Molecular Immunology 113 (2019) 38–42

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

Immunoribosomes: Where's there's fire, there's fire

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ARTICLE INFO

Keywords:
DRiPs
Immunoribosomes
MHC-I
Antigen presentation
Immunosurveillance

ABSTRACT

The MHC class I antigen presentation pathway enables T cell immunosurveillance of cancer cells, viruses and other intracellular pathogens. Rapidly degraded newly synthesized proteins (DRiPs) are a major source of self-, and particularly, viral antigenic peptides. A number of findings support the idea that a substantial fraction of antigenic peptides are synthesized by "immunoribosomes", a subset of translating ribosomes that generate class I peptides with enhanced efficiency. Here, we review the evidence for the immunoribosome hypothesis.

The greatest and noblest pleasure which men can have in this world is to discover new truths; and the next is to shake off old prejudices.

Frederick II of Prussia

At the heart of science is an essential balance between two seemingly contradictory attitudes—an openness to new ideas, no matter how bizarre or counterintuitive, and the most ruthlessly skeptical scrutiny of all ideas, old and new. This is how deep truths are winnowed from deep nonsense.

Carl Sagan of Brooklyn

MHC class I molecules play a central role in T and NK cell immunosurveillance, and other biological processes, including mate selection and neuronal development (Yamazaki et al., 1976; Huh et al., 2000). MHC class I antigenic peptides typically arise from proteasomal products that are transported by TAP (transporter associated with antigen processing) into the lumen of endoplasmic reticulum (ER), trimmed at their NH_2 termini, loaded onto class I molecules, and transported to the cell surface for T cell immunosurveillance.

Peptides from host or viral gene products have two potential sources: "retirees" (Yewdell, 2001) and DRiPs (defective ribosomal products) (Yewdell et al., 1996). Retirees are proteins that reach stable structures and degrade with normal turnover kinetics, *i.e.* a median half-life of 46 h across the entire proteome (Schwanhausser et al., 2011). DRiPs are a substantial subset of nascent gene products that for myriad reasons degrade more rapidly than their corresponding native retiree pools.

DRiPs were originally proposed to explain the rapidity of MHC class I peptide ligand generation from otherwise highly metabolically stable viral proteins (Yewdell et al., 1996). The swift presentation of antigenic peptides enables CD8+ T cells to recognize and kill virus-infected cells before progeny virions are born (Esquivel et al., 1992). In the original incarnation, DRiPs were proposed to represent misfold or prematurely terminated proteins, resulting from inevitable errors and those deliberated enhanced by innate cellular responses to infection (Yewdell et al., 1996). Over the years, numerous studies broadened the concept of DRiPs to include all possible scenarios of defective conversion of genetic information into native translation products (Yewdell, 2003; Yewdell and Nicchitta, 2006; Anton and Yewdell, 2014; Starck and Shastri, 2016), excess subunits of multiprotein complexes (Bourdetsky et al., 2014), errors in tRNA amino acid charging (Netzer et al., 2009); and non-canonical translation or mistranslation products (Schwab et al., 2003). DRiPs appear to play a particularly important role for generating viral peptides, including both RNA and DNA viruses, where peptide class I complexes can be detected simultaneously with, or even prior to, detection of viral proteins (Croft et al., 2015; Yang et al., 2016; Dolan et al., 2010a; Wei et al., 2017).

1. CD8+ T cells do not read molecular biology textbooks

In the late 80's, Boon and colleagues in defining the first tumor-specific peptides found that peptides can be generated from transfected plasmids in which the peptide is encoded by just an exon and upstream intron from the source gene, and is insensitive to inserting upstream stop codon or frameshifting mutations. The spawned the "petpon" hypothesis (Boon and Van Pel, 1989):

> *We suggest a new mechanism for the production of endogenous antigenic peptides that are synthesized in the target cell and are presented to CTL on MHC class I molecules. The minimal formulation of our hypothesis is that these peptides are not degradation products of cellular proteins but are direct products of the translation of very short genetic regions. For the production of antigenic peptides encoded by the cellular genome, we propose that short genetic regions located around the sequence coding for the peptide can be transcribed autonomously. These regions, hereafter*

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https://doi.org/10.1016/j.molimm.2017.12.026
Received 7 September 2017; Accepted 31 December 2017
Available online 17 January 2018
0161-5890/ © 2018 Published by Elsevier Ltd.

called peptons, would have a length of a few hundred bp, thereby exceeding only by a small factor that of the sequence coding for the peptide. Peptons would not be transcribed by polymerase II but by a polymerase that does not require the promoter sequences initiating eukaryotic genes. The resulting "pepton-RNA" (p-RNA) would then be translated to produce a peptide that would either bind directly to the class I MHC molecule or do so after precise cleavage of some segments as observed with peptidic hormones. As a consequence, antigenic peptides could be produced in the absence of classical transcription.

Remarkably insightfully, the authors later added:

If antigenic peptides were produced by degradation of cellular proteins, then the presenting molecules would be flooded with peptides derived from a few heavily synthesized proteins to the detriment of those derived from proteins synthesized in small amounts. This is hardly the way to exert efficient immune surveillance. With peptons, there would be several ways to reduce this bias. Contrary to mRNA synthesis, p-RNA synthesis could be quite uniform throughout the genome, thereby ensuring adequate representation for every gene whether or not it is transcribed. Peptons could also be absent from very highly expressed genes, or present but poorly expressed.

This gets to the heart of the immunosurveillance problem for tumor antigens and for viral antigens in circumstances where viral gene expression is limited relative to cellular genes. Proteomics studies show that a tiny fraction of proteins make up the bulk of the proteome: for CD8+ T cells themselves, 12 proteins constitute 25% of the proteome and 237 proteins make up the next 50% (Hukelmann et al., 2016). The "immunopeptidome" (Istrail et al., 2004), defined by mass spectrometry of class I derived peptides shows much less bias to proteome abundance (Granados et al., 2015; Milner et al., 2006), consistent with a major contribution from processes other than simply bulk synthesis and turnover, with a major contribution from proteins that are unable to assemble into multi-protein structures (Bourdetsky et al., 2014), as well as peptides derived from non-canonical translation of defined mRNAs (Laumont et al., 2016). These peptidome wide studies generalized findings from the pioneering studies that established the principle of peptide generation from unexpected sources: non-AUG initiation codons (Shastri et al., 1995), frame-shifting (Zook et al., 2006) (recently shown to create autoimmune target peptides in human type I diabetes (Kracht et al., 2017; Wei and Yewdell, 2017)), and intronic sequences (Robbins et al., 1997) (see Table 1 in Starck and Shastri (2011) for a comprehensive summary of peptides encoded by non-standard ORFs).

The translation products generated by such processes are unlikely to either fold into stable structures or to find appropriate binding partners, and therefore would have a high likelihood of rapid degradation and access to class I molecules. A critical issue, however, is their abundance, which has been dauntingly difficult to address experimentally with traditional methodology. Fortunately, it is now possible to directly assess the translatome through the method of ribosome profiling (Ribo-Seq) (Ingolia et al., 2011; McGlincy and Ingolia, 2017), which entails the deep sequencing of RNA protected from RNase digestion by the ribosome. Ribo-Seq provides both translation initiation sites, the translation rates of individual mRNAs (the product of the elongation rate and ribosome occupancy), and the reading frames being translated (inferred by the nucleotide periodicity of the reads) (Calviello et al., 2016). Correlating this information with the immunopeptidome should provide definitive information into the contribution of non-canonical translation to immunosurveillance; with the caveat that peptides detected by mass spectrometry must be queried against the translatome revealed by Ribo-Seq, and not the standard exome. The difficulty of correctly identifying peptides is further heightened by a recent study suggesting that as much of a third of the peptide is generated by proteasome mediated peptide splicing (Liepe et al., 2016), make a difficult computational issue even more challenging (exponentially more so if peptides can be spliced from different proteins that are simultaneously degraded in a single proteasome).

2. Why immunoribosomes?

In the broadest terms, there are two possibilities for ribosomal synthesis of class I peptide ligands: either all ribosomes behave similarly or ribosome subsets exist that synthesize translation products with increased access to the class I processing pathway either due to the nature of translation product (*e.g.* truncated polypeptide) or the channeling of the translation product to the proteasomes adept at antigenic peptide generation. Given the typical complexity of biological processes including ribosomes themselves (more than 80 protein subunits), and the sheer number of ribosomes per cell (millions), it seems unlikely that ribosomes are homogeneous peptide generators. This line of reasoning largely prompted the original hypothesis (Yewdell, 2002) that a subset of ribosomes were especially adept at synthesizing antigenically relevant DRiPs (later dubbed "immunoribosomes" to match immunoproteasomes (Yewdell, 2005)). A contributing factor was the finding that targeting proteins for rapid and complete degradation has only a modest effect (3-fold or less) at increasing generation of a number of different class I peptide complexes (Anton et al., 1997; Princiotta et al., 2003; Golovina et al., 2005; Goth et al., 1996), consistent with the interpretation (and others as well) that "dark synthesis" of undetected translation products formed a highly efficient source of antigenic peptides (Goth et al., 1996).

Findings from two subsequent studies are consistent with the existence of a functionally compartmentalized ribosome subset. First, intracellular peptide competition studies showed that while preprocessed peptides (synthesized from cytosolic or ER-targeted minigene or liberated co-translationally from ubiquitin fusion proteins) competed for class I presentation as expected from the law of mass action, they were unable to inhibit presentation by peptides liberated from DRiPs, despite being presented at much higher levels (Lev et al., 2010). This would be expected if the translation products of a DRiP-generating ribosome subset had preferred access to TAP (alternative explanation: NH_2-terminal peptide flanking sequences enhance peptide access to class I sufficiently to limit competition from preprocessed peptides).

Second, visualization of class I peptide complexes with peptide specific probes revealed spatial segregation of the same class I molecule bearing different peptides throughout the secretory pathway and on the cell surface, where each complex exists as cluster of ~50 molecules (Lu et al., 2012). As the complexes differ only by a few residues in their bound peptides, it is difficult to imagine how they could achieve spatial segregation unless they were loaded in distinct locations in the ER. Thus, the idea would be that clusters are generated from peptides synthesized from individual mRNAs degraded *in situ* by associated proteasomes and channeled to TAP and its associated class I molecules.

This evidence for compartmentalized translation is intriguing, but indirect and certainly compatible with other interpretations. There are however two sets of studies that provide direct evidence for enhanced presentation of antigens synthesized through specialized translation, in one case through altered translation initiation, in the other through altered localization of ribosomes.

2.1. Non-canonical initiation via modified translation machinery

In a remarkable series of elegant studies carried out over several decades (Starck and Shastri, 2016; Starck and Shastri, 2011; Shastri et al., 2005; Shastri et al., 1998; Shastri, 1996), the Shastri lab blazed the path for the contribution of alternative translation to class I peptide generation, demonstrating the following salient features:

1. CUG and other non-AUG codons can be used to initiate translation of antigenic peptides (Shastri et al., 1995)
2. CUG can be decoded as Leu, and not Met as previously assumed by

the translation field, and the process is independent of the canonical translation initiation pathway (Malarkannan et al., 1999)
3. Translation from "untranslated" 5′ mRNA sequences occurs constitutively in mice and shapes the TCR repertoire (Schwab et al., 2003)
4. CUG initiated peptide translation occurs independently of eIF2 (Schwab et al., 2004), which is required for standard cap-dependent translation.
5. CUG initiation is unaffected by drugs that block standard AUG initiation, and contributes to a substantial fraction of class I peptides (Cardinaud et al., 2010)
6. CUG initiation of antigenic peptides uses eIF2A in place of eIF2 (note that the two are not closely related by sequence homology), and use *bona fide* "elongator" Leu-tRNA with a Watson-Crick paired anti-codon (Starck et al., 2012).
7. The eIF2-independent pathway is used by non-AUG initiated translation of upstream open reading frames, and greatly enhanced by the integrated stress response that inhibits canonical AUG initiated translation (Starck et al., 2016).
8. The CUG initiation pathway is activated by pro-inflammatory stimuli, including influenza A virus (IAV) infection (Prasad et al., 2016).

Taken together, this highly original body of work demonstrates that antigenic peptides are generated from non-canonical reading frames by an altered translation apparatus utilizing eIF2A to alter ribosome initiation sites. No doubt this translation mechanism is exploited for other biological processes. Indeed, a recent report (Sendoel et al., 2017) concludes that in tumorigenesis eIF2A redirects ribosomes towards unconventional upstream initiation sites to enhance oncogenic mRNA translation. The use of non-canonical translation in peptide generation has been demonstrated in three virus systems.

In the course of characterizing alphavirus (Semliki Forest virus) vaccine vectors, Berglund et al. (2007) serendipitously found that a substantial amount of IAV nucleoprotein (NP) encoded by the vector trafficked not only to the nucleus, as expected, but also to mitochondria. Instructively, detection of mitochondrial NP required the use of antibodies (Abs) capable of recognizing denatured NP. Indeed, in the original description of the vector (Zhou et al., 1995), only a native specific mAb was used, and existence of this mitochondrial form was missed. Biochemical analysis revealed a ladder of NP translation products lacking the NH_2-terminus, suggesting downstream initiation. Indeed, artificially initiating NP on several downstream AUG codons generated truncated NP that localized to mitochondria. Like many viruses, alphaviruses modify the translational machinery to favor synthesis of viral proteins at the expense of host proteins. Berglund et al. found that one of the known alphavirus induced modifications, and inactivation of eIF2α (one of the three eIF2 subunits) is required for downstream initiation of NP, and critically, for increasing peptide generation by increasing DRiPs.

Yang et al. (2016) studied how peptides are generated from the bicistronic IAV M1-M2 gene when the SIINFEKL peptide is appended to the M2 C-terminus. Although blocking M1 mRNA splicing with spliceostatin A, a cellular mRNA splicing inhibitor, completely prevented M2 mRNA and protein synthesis, K^b-SIINFEKL complexes were still robustly expressed at the cell surface. K^b-SIINFEKL complexes were also expressed by cells infected *in vitro* or *in vivo* with a recombinant vaccinia virus expressing the M1-SIINFEKL gene, even though as products of a cytoplasmic virus (IAV mRNA synthesis occurs in the nucleus), vaccinia mRNAs are not spliced. This implied that SIINFEKL was translated by non-canonical initiation in the M2 reading frame, and indeed, synonymous changes in CUG codons upstream of SIINFEKL greatly reduced K^b-SIINFEKL expression in spliceostatin A-treated IAV-infected cells, and reduced K^b-SIINFEKL expression in untreated cells. Further, treating cells with drugs that block Met initiation, spared K^b-SIINFEKL expression from unspliced M1 mRNA. Taken together, these findings extend CUG translation initiation for immunosurveillance from cellular gene products to IAV- and vaccinia virus-gene products, supporting its relevance in multiple circumstances.

2.2. Nuclear translation

At the dawn of molecular biology, initial light microscope autoradiography studies with radiolabeled amino acids pointed to the nucleoplasm and nucleolus as significant sites of protein synthesis (Allfrey et al., 1955; Allfrey, 1954). When it was discovered that ribosomal proteins are synthesized in the cytoplasm and rapidly imported to the nucleolus (Wu and Warner, 1971), the site of ribosome assembly, the concept of nuclear translation lost credence, despite considerable evidence (reviewed in (Kuehl, 1974)). Although Iborra et al. resurrected nuclear translation (Iborra et al., 2004; Iborra et al., 2001), it was quickly reinterred (Dahlberg et al., 2003; Nathanson et al., 2003). Despite this, initial evidence for nuclear translation in immunosurveillance came from blocking the nuclear export of IAV mRNA: this had a much greater effect on translation of the IAV source protein than expression of an encoded antigenic peptide (Dolan et al., 2010b). Shortly thereafter, the concept of nuclear translation gained support from studies visualizing translation sites with improved methodology (David et al., 2012; Al-Jubran et al., 2013; Brogna et al., 2002), (reviewed in (McLeod et al., 2014)).

Firm support for nuclear translation in immunosurveillance came from Apcher et al. (2016, 2013), who found that peptide presentation from both intron and exon mRNA was not affected by blocking their export from the nucleus, which was sufficiently robust to prevent translation of the source protein.

Nuclear translation may also be involved in two studies that demonstrated that antigenic peptides are generated during nonsense mediated decay (NMD), a quality control process that degrades nascent spliced mRNAs with inappropriate stop codons (Apcher et al., 2011; Schmidt, 2009), whose location in nucleus *vs.* cytoplasm is controversial (Varsally and Brogna, 2012). NMD based immunosurveillance requires eIF4G but not eIF4E, the standard cap binding protein for translation initiation (Apcher et al., 2011). It is obvious interest to determine the use of eIF2 *vs.* eIF2A in future studies to relate the NMD and non-canonical start codon findings.

Though the nature of the ribosomes and associated translation factors engaged in nuclear translation remain to be established, even in the unlikely event that this is identical to standard cytoplasmic translation, it clearly represents specialized compartmentalized translation that would semm to have favored access to the class I processing pathway.

3. Modified ribosomes

Ribosomes have been traditionally viewed as monolithic structures, based in large part, on their high conservation, with mechanistic studies on translational control focused on factors associated with the ribosome, (*e.g.* initiation factors, aminoacyl synthetases (David et al., 2011)), rather than ribosome itself. Given the wiliness of evolution (Gesteland and Atkins, 1996), it would be surprising if organisms did not exploit ribosome heterogeneity to increase fitness. Early reports of tissue and developmental differences in ribosomal protein (RP) abundance led to the ribosome filter hypotheses (Mauro and Edelman, 2002) that mRNA binding to scanning ribosome 40S subunits is influenced by RP composition (David et al., 2011).

Indeed, as proteomic technology steadily advances, gross stoichiometric differences in the 80 (or so) RPs are becoming apparent. By standard mass spectrometry, RP copy number can differ from five- to fifty-fold (Wisniewski et al., 2014; Hein et al., 2015). Gross differences in RP composition of ribosomes based on the number of ribosomes present per mRNA has also been reported (Slavov et al., 2015). The considerable heterogeneity in RP composition was confirmed by Shi

et al. (2017) who in meticulously quantitating RPs, identified a number of RPs that are substoichiometric in polysomes. Critically, ribosomes containing specific substoichometric RPs are enriched on certain mRNAs and depleted on others, and are needed to efficiently translate enriched mRNAs, where they positively modulate ribosome binding to internal entry sites.

This work extents prior studies that established that a number of RPs are non-essential in yeast and mammalian cells (reviewed in (Shi and Barna, 2015)). The relevance of these findings to humans is definitively shown by Diamond-Blackfan and other inherited diseases resulting from haploinsufficiency in a number of RPs (Ferretti et al., 2017; Narla and Ebert, 2010). Lee et al. (2013) demonstrated special relevance of ribosome heterogeneity to viral infections by knocking down each RP in cultured cells. This revealed that a number of RPs were not required for either cellular division or viral infection, and that one RP is essential for translation of vesicular stomatitis virus mRNA as well as a subset of cellular mRNAs.

Adding another level of complexity that is no doubt exploited by evolution for translational control, many, if not all, RPs can be post-translationally modified by phosphorylation, ubiquitylation, UFMylation (UFM is ubiquitin like protein) (Simsek et al., 2017; Simsek and Barna, 2017) and likely other molecules, and rRNAs can be methylated at many residues (Marcel et al., 2015). Sorting out how these myriad alterations affect ribosome functions will be a challenging and entertaining exercise.

To examine how ribosome structural heterogeneity might affect peptide generation we used lentivirus vectors to knockdown each of the 80 RPs and examined cells for steady state cell surface class I expression, expression of IAV and vesicular stomatitis virus gene products, and efficiency of generating K^b-SIINFEKL complexes from viral source proteins. As expected (Lee et al., 2013), knockdown of 67 subunits reduced cellular viability, implying they play an essential function in translation of cell critical proteins. Of the remaining subunits, 7 reduced translation of viral proteins, which is interesting, of course, but interferes with studying their effects on peptide generation. We therefore focused on the remaining RPs. Our preliminary findings indicate that some RPs can selectively alter peptide supply on a class I allomorph specific manner, while others selectively modulate K^b-SIINFEKL generation from IAV gene products in a gene product specific manner. Microarray shows that knockdown of peptide modulating RPs has only minor effects on the transcriptome (no effect on known class I processing component mRNAs), consistent with the effects being mediated by specific alterations in the translation apparatus and not global alterations in cellular signaling or stress pathways.

The physiological significance of RP-mediated changes in peptide generation remain to be established. At the very least, however, the alterations will provide insight into peptide generation from DRiPs. Indeed, one of the most glaring holes in our understanding of peptide generation is the extent to which peptides generated by different processes are presented with different efficiencies. Even when peptides are produced from highly similar proteins degraded in the same manner (Ub-dependent proteasome cleavage), their ability to access class I molecules can diverge considerably (Princiotta et al., 2003; Dolan et al., 2012). It seems likely that peptides produced from non-canonical start sites have special access to the class I pathway, but this has not been measured due to the short half-life of non-class I bound peptides, the paucity of peptide specific Abs, and difficulties in accounting for low molecular weight material in biochemical assays. Ribo-Seq offers the first real opportunity for quantitating translation efficiencies of small polypeptides and should provide insight into the critical issue of the efficiency of antigen peptide generation and more generally the efficiency of protein synthesis.

4. Future prospects

It is now well established that the MHC class I processing system exploits non-canonical protein translation to enhance immunosurveillance. The Shastri lab has elegantly and painstakingly documented the important contribution of non-canonical translation initiation to peptide generation, which could account for a substantial fraction of clinically relevant peptide generation in viral infections, cancer, and autoimmunity. It is also likely to be involved in peptide generation from nuclear translation, which at this point, is less well characterized. The possible contribution of ribosome subsets to class I peptide generation, based either on absence of various RPs or post-translational/transcriptional modifications is an exciting area of future research. For all of these avenues of research, Ribo-Seq is poised to make a substantial contribution to DRiP peptide generation, and could have a major impact on finding appropriate target peptides for T cell immunotherapy of cancer.

Acknowledgement

The authors are supported by the Division of Intramurarl Research, NIAID, Bethesda, MD

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