New lane in the information highway: alternative reading frame peptides elicit T cells with potent antiretrovirus activity

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CD8$^+$ T cells rapidly recognize virus-infected cells due to the generation of antigenic peptides from defective ribosomal products (DRiPs) that are encoded by standard open reading frames (ORFs). New data now show that alternative reading frame (ARF) DRiPs can also induce robust CD8$^+$ T cell responses. ARF-specific T cells control retroviral replication and select for viral escape in monkeys, providing the most compelling evidence to date for the biological relevance of ARF immunosurveillance.

Nearly all cells in jawed vertebrates constitutively express surface major histocompatibility complex (MHC) class I molecules. Upon infection with viruses and intracellular pathogens, class I molecules present oligopeptides derived from pathogen gene products for recognition by CD8$^+$ T cells. The generation of antigenic peptides typically begins in the cytosol where proteasomes generate antigenic peptide precursors with amino terminal extensions. These precursors are then trimmed by peptidases before and/or after peptide transport into the endoplasmic reticulum (ER) by transporter associated with antigen processing. Peptides that bind with high affinity to class I molecules trigger the release of the peptide–class I complex from the ER. These complexes are displayed at the cell surface for perusal by CD8$^+$ T cells in virus-infected tissues. A key question in class I antigen processing is which source(s) of proteasome substrates gives rise to class I peptide ligands. Although DRiPs arising from polypeptides translated in-frame are well documented as a source of class I–binding peptides, a new study by Maness et al. in this issue (p. 2505; reference 1) shows that DRiPs derived from ARFs can also induce highly effective antiviral T cell immunity.

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Rethinking immunosurveillance

Key insights into biological systems can be gleaned by considering their evolution. The MHC class I antigen processing system is generally believed to have evolved as a result of selective pressures imposed by intracellular pathogens. New findings, however, have resurrected the idea that cancer immunosurveillance, a process that seeks and destroys cancers of nonviral origin, was a key factor in the evolution of the class I–CD8$^+$ T cell system. Remarkably, lethal tumors can be transmitted in two mammalian species by direct transfer of tumor cells themselves and not by tumor viruses, as was generally assumed (2, 3). The need to reject such transmissible tumors may have provided a strong selective pressure in the evolution of immunosurveillance. Indeed, the constitutive expression of class I molecules (as opposed to their induction by infection) may have evolved primarily to facilitate the detection of transmissible and spontaneously arising tumors (4).

Detection of these tumors would be most efficient if active gene expression was monitored independently of mRNA abundance and protein stability. This would prevent the overloading of class I molecules with peptides from the most abundant cellular proteins (structural elements, chaperones, ribosomes, etc.), which are rarely altered in neoplasms. Indeed, analysis of the class I immunopeptidome reveals little correlation between the abundance of mRNAs and class I–binding peptides (5, 6). Rather, these peptides appear to be selected for presentation by some other metric that favors rare mRNA species and peptides from gene products translated by nonstandard rules (7), perhaps by a subset of ribosomes ("immunoribosomes") whose products have privileged access to the class I processing pathway (8, 9).

The possible evolution of this pathway for detecting peptides from tumor cell gene products that are rare or are rapidly degraded may have influenced the mechanisms used to monitor viral gene expression. Studies in a variety of systems point to an intimate kinetic link between protein synthesis and the generation of viral and cellular peptides that bind to class I MHC (8). This is expected for nonstandard gene products (ARFs, downstream or alternative initiation, stop codon read through), which misfold and are rapidly targeted for degradation. Surprisingly, however, this also applies to peptides derived from standard viral and cellular ORFs (10). Rapid degradation may result from unavoidable mistakes in transcription, translation, protein folding, or assembly of multi-subunit proteins. Alternatively, immunoribosomes (should they exist) may directly target their translation products for rapid degradation to enable immunosurveillance.

Together, the various forms of rapidly degraded polypeptides are termed DRiPs. Surveillance of DRiPs may have evolved to facilitate tumor cell recognition. But this surveillance may also enable the immune system to rapidly detect viral infections by monitoring what is actively being translated, rather than

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what has already been translated. Viral proteins are typically highly stable (half-life in days, not minutes). Antigenic peptides are thus unlikely to be derived from the standard turnover of these proteins, as it would take many hours to generate enough peptides from even the most abundant viral proteins to compete with those generated from the billions of cellular proteins present at the time of infection. Additionally, using DRiPs as a source of peptides provides a mechanism for monitoring the expression of ~25% of cellular gene products (and many viral proteins) that are targeted to the ER for secretion or cell surface display, and that are eventually degraded by extracytosolic proteases.

ARF or ORF?

Although most cellular and viral peptide ligands appear to arise from DRiPs encoded by standard reading frames, increasing numbers of ARF-encoded peptides (ARFPs) have been reported (11). An "ARF" is simply defined as an overlapping reading frame that does not encode a functional gene product. But extreme caution must be exercised when applying this label to uncharacterized gene products. Although some ARFs are synthesized as errors in translation (and are thus DRiPs), proteins encoded by overlapping reading frames can be functional products of natural selection. Many viruses, especially those with small genomes, use overlapping reading frames and/or multiple splice sites to create functional viral proteins. The influenza A virus protein, PB1-F2, is the poster child for ARFs that are really ORFs. This 87-residue protein was serendipitously discovered in the first systematic survey for viral ARFPs and was identified as the source of a highly immunogenic peptide in influenza virus–infected B6 mice (12).

PB1-F2 is encoded by the +1 reading frame of the PB1 polymerase genes in >90% of human and avian influenza viruses. PB1-F2 is an abundantly synthesized mitochondrial protein that plays an important role in viral pathogenesis (13). Had PB1-F2 been discovered before PB1, it would have been considered the ORF, and PB1 the (very long) pseudo-ARF. The first viral ARF that was reported to have $CD8^+$ T cell immunogenicity is probably also an ORF with an important function in viral pathogenesis (14). Similarly, immunogenic ARFs from the hepatitis C virus, which elicit $CD4^+$ T cell and antibody responses (15), now have ascribed functions (16), bestowing ORFhood on these gene products.

ARFs created by genetic manipulation of viral (17, 18) or plasmid ORFs (19) have also been shown to demonstrate antigenicity and immunogenicity. Several immunogenic ARFPs have been reported for tumor antigens (11), but it is difficult to establish that their expression (and immunogenicity) is not based on legitimate splicing events that only occur in vivo. The best case for natural ARFPs comes from studies of immunodeficiency viruses. In a seminal study, Cardinaud et al. (20) described six ARFPs encoded by human immunodeficiency virus (HIV) and provided evidence for their immunogenicity in humans. These six peptides were encoded by three ARFs ranging from 33 to 52 residues, which is much shorter than is typically needed to generate a stably folded, functional protein. There is also no evidence that these gene products are functional. Although it is impossible to prove the lack of function of an ARF gene product, documenting that a translated gene product is rapidly degraded is a good start (though perversely, a sizable fraction of PB1-F2 is rapidly degraded; reference 12).

ARFs as sources of biologically important determinants

In this issue, Maness et al. (1) use the Rhesus macaque simian immunodeficiency virus (SIV) model to establish the in vivo relevance of viral ARFPs. Using computer algorithms to predict SIV ARFPs with high affinity for the Mamu B*17 class I molecule, they identified a potential immunogenic peptide. This peptide originates from an ARF of the viral Env protein (or the incompletely spliced product of the viral protein Rev, as these genes occupy overlapping reading frames). A synthetic version of the peptide, they show, binds to class I molecules with high affinity (K_d = 32 nM), and SIV-infected Mamu-$B*17^+$ macaques mount a strong B*17-restricted $CD8^+$ T cell response against this peptide.

The biological relevance of this response was elegantly evinced by two findings. First, $CD8^+$ T cells specific for the peptide inhibited replication of virus in vitro in B*17-expressing cells. Second, and most remarkably, 5 of 20 $B*17^+$ SIV-infected animals selected for viral escape mutants with the identical point mutation, which recodes the C-terminal anchor residue of the peptide from W to K (and is synonymous in Env). This substitution caused a 30-fold reduction in the binding of the mutant peptide to B*17 and prevented peptide-specific $CD8^+$ T cells from recognizing cells infected with the escape mutant. As the first report of an ARF-encoded determinant that facilitates viral evolution in response to immune pressure, these findings provide the smoking gun for the in vivo relevance of ARFPs.

The list of bona fide viral ARFPs is short. This undoubtedly reflects, in part, investigator bias in searching for immunogenic determinants in only the most obvious places. It is now possible to predict in silico high affinity class I–binding peptides with an accuracy as high as 90% (21). The success of Maness et al. in using this approach should spur investigators to systematically search for ARFPs in other viruses. Moreover, as improvements in mass spectroscopy enable more rapid and sensitive identification of peptides recovered from MHC class I molecules, spectroscopists should compare detected masses with all six possible reading frames for potential matches and not forget about nucleic acid or direct proteasome-mediated peptide splicing, which can even reorder peptide sequences (22).

Alternative peptides recognized by virus-induced $CD8^+$ T cells may also be encoded by the host. The initial study that characterized HIV class I peptide ligands using mass spectroscopy identified three peptides that were not present on uninfected cells (23). Remarkably, however, each of these peptides was encoded by the human vinculin gene, whose expression is enhanced by HIV

infection. Even more remarkably, $CD8^+$ T cells obtained from several HIV-infected individuals, but not from uninfected hosts, recognized these self-determinants. Similarly, measles virus infection induces human autoreactive $CD8^+$ T cells that recognize up-regulated self-peptides (24). The latest mass spectrometric sequencing study of HIV-induced class I peptide ligands identified no fewer than 15 host-derived peptides that were absent in uninfected cells (25). Although acute viral infections are frequently suspected of initiating chronic autoimmune diseases (with little supporting evidence to date), these findings raise the important but frequently overlooked question regarding the extent to which self-limited autoreactive $CD8^+$ T cells might contribute to antiviral responses.

ARFP pragmatics

It is obvious (but still needs to be stated) that the induction of self-peptide–specific $CD8^+$ T cell responses, regardless of their physiological function, is too dangerous to be considered in vaccine design. But what about viral ARFPs? In theory, the most effective $CD8^+$ T cell–based vaccines should encompass the widest possible range of viral peptides that are naturally displayed by class I molecules on infected cells. Maness et al. clearly demonstrate that ARF-derived peptides can generate robust, highly protective responses. It is possible to design vaccines that deliberately (over)express all possible reading frames of viral proteins, even targeting them for rapid degradation in vectors designed for direct priming.

Unless further research demonstrates the ubiquitous targeting of viral ARFPs by $CD8^+$ T cells, however, it is probably best not to pursue this approach. To some extent, competition limits the immunogenicity of immunogens in individual lymphoid organs (particularly via immunodomination; reference 26). Therefore, generating $CD8^+$ T cells specific for peptides that are never actually produced during infection would be counterproductive. Moreover, the findings of Maness et al. uncover the Achilles heel of ARFPs: if the source ARFP is a genuine ARF, it is under no selective pressure to maintain its function. An effective $CD8^+$ T cell response, therefore, will result in rapid mutation of the targeted peptides, particularly in RNA viruses, which have extremely high mutation rates. It is important to note that this argument is much less relevant for tumor ARFPs, as tumor-specific determinants are both more difficult to identify and much more genetically stable than viral determinants.

But doubts about practical uses for viral ARFPs should in no way dampen enthusiasm for their further discovery and characterization. Understanding immunity to viruses requires the characterization of the full breadth of the immune response to viral peptides and, as Maness et al. demonstrate, ARFPs can be crucial in controlling viral replication and in driving viral evolution. Moreover, understanding the mechanisms for ARFP generation will no doubt provide unique insights into the varied processes by which the information encoded by nucleic acids is converted into polypeptides. Time and time again, such curiosity-driven explorations into basic cell biology have generated practical applications in diverse areas that extend beyond the immediate topic of interest.

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