Cancer's Dark Matter: Lighting the Abyss Unveils Universe of New Therapies

Bernard A. Fox[1,2,3], Walter J. Urba[1], Shawn M. Jensen[1], David B. Page[1], Brendan D. Curti[1], Rachel E. Sanborn[1], and Rom S. Leidner[1]

SUMMARY

The authors of a recent study identified noncanonical peptides (NCP) presented by cancer cells' HLA and observed lack of reactivity to these antigens by endogenous tumor-reactive T cells. *In vitro* sensitization generated NCP-reactive T cells that recognized epitopes shared by a majority of cancers tested, providing opportunities for novel therapies to shared antigens.

See related article by Lozano-Rabella et al., p. 2250

In this issue of *Clinical Cancer Research*, Lozano-Rabella and colleagues (1) identified noncanonical peptides (NCP) presented by the HLA class I molecules of human tumor cell lines and demonstrated that these peptides were immunogenic (1). While other publications support these findings of HLA presentation of noncanonical, aberrantly translated peptides derived from upstream open reading frame (uORF), 5′UTRs, ncRNAs, endogenous retroelements (ERE), pseudogenes and out-of-frame transcripts, from tumor cells, there has been little evidence that these rapidly degraded NCPs, or Dark Matter, were targets of the endogenous anticancer immune response (2–5).

The authors' explore this question by determining whether tumor-reactive T cells from the peripheral blood or tumor-infiltrating lymphocytes (TIL) recognized this HLA-bound Dark Matter, which they characterized as noncanonical tumor ligands (nonC-TL). While there was no evidence of an endogenous immune response to the NCPs, in some instances peripheral blood lymphocytes (PBL) *in vitro* sensitized (IVS) to the nonC-TL recognized nonC-TL pulsed onto antigen-presenting cells (APC) and secreted IFNγ or upregulated 4–1BB (**Fig. 1**). These sensitized T cells also recognized the NCP naturally presented by the patient's tumor cell line as well as other cancer cell lines that shared the same HLA restriction element but represented different tumor types. Upon transfection with the appropriate restricting HLA molecule that presented the specific nonC-TL, two of three nonC-TL–reactive T cell cultures recognized a majority of the 20+ tumor cell lines tested. This included endometrial, ovarian, gastrointestinal, and melanoma cancer cell lines and provided evidence for this HLA-bound Dark Matter to be shared by a spectrum of different cancers.

The inability of short-lived noncanonical proteins to be picked-up, processed and cross-presented by professional APCs has been described previously (4). Most of the noncanonical proteins/peptides are short-lived proteins (SLiP) or defective ribosomal products (DRiP), not subject to thymic selection, typically degraded within 30 minutes, but efficiently captured by MHC class I and thereby widely expressed on the surface of tumor cells (**Fig. 1**; refs. 4, 6). While the stability provided by MHC binding allows the peptide to be recognized by LC/MS-MS and a spectra of amino acids generated, decoding the LC/MS-MS spectra requires either a computational approach, which the authors used, or a database of expected sequences determined by whole transcriptome or ribo-seq (7–9). Both analytical methods are in a state of evolution and it is expected that additional nonC-TL will be identified as these processes mature.

The authors' data help explain why we are only now discovering the immunogenic nature of these nonC-TL. For instance, the detection of the first MHC-restricted tumor antigen in mice was made using cytotoxic T lymphocytes from spleen cells of animals vaccinated with P815 tumor cells (10). Similarly, tumor-reactive PBL or TIL were used to facilitate the cloning of the early human melanoma antigens (11, 12). On the basis of Lozano-Rabella's work, neither of these sources of tumor-reactive T cells would be expected to harbor T cells capable of recognizing nonC-TL derived from SLiPs or DRiPs. In retrospect, these findings may also explain the work of Prehn and Main, who more than 60 years ago demonstrated that vaccination with chemically induced tumors was exquisitely specific, providing protection against a live tumor challenge with the same tumor used as the vaccine, but not syngeneic sarcomas induced by the same carcinogen (13). Today we postulate that only the neoantigens contained in the long-lived proteins are retained in the tumor cells used as a vaccine, the nonC-TL, anticipated to be present, because of their short-lived nature, are not cross-presented and thus fail to induce a therapeutic anticancer immune response. However, Twitty and colleagues employing the same chemically induced sarcoma model developed a vaccine enriched for SLiPs and DRiPs and found it could provide significant cross-protection in 8 of 9 combinations tested (14). Their vaccine strategy blocks the proteasome and thereby shunts the SLiPs and DRiPs to the autophagosomes, which are harvested and used as a vaccine. Thus, we postulate that Twitty's vaccine actually enriched for nonC-TL, allowed for cross-presentation and ultimate induction of an immune response to these noncanonical antigens. Currently we are characterizing the NCPs contained in the human version of this vaccine, which has moved into a combination immunotherapy trial for patients with advanced cancer (NCT04470024; refs. 15–17)

Are these nonC-TL relevant cancer antigens? It is becoming increasingly clear that many of the uORFs, 5′UTRs, and ncRNAs unexpectedly code for peptides associated with malignant processes,

[1]Robert W. Franz Cancer Center, Earle A. Chiles Research Institute, Providence Cancer Institute, Providence Portland Medical Center, Portland, Oregon. [2]Department of Molecular Microbiology and Immunology, and Knight Cancer Institute, Oregon Health and Science University, Portland, Oregon. [3]UbiVac, Portland, Oregon.

Corresponding Author: Bernard A. Fox, Harder Family Chair for Cancer Research, 2N56 North Pavilion, Earle A. Chiles Research Institute, Providence Portland Medical Center, 4805 NE Glisan, Portland, OR 97214. E-mail: Bernard.fox@providence.org

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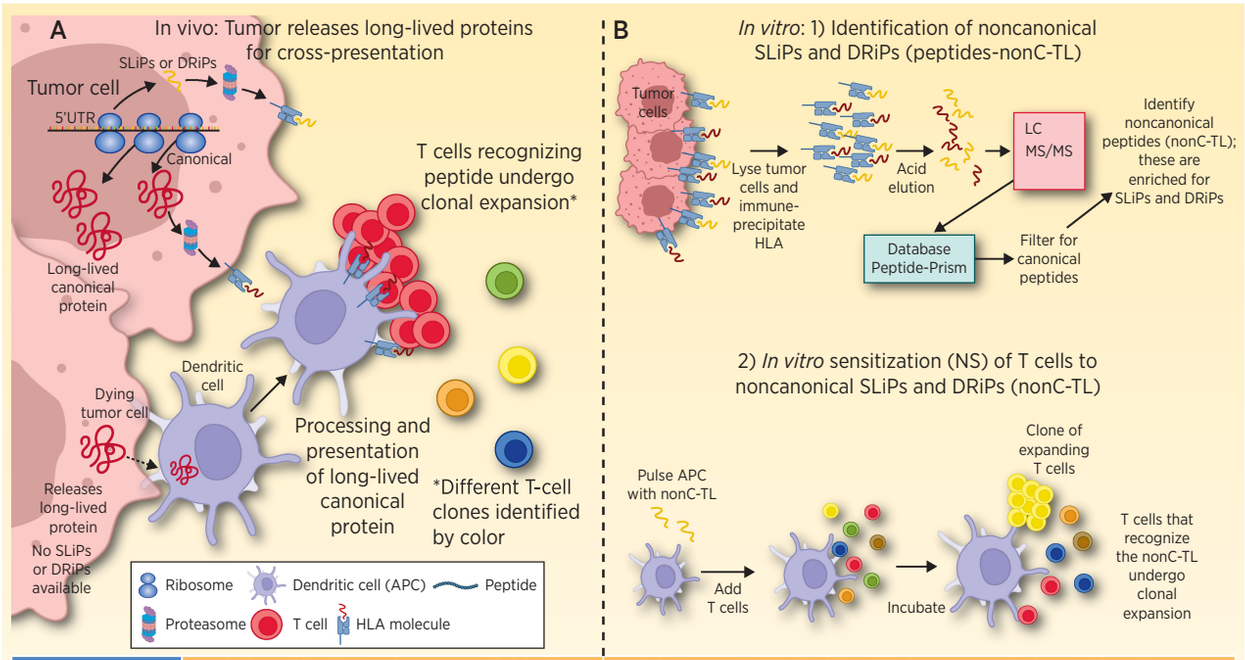

Antigen processing and presentation	**Only long-lived proteins are available for processing and cross-presentation by APCs.**	**Pulsing APCs with the identified noncanonical peptides allows the APCs to present these SLiPs and DRiPs (or nonC-TL) to T cells *in vitro*.**
T cells recognize	**T cells clonally expand to the peptides that their TCR can recognize. These T cells recognize long-lived proteins.**	**T cells that can recognize the noncanonical peptides undergo clonal expansion. These T cells recognize peptide-pulsed targets as well as peptide naturally presented by the tumor.**
Clinical application	**T cells recognizing canonical peptides or neoantigens contained in long-lived proteins are currently used for clinical trials of tumor-infiltrating lymphocytes (TIL). Whole tumor cell vaccines stimulate these T cells. TCRs to limited number of canonical or mutation-specific T cells are being studied in adoptive immunotherapy trials.**	**Shared noncanonical peptides will be used as cancer vaccines and to *in vitro* expand tumor-reactive T cells from PBL for adoptive immunotherapy. Vaccines containing noncanonical and canonical peptides will be used post adoptive immunotherapy with TIL or expanded PBL to boost T cells *in vivo* and prime new T cells. TCRs specific for shared noncanonical peptides will be used for adoptive immunotherapy, *in vivo* gene therapy, and as TCR-drug conjugates.**

Figure 1.

A, Progressively growing tumors are transcribing and translating canonical proteins. At the same time, NCPs derived from uORFs, 5′UTRs, ncRNAs, EREs, pseudogenes, and out-of-frame transcripts may also be generated, some of which can contribute to malignant properties of the cancer. The majority of these NCPs are SLiPs and DRiPs that will be rapidly degraded, and presented by HLA, but because of their short-lived nature are unable to be picked up, processed, and presented by APCs. Thus, the patient is unable to generate an immune response to these short-lived noncanonical proteins. When tumor cells die, the proteins that are available to be picked up and processed are predominantly long-lived proteins. Peptides derived from these long-lived proteins are the dominant antigens that the patient makes a T-cell response against. **B,** 1) *In vitro* isolated tumor cells are lysed, antibody to HLA class I is incubated with the lysate, and HLA molecules are immunoprecipitated. The peptides are next eluted from the HLA molecule and run through LC/MS-MS to identify peptide sequences. These data are then queried using a database (Peptide-PRISM) that identifies the peptides that were bound to HLA. These data are then filtered for canonical proteins. The result is a list of NCPs presented by HLA. 2) These NCPs (nonC-TL) were used in IVS assays to prime and expand T cells able to recognize the NCPs. In some cases, the expanded T cells recognized the NCP pulsed onto APC. These also recognized naturally presented peptide on tumor cells and secreted IFNγ or upregulated 4–1BB expression. TCRs isolated from these expanded T cells also recognize the NCP.

providing a basis for their presence in cancer and strengthening the rationale for developing therapies that target these peptides (18). Some of these nonC-TL might be compared with truncal cancer neoantigens that are driver mutations for cancer development. In this case, an increasing number of NCPs are being found to facilitate malignant processes. Targeting cancer cells that express these NCPs, with expression restricted to or substantially increased in cancer, has a solid footing for translation into humans. These findings have relevance for both vaccine and adoptive cell immunotherapy approaches.

In addition to the short-lived nonC-TL described above, NCPs can also be derived from alternative splicing junctions of transposable elements and exons in proteins (19). In some cases these are selectively expressed in cancer and, unlike nonC-TL derived from uORFs, 5′UTRs, and ncRNAs, are apparently long-lived proteins that can be

cross-presented and prime an endogenous immune response to this class of NCPs. It will be interesting to see which class of nonC-TL is most effective and whether T cells targeting these long-lived peptides will represent a more exhausted pool of T cells as compared with the short-lived nonC-TL.

As tumor-specific nonC-TL are identified, vaccine strategies should be developed and trials initiated. In acute myeloid leukemia (AML), Ehx and colleagues have already identified 58 AML-specific nonC-TL; approximately one-half represent intron retention (20).They predict that a vaccine with these 58 antigens would provide at least one tumor-specific antigen to 99% of patients with AML. One should expect to see additional reports of nonC-TL that are shared by cancers of the same, and possibly different histologies in the near future.

TCR adoptive immunotherapy or the generation of nonC-TL-reactive T cells by IVS and expansion *ex vivo*, represent two additional strategies that can take advantage of these findings. The preliminary data shown for HLA-A 11.01 suggests that multiple tumor types could be targeted with a single TCR. While it will take some time, it is not unreasonable to expect that clinical adoptive immunotherapy with peripheral blood T cells transduced with three or more TCRs specific for nonC-TL will be attempted. Similar to advances in CAR T cells, these strategies might involve short-term *in vitro* transduction and reinfusion in 24 to 48 hours. Alternatively, *in vivo* transduction strategies with these TCRs could also change the landscape of cancer treatment while reducing costs.

It is clear that we have entered a watershed period for cancer immunotherapy. The discovery of NCPs appears certain to grow as improved libraries of the transcribable genome become available. This also represents a watershed period for pharmacology approaches to target noncanonical uORFs, 5′UTRs, and ncRNAs that are involved in driving the malignant properties of cancer cells. This realm need not be restricted to immunotherapists; there is space for multiple approaches to address the apparent broad universe of targets presented by Cancer's Dark Matter.

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