotated as the lncRNA *LINC00998*.[127] MAVI1 is downregulated via an undetermined pathway following RNA virus infection.[127] It interacts with and inhibits MAVS aggregation and subsequent type I interferon induction (Figure 3C).[127] Consistent with this, *Mavi1* knockout mice are more susceptible to RNA virus infection, and inhibitors that disrupt the interaction between MAVI1 and MAVS boost *in vitro* antiviral responses.[127] These data show how noncanonical microproteins in non-immune cell types can profoundly affect antiviral immune responses and may be targets for preventing or treating viral infections.

MAVI1/*LINC00998* is also an example of a bifunctional gene, but its noncoding function has thus far only been evaluated in tumor cells where it appears to exert a tumor-suppressive effect in glioma and acute myelogenous leukemia.[144,145] By contrast, the MAVI1 microprotein (also called small integral membrane protein 30 [SMIM30]) promotes HCC and hepatoma growth.[128,129] The reasons for the divergent effects of the MAVI1 microprotein and the *LINC00998* RNA molecule on tumor growth are intriguing and suggest cell-type-specific translation of MAVI1, which, along with determining whether the *LINC00998* RNA molecule regulates innate antiviral pattern recognition receptor pathways, will require careful examination.

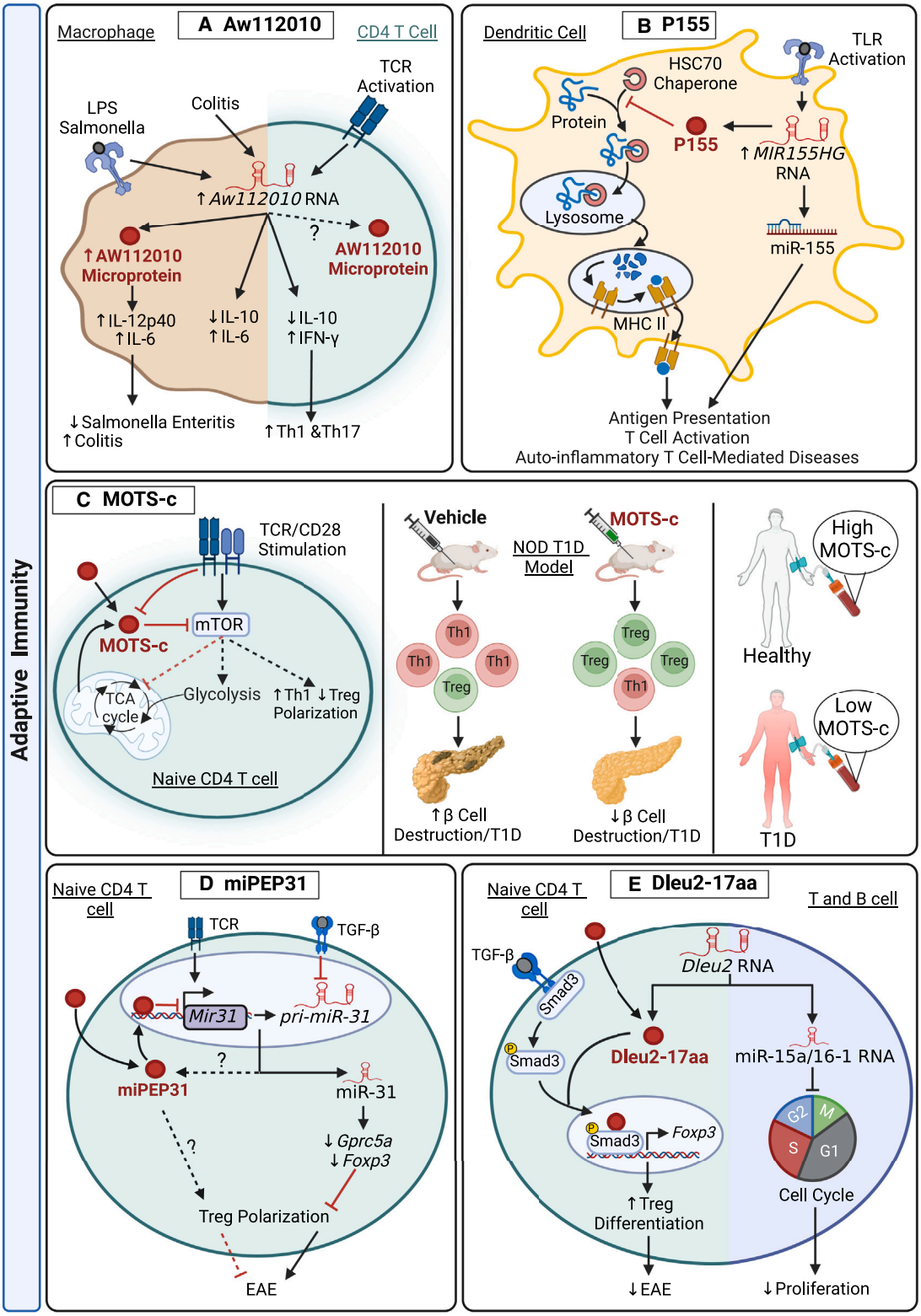
U9-ORF, a regulator of systemic inflammation

Macrophages and dendritic cells (DCs) are central components of the innate immune system. They are equipped to detect microbial threats and rapidly respond by producing inflammatory cytokines and alerting the rest of the immune system, yet their dysregulation also contributes to immune-mediated pathology and fatal systemic shock.[157] U9-ORF, encoded by the lncRNA *U90926*, is an 87-amino-acid murine microprotein secreted by activated macrophages and DCs that dampens systemic inflammatory responses (Figure 3D).[112] It is induced by TLR1, 2, 4, 7, and 9 ligands in a myeloid differentiation primary response 88 (MyD88) and p38α mitogen-activated protein kinase-dependent manner.[112] Its expression is enriched in myeloid cells,[112] but it is also expressed in a variety of non-hematopoietic tissues.[130,146,147] Deletion of *U90926* minimally alters leukocyte development and macrophage function.[112] By contrast, *U90926* knockout mice are more susceptible to endotoxic shock in an IL-6-dependent manner.[112] Because the entire *U90926* locus was deleted in this model, it was not able to attribute the endotoxic shock susceptibility directly to macrophages nor distinguish U9-ORF microprotein-dependent from *U90926* transcript-dependent effects.[112]

The *U90926* RNA molecule also regulates microglia responses in murine models of stroke.[130] Following microglia-selective *U90926* lentivirus knockdown, ischemic brain damage decreases due to less inflammatory neutrophil infiltration.[130] Like that observed for macrophages, *U90926* does not alter microglia function.[130] Instead, neutrophil recruitment increases in part due to the *U90926* RNA molecule stabilizing the mRNA of C-X-C motif chemokine ligand 2 (*Cxcl2*), a neutrophil chemoattractant.[130] The *U90926* RNA molecule stabilizes *Cxcl2* mRNA by competitively inhibiting the binding of *Cxcl2* to malate dehydrogenase 2 (MDH2), an RNA binding protein that promotes RNA decay.[130] While the role of the microprotein was not specifically assessed in microglia following IR, combined, these two studies suggest an interesting contradiction in which *U90926* promotes microglia-dependent inflammation following IR but decreases inflammation during endotoxic shock. Therefore, *U90926*'s effect is also cell and context specific, a conclusion further supported by its diverse roles in non-immune cells.[146,147,158] However, whether the U9-ORF microprotein definitively contributes to any of these other phenotypes has not been specifically tested, and it is unknown if the syntenic human putative lncRNA homolog, *AC110615.1-201*,[148] which lacks microprotein

Figure 3. Microprotein modulators of innate immunity

Microproteins that regulate various components of the innate immune system including inflammasome activation, cytokine production, pattern recognition receptor (PRR) signaling, immune cell recruitment, and phagocytosis are depicted. (A) Mm47 positively regulates the NLRP3 inflammasome pathway in macrophages and microglia. It is encoded by the noncoding RNA, *1810058124Rik* (*181-Rik*). The expression of *181-Rik* is reduced by activation of the TLR4 pathway, whereas ischemia-reperfusion (IR)-mediated inflammation induces its expression. In LPS-treated macrophages, Mm47 enhances NLRP3 activation and IL-1β maturation and secretion. In IR-stimulated microglia, Mm47 induces expression of Nlrp3 inflammasome components, inflammasome activation, Nlrp3-driven inflammatory cytokine production, reactive oxygen species (ROS) production, and mitochondrial fission. (B) MAVI1 is an ER-localized microprotein that inhibits antiviral PRR signaling by inhibiting the aggregation of MAVS on mitochondria. (C) MOCCI is a mitochondrial microprotein, encoded by the *C15ORF48* RNA. It negatively regulates macrophage/monocyte inflammatory responses by displacing NDUFA4 from complex IV of the electron transport chain and lowering complex IV activity. MOCCI also has cyto-protective effects in stimulated macrophages due to its ability to decrease mitochondrial membrane potential and ROS production. *C15ORF48* also produces miR-147b, which targets the *NDUFA4* transcript and, similar to MOCCI, reduces complex IV activity and suppresses inflammation. Unlike MOCCI, miR-147b does not affect membrane potential nor ROS production. In addition, miR-147b enhances antiviral PRR signaling through RIG-I/MDA-5 independent of MOCCI. (D) The U9-ORF microprotein regulates systemic inflammation. Its transcript is induced by TLR1, 2, 4, 7, or 9 ligands via a MyD88 and p38α MAPK-dependent pathway in macrophages. The cellular function of the U9-ORF microprotein is still unknown, but its secretion lowers serum IL-6 and protects against endotoxic shock in pre-clinical models. The *U90926* RNA molecule also influences innate immunity. Specifically, it worsens ischemic brain damage by promoting neutrophil recruitment through the stabilization of the *Cxcl2* transcript, a neutrophil chemoattractant, in microglia. (E) Hemotin is a transmembrane microprotein located in early endosomes that positively regulates phagocytosis in macrophages. It promotes early-to-late endosomal maturation, and its deficiency shortens the lifespan of bacteria-challenged *Drosophila*.



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amino acid conservation, performs similar functions. Thus, much remains unknown about this bifunctional locus important for coordination of inflammation.

Hemotin, a phagocytosis regulator

Phagocytosis by innate immune cells is critical for tissue repair and antimicrobial defense.[159] The microprotein Hemotin is a conserved 88-amino-acid transmembrane protein that localizes to early endosomes and promotes endosomal maturation during phagocytosis in *Drosophila* macrophages (Figure 3E).[126] *Hemotin* mutants accumulate phagocytosed material and have defective anti-bacterial responses resulting in a shortened lifespan.[126] Mechanistically, Hemotin indirectly reduces the development of early endosomes by binding to the adaptor protein 14-3-3ζ and attenuating its effect on phosphatidylinositol 3-kinase 68D (P1KD68D), which is important for early endosome formation and function.[126] Stannin is the human functional homolog of Hemotin and rescues *Hemotin* mutant hemocytes.[126] However, future studies will need to address whether Hemotin is also necessary for *in vivo* mammalian immunity.

Microprotein regulation of adaptive immunity

Aw112010, a myeloid and potentially adaptive pro-inflammatory microprotein

The 84-amino-acid murine microprotein encoded by *Aw112010* is essential for mucosal immunity (Figure 4A).[132] It is induced following either LPS or *Salmonella* infection and promotes production of the inflammatory cytokines IL-12p40 and IL-6 in macrophages.[132] Consistent with its inflammatory role, mice with a mutated *Aw112010* start codon ($Aw112010^{Stop}$) are more susceptible to *Salmonella* infection but less susceptible to a dextran sodium sulfate model of colitis.[132] The precise mechanism for how the *Aw112010* microprotein enhances IL-12p40 production or how this affects mucosal immunity are not known. One possibility that would be congruent with *Aw112010*'s RNA-dependent function (described below)[131] is that IL-12p40, an inducer of antibacterial and pro-inflammatory type I helper T cell (Th1) differentiation, promotes mucosal Th1 polarization.

The *Aw112010* RNA molecule is induced in $CD4^+$ T cells upon T cell receptor (TCR) stimulation and enhances polarization of Th1 and Th17 cells.[131] Its depletion decreases production of the inflammatory cytokine IFN-γ while increasing expression of the anti-inflammatory cytokine IL-10.[131] At the molecular level, the *Aw112010* RNA associates with the IL-10 promoter and the epigenetic transcriptional repressor lysine demethylase 5A (KDM5A).[131] *Aw112010* depletion increases activating epigenetic marks near the *IL-10* promoter.[131] These data imply that the *Aw112010* RNA molecule may suppress IL-10 expression by removing activating epigenetic marks. To further test the RNA-dependent role of *Aw112010*, a region downstream of the ORF was mutated with CRISPR-Cas9 in a murine macrophage cell line resulting in less IL-6 but more IL-10 being produced in response to LPS.[131] While suggestive of an RNA role for these effects on IL-6 and IL-10, this CRISPR-generated mutant could not rule out a microprotein-dependent effect. Regardless, the function of both the *Aw112010* RNA molecule and its microprotein seem to converge on inflammatory myeloid and helper T cell responses.

P155, a regulator of antigen presentation

Professional antigen-presenting cells have specialized machinery for internalizing exogenous antigens and presenting them on MHC class II molecules to $CD4^+$ T cells.[160] In humans, the lncRNA *MIR155HG* encodes a 17-amino-acid microprotein that reduces MHC class II antigen presentation in mouse and human DCs (Figure 4B).[45] It reduces MHC class II presentation by interacting and interfering with the chaperone heat shock cognate protein 70 (HSC70), which is required for MHC class II antigen trafficking and presentation.[161] Treatment of DCs with P155 (also called miPEP155) reduces MHC class II expression, antigenic peptide transportation, T cell proliferation, and helper T cell polarization toward Th1, Th2, and Th17 cells.[45] Intravenous administration of P155 ameliorates murine models of DC-driven auto-inflammation

Figure 4. Microprotein regulation of adaptive immunity

Microproteins that regulate components of adaptive immunity including cytokine production, antigen presentation, and $CD4^+$ T cell polarization are shown.

(A) The Aw112010 microprotein in macrophages promotes IL-12p40 and IL-6 production following stimulation with LPS or in models of bacterial infection and colitis. The *Aw112010* RNA molecule in macrophages also promotes inflammatory cytokine transcription. In $CD4^+$ T cells, the *Aw112010* RNA molecule is induced following TCR activation and enhances polarization of inflammatory Th1 and Th17 cells possibly by suppressing anti-inflammatory *IL-10* transcription. The translation and function of the AW112010 microprotein in T cells are unknown (dotted line). (B) The P155 microprotein, encoded by the *MIR155HG* RNA, is an important regulator of MHC class II antigen presentation in dendritic cells (DCs). P155 binds and inhibits the chaperone HSC70, a critical component of MHC class II antigen trafficking and presentation, leading to reduced MHC class II antigen presentation, reduced $CD4^+$ T cell activation, and reduced severity of auto-inflammatory T cell-mediated disease models. The *MIR155HG* RNA also generates the anti-inflammatory micro-RNA, miR-155. In DCs, miR-155 promotes T cell activation, proliferation, and in some contexts Th1 cytokine production. In T cells, miR-155 promotes inflammatory Th1 polarization. (C) MOTS-c is a mitochondrial microprotein that regulates T cell self-tolerance. Its expression declines with TCR stimulation. However, it interacts with and negatively regulates the mTOR complex downstream of TCR activation. This is consistent with its ability to promote Treg polarization and limit glycolysis, both of which are positively regulated by mTOR signaling (dotted lines are functions of MOTS-c not definitively dependent on MOTS-c's effect on mTOR). In a type 1 diabetes (T1D) mouse model, systemic delivery of MOTS-c protected against pancreatic β cell destruction and subsequent T1D development by reducing Th1 and increasing Tregs in the spleen and pancreas. In humans, patients with T1D have lower serum levels of MOTS-c than do healthy controls. (D) The *pri-miR-31* transcript encodes both the microprotein miPEP31 and the micro-RNA miR-31. TCR stimulation induces miR-31, whereas TGB-β signaling decreases it. Treg differentiation is inhibited by miR-31 targeting of the *FOXP3* and *Gprc5a* transcripts. Exogenous miPEP31 enhances Treg differentiation and alleviates murine experimental autoimmune encephalitis (EAE) potentially through its ability to inhibit *pri-miR-31* transcription and miR-31 production. The translation and function of endogenous miPEP31 are not known (dotted lines). (E) The Dleu2-17aa microprotein promotes Treg differentiation by binding phosphorylated Smad3 and enhancing its recruitment to the *Foxp3* promoter following TGB-β signaling. The *Dleu2* transcript also encodes miR-15a/16-1, which limits T cell proliferation, survival, and memory differentiation.

(psoriasis and experimental autoimmune encephalitis [EAE]) by decreasing inflammatory Th17 and increasing regulatory $CD4^+$ T cell infiltration in diseased tissues.[45] Altogether, these data show that P155 is a modulator of DC antigen presentation that leads to blunted inflammatory T cell responses.

The *MIR155HG* gene also encodes the well-described pro-inflammatory micro-RNA, miR-155.[138] Micro-RNA 155 expression is induced upon DC activation where it promotes T cell activation, proliferation, and in some contexts Th1 cytokine production.[139–141] Overexpression of miR-155 in T cells promotes Th1 polarization,[162] and miR-155 expression in multiple immune cells contributes to the development and severity of inflammatory disorders.[138,142] Thus, P155 and miR-155 have anti- and pro-inflammatory effects, respectively. Because nuclear micro-RNA processing and cytoplasmic translation are mutually exclusive,[163] a bidirectional fate for the *MIR155HG* transcript likely exists in which either the pro-inflammatory miR-155 is produced or the anti-inflammatory P155 is translated. As with MOCCI and miR-147b, future studies will also need to determine the mechanisms governing fate decisions of bifunctional transcripts.

MOTS-c, an inflammatory helper T cell inhibitor and regulatory T cell enhancer

T cell-mediated adaptive immune responses are intricately regulated and provide specific protection against infection and malignancy.[164] In autoimmune disorders, T cell responses are often altered leading to a loss of self-tolerance and tissue damage.[164] Type 1 diabetes (T1D) is an autoimmune disease that results in part from autoreactive T cell-mediated destruction of pancreatic β cells.[165] MOTS-c is a conserved 16-amino-acid microprotein encoded by the mitochondrial 12S rRNA that is downregulated in T cells following TCR stimulation and promotes T cell self-tolerance (Figure 4C).[83,111] In the non-obese diabetic T1D mouse model, systemic delivery of MOTS-c prevents autoimmune diabetes by inhibiting T cell infiltration of pancreatic islets and preserving β cells.[111] MOTS-c administration directly increases regulatory T cell (Treg) and decreases inflammatory Th1 cell accumulation in islets.[111] Mechanistically, MOTS-c interacts with the mammalian target of rapamycin (mTOR) signaling pathway molecule Raptor to negatively regulate mTOR signaling. This is consistent with the mTOR pathway's established role in promoting Th1 and inhibiting Treg differentiation.[166] Tregs favor oxidative phosphorylation (OXPHOS) over glycolysis, and mTOR inhibition can promote OXPHOS.[167] In line with this, MOTS-c blocks glycolysis and promotes OXPHOS in T cells.[111] Importantly, serum levels of MOTS-c are lower in T1D patients versus healthy controls.[111] Although further studies are needed to confirm the protective effect of MOTS-c in humans, these data collectively suggest that MOTS-c may be a useful inhibitor of T cell-mediated autoimmune disorders.

miPEP31, a promoter of Treg differentiation

The conserved 31-amino-acid microprotein miPEP31, encoded by the *Mir31/MIR31HG* gene, is enriched in Tregs relative to conventional $CD4^+$ T cells, and its exogenous administration enhances Treg differentiation (Figure 4D).[43] miPEP31 is passively taken up by T cells, and intravenous administration of recombinant miPEP31 diminishes EAE by enhancing Treg differentiation.[43] Like P155, the transcript that encodes miPEP31 also encodes a micro-RNA, miR-31, that has the opposite phenotype of its microprotein.[136] Specifically, miR-31 inhibits Treg differentiation by targeting the forkhead box protein 3 (*FOXP3*)*164* and retinoic acid-inducible protein 3 (*Gprc5a*) transcripts, and miR-31 deletion in T cells ameliorates EAE.[136] Exogenous miPEP31 may promote Treg differentiation through its ability to decrease miR-31 expression by binding the *Mir31* promoter and enhancing the removal of transcriptionally permissive epigenetic marks.[43] Consistent with this, exogenous miPEP31 is unable to enhance Treg differentiation or decrease EAE severity in mice with a T cell-specific deletion of *Mir31.36* Altogether, these data suggest a model where the *pri-miR-31* transcript generates the anti-Treg miR-31 or the pro-Treg miPEP31. This model also suggests that exogenous miPEP31 inhibits *pri-miR-31* transcription, which, by decreasing miR-31, may contribute to its ability to enhance Treg differentiation. How the balance of endogenous miPEP31 versus miR-31 expression is regulated will need further study, but one possibility may be related to transforming growth factor β (TGF-β), a cytokine critical for Treg polarization. *In vitro* Treg polarization with TGF-β diminishes miR-31 expression,[136] whereas miPEP31 expression remains elevated in Tregs, suggesting that TGF-β signaling may regulate this balance.[43] Generating T cells with a miPEP31 start codon mutation would help clarify endogenous miPEP31-dependent regulation of its own bifunctional transcript's expression as well as clarifying the role of endogenous miPEP31 on Treg differentiation.

Dleu2-17aa, an inducer of Tregs

Dleu2-17aa is a conserved 17-amino-acid microprotein encoded by the *Dleu2* lncRNA.[44] The Dleu2-17aa microprotein is a cell-intrinsic promoter of Treg differentiation (Figure 4E).[44] It is also readily taken up by T cells, and exogenous administration of recombinant Dleu2-17aa promotes Treg differentiation and relieves EAE.[44] By contrast, mice with a mutation in the start codon of *Dleu2-17aa* are more susceptible to EAE due to decreased induction of Tregs.[44] Dleu2-17aa promotes Treg differentiation by binding SMA- And MAD-related protein 3 (Smad3), a transcription factor downstream of the TGF-β receptor, and enhancing its recruitment to the Treg-defining transcription factor *Foxp3*.[44] The *Dleu2* transcript also encodes miR15a/16-1, which restricts B cell and conventional T cell proliferation and is deleted in several malignancies.[133–135] Similar to the bifunctional transcripts described above, the mechanisms determining the coding versus noncoding fate of *Dleu2* are not known.

Microproteins and cancer immuno-surveillance

In addition to their regulatory role in immune cells, some noncanonical microproteins may be also potent tumor-specific targets for T cell-mediated tumor immuno-surveillance. As an example, MHC class I presentation of the HF10 peptide, generated from a novel ORF in the lncRNA plasmacytoma variant translocation 1 (*PVT1*),[168] is enriched in human colorectal cancer.[168] HF10 is recognized by peripheral blood lymphocytes as well as tumor infiltrating

lymphocytes and triggers the cytolytic activity of patient-derived HF10-specific $CD8^+$ peripheral blood T cells against colon cancer cell lines.[168] Thus, because HF10 is commonly expressed in colon cancer and generates an endogenous immune response, it is an example of how microproteins may be important for T cell-mediated tumor immune surveillance. However, further work will be required to determine how many tumor-associated microproteins promote tumor immuno-surveillance.

Noncanonical microproteins may also be useful for designing anti-tumor immunotherapies. For example, high-throughput MHC class I immunopeptidomics identified several colon cancer-specific murine and human peptides encoded by differentially expressed lncRNAs.[58] In a murine model, a viral vector-based vaccine containing a consortium of these peptides induced antigen-specific $CD8^+$ T cell responses and, when administered prophylactically, delayed colon cancer growth.[58] Furthermore, a DC vaccine pulsed with this peptide consortium effectively delayed established colon cancer growth.[58] Like lncRNA-derived microproteins, peptides from uORF-derived microproteins also stimulate efficacious anti-tumor T cell responses. For instance, the ring finger 10 (*RNF10*) uORF microprotein is expressed in murine colon cancer cells and human pancreatic cancer.[169] Peptides derived from it induced antigen-specific T cell responses in both mouse and human T cells, and mouse and human peptide vaccines delayed *in vivo* growth of murine colon cancer and pancreatic cancer patient-derived xenografts, respectively.[169] Collectively, these studies suggest that tumor-associated microproteins may be useful targets for anti-tumor immunotherapies.

POTENTIAL CLINICAL APPLICATIONS OF IMMUNOMODULATORY MICROPROTEINS

The diverse and critical functions of immunomodulatory microproteins may present opportunities to translate microprotein immunobiology to the clinic. Their increased tissue- and context-specific expression relative to canonical protein-coding genes may be particularly attractive for clinical translation.[15,31,36] This specificity might lead to more precise diagnostics or therapeutics with less off-target side effects. Their small size and other biochemical properties may enable passive cellular uptake implying that some microproteins could be directly employed as therapeutic modulators of immune responses. Microproteins are also aberrantly expressed by tumor cells and can be targeted by cytotoxic T cells, indicating that they may be clinically translated as tumor vaccines or targets of engineered anti-tumor therapeutics. Next, we discuss the potential clinical applications of microprotein modulators of immunity.

Microproteins as biomarkers

Microproteins may be useful biomarkers for immune-related disorders, particularly secreted microproteins that are easily sampled. Examples include MOCCI for inflammatory conditions[73] and Humanin for conditions associated with age-related inflammation (Figure 5A).[170–172] Clinically, it is difficult to conflate general markers of inflammation with specific diseases, but as the noncanonical microprotein field advances, their superior tissue- and context-specific expression may lead to the development of more precise diagnostics for inflammatory disorders.

Potential prognostic microprotein biomarkers have also been identified for a number of malignancies.[66,173–176] Those predictive of overall survival in patients include *HOXB-AS3* (Homo sapiens HOXB cluster antisense RNA 3)[176] and ASAP (*LINC00467*-encoded ATP synthase-associated peptide)[66] for colon cancer; APPLE (a peptide located in ER) for acute myeloid leukemia[174]; RASON (RAS-ON, encoded by *LINC00673*)[177] for pancreatic cancer; and the microprotein encoded by the TINCR (terminal differentiation-induced non-coding RNA) lncRNA for cutaneous squamous cell carcinoma.[178] Since the above microprotein tumor biomarkers were sampled from tumor biopsies, further research is needed to identify microprotein tumor biomarkers sampled from more accessible sites such as peripheral blood. In support of this approach, microproteins uniquely secreted in peripheral blood extracellular vesicles of patients with glioblastoma have recently been identified.[173] Furthermore, future studies will also need to determine whether microproteins may be useful for predicting endogenous or immunotherapy-driven anti-tumor responses.

Microproteins as therapeutic targets

Targeting microprotein expression may also be useful for modulating dysfunctional immune responses. For instance, a pro-inflammatory microprotein might be depleted to treat inflammatory disorders or overexpressed to promote anti-tumor immuno-surveillance. Lentivirus or adeno-associated viral vectors may be used to modulate microprotein expression (Figure 5B).[179] To reduce the risk of side effects caused by unintended viral transduction of off-target tissues, tissue-specific promoters and approaches that alter viral vector tissue tropism could be employed.[179] Microprotein expression may also be modulated using antisense oligonucleotides and nanoparticle delivery systems; although, achieving tissue-specific delivery may be more challenging with these approaches.[180,181] The feasibility of altering microprotein expression *in vivo* has been demonstrated in multiple murine models of malignancy.[66,175,177] Future work will need to explore if such approaches are also effective in murine models of immune-related disorders.

Microprotein function may also be targeted by small molecules (Figure 5B),[104] but this may be difficult due to limited sites for small molecule binding. Another approach to disrupt microprotein function is with cell-penetrating peptides (CPPs). CPPs have an intrinsic ability to transduce cells and can be linked with various therapeutic molecules including rationally designed inhibitory peptides.[182] Shi et al. used an inhibitory peptide linked to a CPP to disrupt the interaction between the transmembrane domain of the microprotein MAVI1 with that of its binding partner MAVS. This prevented MAVI1 inhibition of antiviral innate immune signaling resulting in resistance to viral replication *in vitro*.[127] The MAVI1 inhibitory peptide CPP was not tested *in vivo*, but the efficacy of other CPPs has been demonstrated in models of heart disease, amyotrophic lateral sclerosis, and cancer.[182–185] As a group, CPP therapeutics are still in the early phases of development,[186] but the ability to rationally design peptides to

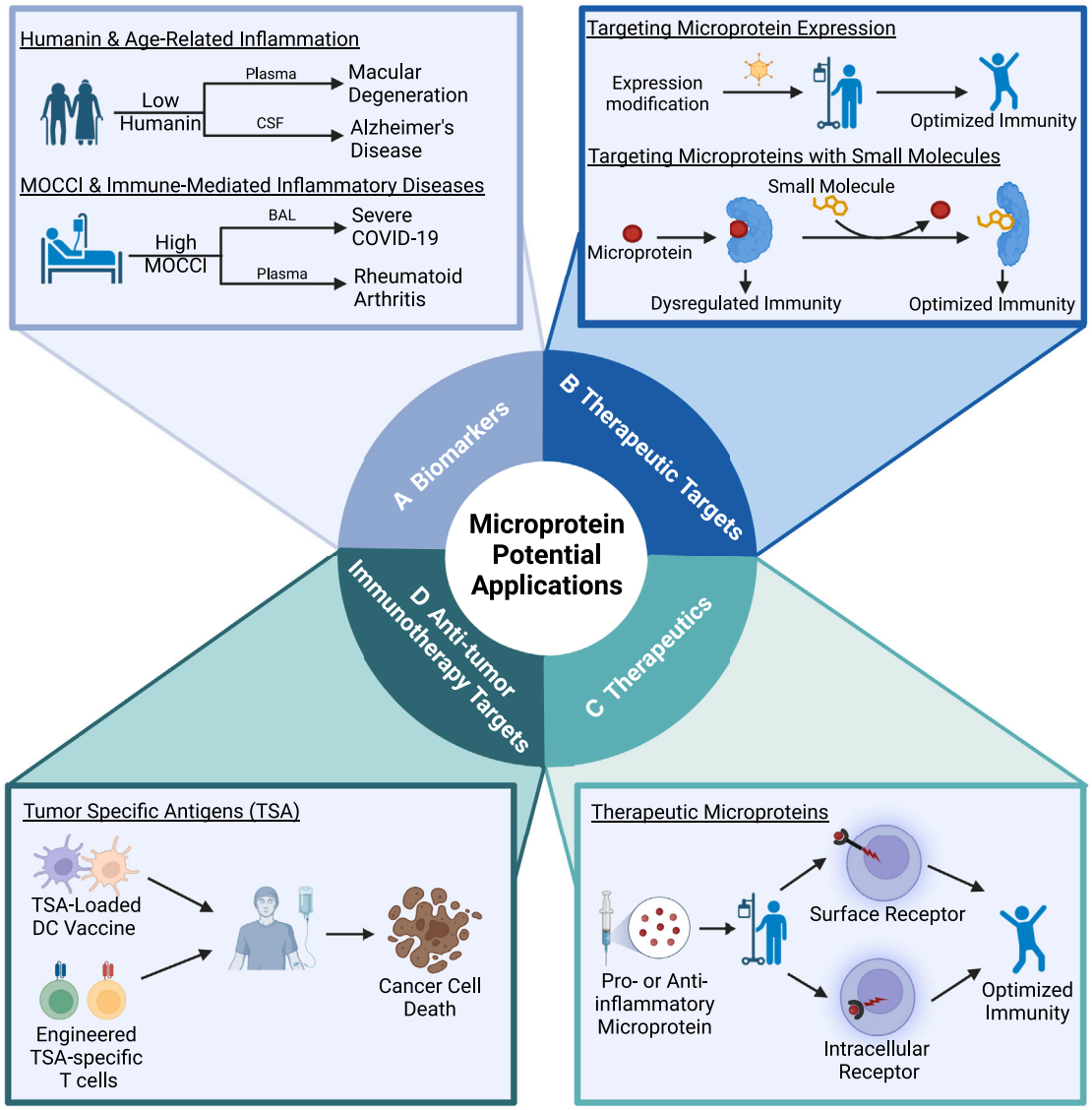

Figure 5. Potential clinical applications of immunomodulatory microproteins

Due to their diverse and critical functions, immunomodulatory microproteins may have several useful clinical applications, which are depicted here. (A) Potential immunomodulatory microprotein biomarkers for immune-related disorders are pictured. Humanin is decreased in the plasma of patients with macular degeneration and the cerebral spinal fluid (CSF) of patients with Alzheimer's disease. MOCCI is increased in bronchiolar lavage (BAL) fluid from patients with severe COVID-19 as well as in peripheral blood monocytes and synovial macrophages in patients with rheumatoid arthritis. (B) Immunomodulatory microproteins as potential therapeutic targets for optimizing immunity are shown. One strategy is to modify their expression using overexpression viral vectors or to deplete them using CRISPR-Cas9, shRNA, or siRNA methods. Another potential approach is to target microprotein function with small molecules. (C) The potential for some immunomodulatory microproteins to be directly applied as therapeutic molecules is shown. Pro- or anti-inflammatory therapeutic microproteins that target surface or intracellular targets are illustrated. (D) Some microproteins generate TSAs. Two potential applications of microprotein TSAs, anti-tumor microprotein-specific engineered cellular therapies, and microprotein DC vaccines, are depicted.

specifically disrupt a wide array of interactions could prove useful for many disorders.

Microproteins as immunomodulatory therapeutics

Microproteins themselves may also directly be used as immunomodulators. For instance, a pro-inflammatory microprotein might be administered to augment anti-tumor immunity or an anti-inflammatory microprotein might be used to treat inflammatory disorders (Figure 5C). The direct administration of an immunomodulatory microprotein as a drug is most straightforward for secreted microproteins that act on cell surface receptors.[96] As an example, exogenous Humanin rescues age-related inflammatory marker

elevation, protects retinal pigment epithelia cells from mitochondrial inflammation, and attenuates various aspects of Alzheimer's disease.[171,187,188]

In contrast to modulating cell surface targets, modulating intracellular targets with immunomodulatory microproteins requires their ability to cross membranes. The biochemical features of many microproteins (i.e., small size, positive charge bias, and amphipathic α helix) are similar to that of CPPs.[8,9,143,158] Hence, microproteins with these features may be well suited to modulate intracellular targets when delivered as exogenous therapeutics as demonstrated in multiple pre-clinical models of non-immune-related disorders.[14,15,83,182,188–190] Several immunomodulatory microproteins also have biochemical characteristics similar to CPPs, are readily taken up by cells, and are subsequently able to interact with their intracellular targets.[43–45,111] Importantly, systemic delivery of these immunomodulatory microproteins ameliorates murine models of autoimmunity.[43–45,111] Still, there will likely be challenges to developing some microproteins as therapeutics. Principal among these challenges are cell-type-specific uptake[191,192] and stability,[191,192] which often requires chemical modification to overcome.[191,192] However, approaches to address these issues are steadily improving.[189,191,192] Altogether, the studies here suggest some microproteins may be useful immunomodulatory therapeutics.

Microproteins as anti-tumor immunotherapy targets

Tumor vaccines and engineered T cells targeting tumor-specific antigens (TSAs) are promising anti-tumor immunotherapy approaches.[193] TSAs are typically derived from tumor-specific somatic mutations, referred to as neoantigens; however, some tumors have a low somatic mutation burden in their protein-coding genome and are thus "mutationally silent."[194] Noncanonical microproteins are emerging as an alternative source of immunogenic TSAs that may lead to improved immunotherapy against mutationally silent tumors. Relative to canonical proteins, noncanonical microproteins are aberrantly expressed in tumors[36,38,54,195,196] and may be enriched with tumor-specific somatic mutations.[38] Furthermore, they are efficiently presented by MHC molecules,[36] generate tumor-specific cytotoxic T lymphocyte responses *in vitro* from patients with malignancies,[21,48,143] generate anti-tumor cytotoxic $CD8^+$ T cells that are effective against patient-derived xenografts,[169] and are effective tumor vaccine antigens in murine models.[58,168,169,195] In addition, microprotein-derived TSAs shared across tumor types and individual patients have been identified suggesting that immunotherapies targeting these antigens may be useful for a broad group of patients.[38,54,168,196]

One challenge of developing TSAs from noncanonical microproteins is identifying the small fraction of microproteins (approximately 1%) that are immunogenic.[54,59] Previous efforts identified characteristics associated with those microproteins more likely to be antigenic including being shared among tumors,[59] being robustly expressed in tumors,[195] being poorly expressed in normal tissue,[59] and being poorly expressed in the thymus thereby limiting central tolerance.[58,195] These characteristics were identified using low-throughput validation techniques; however, recently developed multiplexed, genome-scale platforms[197,198] might now facilitate the high-throughput identification and validation of antigenic tumor-specific microproteins. Collectively, these data indicate that noncanonical microproteins may be an untapped source of TSAs (Figure 5D).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Thousands of microproteins derived from sORFs in both coding and noncoding transcripts have now been identified.[1] While most have not been functionally characterized, those that have perform critical functions at both the cellular and organismal levels.[81,84,85,92,94–98,110,199–201] Recently, several microproteins important for immune cell function were identified (Table 2), but these are likely just the tip of the iceberg because thousands of others remain uncharacterized.[10,104,202] We hope this review generates interest among immunologists and molecular biologists who can help characterize these putative immunomodulatory microproteins. As the field advances and the role of microproteins in protective and pathologic immune responses becomes clearer, their cell- and context-specific expression may offer opportunities for developing more specific diagnostics and therapeutics for immune-related disorders.

To realize these opportunities, several unknown aspects of immunomodulatory microprotein biology need to be addressed. First, to avoid any unintended side effects, more study is needed to fully characterize the functions and underlying mechanisms of known immune-related microproteins. Second, the role of microproteins in immune cells is still largely unknown and will need to be systematically determined. Third, the influence of microproteins in protective and pathogenic immune responses will also need to be carefully examined to understand the full spectrum of context-dependent effects. Fourth, how immunity is regulated by microproteins derived from sORFs in protein-coding transcripts needs to be determined. Thus far, validated immunomodulatory microproteins are all derived from sORFs in noncoding transcripts (Table 2), but the majority of sORFs are present in protein-coding transcripts.[1] Fifth, the mechanisms governing coding versus noncoding fate decisions of bifunctional transcripts will need to be better understood. While it is likely these fate decisions are in part controlled by mechanisms that govern RNA localization[203] as well as secondary structures in 5′ and 3′ UTRs that regulate translation,[127,204,205] an improved understanding and ability to predict how RNA fates change in varying contexts will likely help avoid unintended consequences of targeting bifunctional transcripts. Finally, to facilitate rapid characterization, improved methods to predict microprotein function from amino acid sequence will be vital. Through these efforts, we anticipate the tissue- and context-specific expression of noncanonical microproteins will help advance understanding of complex immune processes and improve outcomes for immune-related disorders.

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AUTHOR CONTRIBUTIONS

C.N., V.A.D.-T., and D.C.P. performed the literature review and wrote and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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