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Commentary

Dark genome, bright ideas: Recent approaches to harness transposable elements in immunotherapies

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Transposable elements (TEs), which make up almost half of the human genome, often display altered expression in cancers. Here, we review recent progress in elucidating the role of TEs as mediators of immune responses in cancer and discuss how novel therapeutic strategies can harness TE immunogenicity for cancer immunotherapy.

Transposable elements (TEs) are current or previous mobile elements within the genome. In humans, they constitute 46% of the genome and are classified into two main types: DNA transposons and retroelements. Retroelements are further divided into three broad subclasses: long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), and long terminal repeat (LTR)/endogenous retrovirus (ERV) elements (Figure 1A).

TE-derived nucleic acids expressed in cancer are immunogenic

A growing body of research has now established that TE-derived nucleic acids are ligands of innate immune sensors in the tumor microenvironment (TME). Specifically, the remodeling of the epigenetic landscape concomitant with cancer cell transformation can lead to reactivation of TEs, which have the potential to activate nucleic acid-sensing pathways. These pathways include sensing of RNA through RIG-I-like receptors (RLRs) and Toll-like receptor 3 (TLR3) and sensing of reverse-transcribed complementary DNA (cDNA) through cyclic GMP-AMP synthase (cGAS).

Activation of RNA sensors

Two RLRs, RIG-I and MDA5, are primarily responsible for sensing immunogenic intracellular RNA. Upon activation, they interact with mitochondrial antiviral signaling (MAVS) protein and induce IRF3, IRF7, and NF-κB translocation to the nucleus, leading to the induction of type I interferon (IFN-I) and pro-inflammatory cytokines. RIG-I is preferentially activated by short double-stranded RNA (dsRNA) with 5′-triphosphate moieties, while MDA5 primarily binds longer dsRNAs. Importantly, several classes of TEs can harbor these structural features typically found in viruses. Experimental approaches such as dsRNA antibody capture or dsRNA enrichment through single-stranded-specific RNase treatment showed that several classes of TEs expressed in cancer cell lines—including families of ERV, LINEs, or SINEs—can lead to the formation of dsRNA (Sheng et al., 2018; Tunbak et al., 2020; Choi et al., 2021). While the role of MDA5 in sensing these dsRNA populations had been previously demonstrated through genetic depletion of this sensor, inverted repeats (IR)-Alu (a family of SINEs) was identified as the primary source of TE-derived dsRNA binding MDA5 in patient-derived colorectal cancer cells (Mehdipour et al., 2020). IR-Alu, likely forming stem-loops from unidirectionally transcribed repeats, made up to 53% of MDA5-bound RNA at baseline and 73% after treatment with decitabine, an inhibitor of DNA methyltransferase (DNMT) that suppresses IR-Alu expression by DNA methylation (Mehdipour et al., 2020).

Investigation of RIG-I as a sensor of TE expression has been more limited than MDA5, and reports have been conflicting, with some studies demonstrating an absence or little impact on interferon stimulated genes (ISGs) activation following RIG-I downregulation (Sheng et al., 2018; Tunbak et al., 2020; Choi et al., 2021). However, ISG activation in triple-negative breast cancer cell lines treated with a protein arginine methyltransferase (PRMT) inhibitor, which alters mRNA splicing and induces intronic retention of IR-Alus, was significantly reduced after RIG-I silencing (Wu et al., 2022). More generally, the fact that several types of TEs, including most evolutionarily recent Alu families, are primarily transcribed by RNA polymerase III, which generates uncapped RNA that can retain 5′-PPP moieties, suggests a possible role of RIG-I in sensing these TE families.

TLR3, a cell surface and endosomal receptor that can also sense dsRNA, was also shown to sense TE-derived RNA. In human breast cancer cell lines MCF7 (Sheng et al., 2018) and MDA-MB-468 (Wu et al., 2022), silencing TLR3 impaired ISG activation induced by the knockdown of LSD1, an epigenetic suppressor of ERVs (Sheng et al., 2018) or by the inhibition of PRMT1 (Wu et al., 2022), respectively. Importantly, TLR3 is a sensor found both at the cell surface and in endosomes, and its activation indicates the uptake of TE-derived dsRNA from other cells or translocation of dsRNA from the cytosol into endosomes.

Reverse transcription-dependent activation of DNA sensors

A very limited subset of TEs in humans can encode functional reverse transcriptase (RT), including elements from the HERV-K and LINE-1 families. RT

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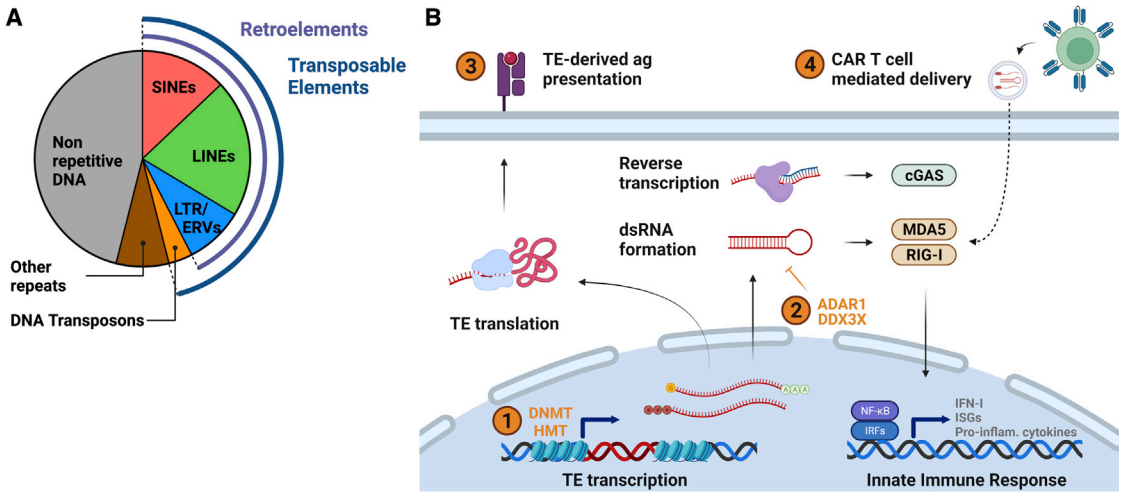

Figure 1. Transposable elements have the dual ability to form nucleic acid ligands that activate innate immune responses and generate tumor antigens recognized by adaptive responses

(A) Distribution of repetitive sequences in human genome. Other repeats include satellite, simple repeats, and low complexity repeats.

(B) Therapeutic approaches to harness the immunogenicity of TEs currently explore: 1) modulating their expression through inhibition of epigenetic modulators; 2) interfering with post-transcriptional mechanisms that modulate their immunogenicity; 3) targeting TE-derived antigen (ag) by adaptive responses; and 4) delivering immunogenic nucleic acids by CAR T cells.

expression can lead to the formation of RNA:DNA hybrids and cytosolic DNA, both of which can activate the innate immune sensor cGAS. Binding of cGAS to RNA:DNA hybrids or cDNA of sufficient length induces downstream activation of the adaptor protein stimulator of interferon genes (STING), promoting translocation of IRF3 and NF-κB to the nucleus and the initiation of IFN-I and pro-inflammatory cytokines response. A recent study showed that the cGAS-STING pathway is strongly activated in hyperproliferative cells from the blind mole rat, a species of rodents known for its ability to resist tumor development (Zhao et al., 2021). Blind mole rats express very low level of DNMT at baseline, and precancerous cells undergoing hyperproliferation further reduce DNA methylation levels, thereby derepressing several TEs, including SINEs, ERVs, and LINEs. These TEs are then reverse-transcribed, forming RNA:DNA hybrids that activate cGAS, inducing an IFN-I-dependent necrotic cell death in hyperplastic premalignant cells and preventing cancer development (Zhao et al., 2021). In humans, repression of the histone methyltransferase EZH2 in prostate cancer patients correlated with increased ERV expression, and genetic knockout of STING in cell lines significantly prevented IFN-dependent upregulation of major histocompatibility complex (MHC)-I and PD-L1 that followed EZH2 inhibition (Morel et al., 2021).

Deciphering the impact of TE expression on antitumor immunity

A widespread analysis of TE expression in the Cancer Genome Atlas (TCGA) samples found that stomach, bladder, liver, and head and neck were the cancer types with the most significantly overexpressed TEs compared to matched healthy tissue, indicating that TE expression in tumor is impacted by tissue type (Kong et al., 2019). Further, the origin and consequences of innate immune activation will vary depending on the cellular origin of TEs and which TE classes are expressed.

Common and specific immune properties of TE classes

Altered TE expression in transformed cells is primarily a consequence of broad dysregulation of epigenetic control mechanisms, making it complex to link general innate immune activation with specific TE classes. Therefore, defining common and specific immunogenic properties of TEs will require careful experimental approaches which connect immunogenic patterns with TE classes, the epigenetic mechanisms controlling their expression, and the pathways they activate. In this context, the aforementioned study performed a correlation analysis of TE upregulation with changes on different cellular pathways, including DNA damage response (DDR) pathway or type I and type II IFN signaling. TEs from the Gypsy, ERVL, and ERV1 families (all members of the LTR/ERV class) showed the strongest positive association with IFN-I response across cancer type, while other families from ERV1, ERV3, and DNA transposons had the highest association with DDR (Kong et al., 2019).

However, despite the strong correlation across tumor types between ERV expression and immune responses, when both ERVs and Alu elements were induced through inhibition of DNMT1 in patient-derived colorectal cancer cells, Alus were found primarily responsible for dsRNA accumulation and activation of MDA5 (Mehdipour et al., 2020). In another study, the depletion of MPP8 (a human silencing hub [HUSH] complex component) led to simultaneous expression of LTRs (including ERV9 and HERVH) and LINE1, while LINE1 was the primary driver of the type I IFN response in HEK293 cells (Tunbak et al., 2020). Finally, Griffin et al. found that widespread induction of several TE families—including SINE, LINE, and a majority of LTR, consecutive to knockout of histone methyltransferase SETDB1—did not induce IFN-I signature in models of murine melanoma and lung carcinoma (Griffin et al., 2021). Altogether, recent results seem to point toward the

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cancer cell type as a key factor in controlling the immunogenicity of different TE classes and indicate that thorough molecular assays are required to quantify their respective contribution to immune activation.

Dichotomous effect of IFN-I in the tumor microenvironment

The widespread upregulation of TEs with immunogenic potential during cancer development can at first appear paradoxical, and one could wonder how cancer growth can tolerate innate immune activation. However, while TE expression can lead to the induction of innate immune responses, the activation of these pathways can ultimately have both pro- and anti-tumoral effects. Acute IFN-I induction contributes to increased MHC expression, activation of antigen-presenting cells, and differentiation of $CD4^+$ Th1 cells and promotes $CD8^+$ T cells function and cytotoxicity, all of which are beneficial to stimulating antitumor immune responses. Conversely, IFN-I can also upregulate PD-L1 expression on tumor cells, a key T cell-inhibiting checkpoint, and chronic IFN-I stimulation contributes to $CD8^+$ T cell exhaustion. Further, chronic IFN-I signaling can induce the production of ISGs that promote epithelial-mesenchymal transition, tumor invasion, and metastasis or alter tumor metabolism. Ultimately, either a complete loss or prolonged IFN-I signaling can prove deleterious to the antitumor immune response, highlighting a need for better immunotherapeutic strategies to control IFN-I responses in the TME.

Many studies have demonstrated overall beneficial effects of inducing TE expression in tumors (Sheng et al., 2018; Kong et al., 2019; Choi et al., 2021; Morel et al., 2021; Zhao et al., 2021); however there are also discrepancies in the responses to expression of the same TE class in different cell populations or tumor types. This demonstrates a need to further investigate whether anti-tumoral effects of TE expression can be optimized therapeutically by selecting specific locations for TE expression (e.g., preferentially in immune cells vs. tumor cells) or the class of TE expressed and their timing of induction. Further, we still lack a complete understanding of the landscape of immunogenic TEs and what differentiates immunogenic TEs from those that don't trigger an immune response. This will be required to determine whether TEs that are overexpressed in tumors have the potential to stimulate IFN-I responses. Additionally, baseline TE expression in cancer cells and the extent to which cancer cells have adapted to TE expression would impact the cancer-intrinsic and the TME response to TE expression induced by therapy.

TE-derived antigens provide shared targets for adaptive responses

Tumor specific antigens (TSAs) are peptides bound to MHC presented on the surface of cancer cells and not healthy tissue. TSAs typically result from mutations in cancer cell DNA leading to the synthesis of mutated proteins uniquely expressed in tumor tissue. However, tumor-specificity can also result from the unique expression of non-mutated regions of the genome that are normally silenced, such as TEs, making TE-derived antigens an emerging class of TSAs considered for therapy. Further, despite being characterized as "noncoding" RNA, a subset of TEs contain open reading frames (ORFs), like ORF1/ORF2 within LINE1 elements or the *gag*, *pol*, and *env* genes present in intact ERVs. These protein-coding regions of TEs have the potential to be translated and presented on MHC and serve as targets of antitumor immune responses.

Two studies examined two murine cancer cell lines and 26 primary human tumor samples to develop a proteogenomic approach that identifies tumor-specific transcripts that could be translated into novel TSAs (Laumont et al., 2018; Ehx et al., 2021). Cancer-specific RNA expression was first identified from RNA-sequencing data by comparing cancer samples to healthy tissue—either thymic epithelial cells (Laumont et al., 2018) or hematopoietic progenitors (Ehx et al., 2021). These cancer-specific sequences were then translated *in silico* across three potential ORFs to create a cancer-specific proteome used as a database for immunopeptidomic mass spectrometry data. Using this approach, the authors identified TSAs originating from noncoding regions of the genome in primary B cell acute lymphoblastic leukemia and lung cancer samples and acute myeloid leukemia (Laumont et al., 2018; Ehx et al., 2021). Kong et al. also identified TE-derived peptides presented on MHC using matched transcriptome and immunopeptidome data from a glioblastoma cell line. The authors took a more TE-focused approach, first identifying differentially expressed TE subfamilies in epigenetic drug-treated cell lines from RNA-sequencing, then translating *in silico* all transcripts from those subfamilies across six ORFs to create their TE-specific database to search immunopeptidomic data (Kong et al., 2019). Interestingly, they discovered peptides that originated from SINE-VNTR-Alu elements, which do not contain any previously characterized ORFs, indicating that the presence of known ORFs was not a prerequisite for encoding TE-derived antigens and implying a larger pool of potential antigen-encoding transcripts (Kong et al., 2019). Finally, Griffin et al. took yet a different approach, searching RNA-sequencing data for previously annotated TE ORFs and restricting to 8–10mer peptides with predicted MHC binding to use in their immunopeptidomic mass spectrometry search library. This approach allowed the authors to identify TE-encoded peptides presented on MHC in murine cancer cell lines, either present at baseline or upregulated after silencing H3K9 methyltransferase SETDB1 (Griffin et al., 2021).

These studies demonstrated that transcripts from non-mutated regions of the genome, including TE, can serve as a source of peptides presented on MHC uniquely on tumor cells. These TSAs serve as attractive targets since their antigenicity does not rely on specific amino acid mutations and therefore can be shared across different tumors from different patients (Ehx et al., 2021).

To validate the immunogenicity of TE-derived antigens, Laumont et al. examined frequencies of $CD8^+$ T cells against viral epitopes, TE-derived TSAs, and non-TE-derived TSAs in murine models of colon cancer and lymphoma. In naive mice, while T cells recognizing non-TE-derived TSAs were rare, T cells recognizing TE-derived TSAs had a similar frequency to those specific to viral peptide controls, though relatively few TSAs were examined overall (Laumont et al., 2018). In humans, Ehx et al. emphasized the potential immunogenicity of intron-derived TSAs in acute myeloid leukemia. High T cell response to TSAs was predicted by a machine learning model

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trained on public T cell receptor data, and functional T cell responses were demonstrated by IFNγ secretion and expansion of T cell clones from peripheral blood in response to stimulation with intron-derived TSAs (Ehx et al., 2021). Griffin et al. performed TCR-sequencing and tetramer staining of murine melanoma tumor-infiltrating lymphocytes to show that TE-derived antigen-specific $CD8^+$ T cells upregulated genes associated with T cell activation and cytotoxicity (Griffin et al., 2021). Saini et al. additionally discovered higher T cell reactivity to antigens derived from transcribed ERVs in patients with myeloid malignancies compared to healthy donors (Saini et al., 2020). Further, they demonstrated functional patient T cell responses targeting ERV peptides presented on MHC, as measured by cytokine release upon co-culture with ERV-peptide-loaded leukemia cells.

Finally, it is important to note that TEs are not necessarily transcribed in an autonomous fashion, and a large fraction are found in introns. Moreover, *de novo* integration of TEs into exons or alternative splicing events allowing readthrough of intronic TEs (Wu et al., 2022) can generate fusion proteins that could serve as targetable antigens. Overall, TEs represent a specific source of immunogenic tumor antigens that can be recognized by T cells and induce a functional response (Laumont et al., 2018; Saini et al., 2020; Ehx et al., 2021) (Griffin et al., 2021). They represent an exciting class of immune targets, but our ability to harness TE-TSAs as immunotherapeutic targets is currently limited by an incomplete knowledge of the TE-derived peptidome.

Therapeutic approaches to leverage noncoding RNA immunogenicity

TEs display multiple properties that make them attractive targets in immunotherapy, starting with their ability to stimulate innate sensors and provide a source of tumor antigens. Here, we discuss strategies currently explored to modulate TE expression and immunogenicity.

Mediating TE expression

Organisms have developed multiple mechanisms to tightly control the expression of repetitive elements. Among these, epigenetic modulators have been a primary target for therapeutic drug research that seeks to induce TE expression, predominantly through inhibiting enzymes that keep TEs in a heterochromatin state, such as DNMT, histone methyltransferases (HMT), and histone deacetylases. Consequently, drug targeting these epigenetic enzymes remains the primary approach for modulating TE expression broadly.

There is now a large body of literature that have used DNMT inhibitors such as azacytidine or decitabine to induce widespread TE expression in preclinical models. Building on previous studies, Kong et al. showed that treatment of glioblastoma cell lines with decitabine triggered several innate immune pathways but also led to presentation of novel TE-derived peptides on MHC, demonstrating the dual ability of DNMTi to engage innate and adaptive immune responses (Kong et al., 2019).

In addition to DNMTi, other recent studies have uncovered the role of specific histone methylation marks in the silencing of TEs, providing additional druggable targets to induce TE expression. The HUSH complex—comprised of TASOR, MPP8, and periphilin—localizes to genomic loci rich in the repressive histone modification H3K9me3 and is a known repressor of TE. Depletion of MPP8, a phosphoprotein with a chromodomain necessary for the establishment of suppressive marks by HUSH, induced expression of repeats from the LTR, LINE, and SINEs subclasses in human cell lines, with the family L1PA1/2 demonstrating bidirectional transcription, constituting a potential source of immunostimulatory dsRNA (Tunbak et al., 2020). In another study performed on mouse models of melanoma or lung carcinoma, the genetic depletion of H3K9 methyltransferase SETDB1 derepressed TE expression and led to the induction of TE-specific T cell response (Griffin et al., 2021). In an earlier study, an inhibitor of the histone H3K4 demethylase LSD1 was identified in a screening of compounds for upregulation of randomly selected ERV transcripts, IFN-Is, and select ISGs (Sheng et al., 2018). In a mouse model of melanoma, genetic depletion of LSD1 decreased tumor growth, increased T cell infiltration, and sensitized resistant tumors to treatment with PD-1 blockade, defining LSD1 as an attractive target for induction of TE-mediated immune responses in the TME (Sheng et al., 2018). Inhibition of EZH2, another methyltransferase acting on H3K27me3, induced dsRNA and ISGs expression and reversed resistance to anti-PD-1 therapy in a murine model of prostate cancer (Morel et al., 2021). Together, these studies focusing on histone methylation argue for a promising role of histone methylation modulators as a class of drugs to induce TEs.

These recent advances in our understanding of TE silencing also indicate that different epigenetic modulators might control different classes of TEs. Importantly, the characterization of TE expression in TCGA showed that the most widely expressed TEs were also evolutionarily the youngest and the ones that associated most with innate immune responses (Kong et al., 2019). Further, emerging concepts suggest that the dominant silencing mechanisms could depend on the evolutionary age of each TE class: in the case of LTR/ERVs, the youngest are primarily silenced through DNA methylation whereas older LTRs families are repressed through histone methylation (Ohtani et al., 2018). The overlap of silencing mechanisms provides a window of opportunity for therapeutic strategies aimed at inducing particular TEs based on their age, especially if this correlates with their immunogenicity (Ohtani et al., 2018). However, this link between the modes of epigenetic silencing and specific TEs is not fully established, and additional work is required to parse differences in TE classes that can be induced by targeting distinct epigenetic enzymes.

Alternative targets can also impact epigenetic gene regulation. Thus, an unbiased screening system designed to uncover regulators of heterochromatin found cyclin-dependent kinase 9 (CDK9) as a modulator of chromatin accessibility. Zhang et al. demonstrated that inhibition of CDK9 led to the dephosphorylation of BRG1, a regulator of heterochromatin, and resulted in increased global gene expression, including expression of ERVs and IFN-I-associated signature (Zhang et al., 2018). Further, Wu et al. demonstrated that inhibition of type I PRMTs in triple-negative breast cancer cell lines triggered aberrant splicing events, including the retention of introns containing IR-Alus that can form dsRNA, contributing to the induction of an IFN-I response (Wu et al., 2022). This work

demonstrates how splicing represents another potential intervention point in controlling TE expression and immunogenicity.

Mediating TE immunogenicity

Epigenetic suppression prevents widespread transcription of TEs, but expression of specific TE classes happens in homeostatic conditions, and cells have evolved post-transcriptional mechanisms to prevent aberrant innate immune activation in healthy cells. Importantly, TE expression is further increased as a consequence of the dysregulation of epigenetic landscape concomitant to cellular transformation, and RNA-modifying enzymes can then act as tumor protection mechanisms by limiting TE immunogenicity (Choi et al., 2021). Recent strategies have evaluated the targeting of these RNA-modifying enzymes, demonstrating that their inhibition can result in anti-tumoral innate immune responses driven by baseline TE transcription.

ADAR1 is an RNA-editing enzyme that converts adenosine to inosine, destabilizing A:T pairing and dsRNA structures, which limits their subsequent binding to MDA5. Mehdipour et al. showed that depletion of ADAR1 in patient-derived colorectal cancer cells increased accumulation of inverted-repeat Alu elements bound to MDA5. Further, the combination of decitabine treatment with ADAR1 depletion resulted in enhanced MAVS activation and ISG induction, compared to decitabine treatment alone (Mehdipour et al., 2020). ADAR1 depletion also demonstrated synergy with CDK4/6 inhibition, as CDK4/6 inhibitors were previously found to stimulate a viral mimicry response through DNMT1 depletion (Mehdipour et al., 2020). These results further suggest that synergistic treatment strategies could improve TE-dependent IFN-I responses. DEAD-box RNA helicase 3X (DDX3X) is another enzyme acting post-transcriptionally by unwinding dsRNA. Overexpression of DDX3X in cancer cells is also associated with decreased survival in breast cancer patients. A recent study demonstrated that knockdown of DDX3X in the human breast cancer cell line MCF7 induced MDA5-dependent sensing of endogenous dsRNA (Choi et al., 2021). Additionally, knocking down DDX3X in a mouse model of breast cancer suppressed tumor growth, concomitant with an induction of IFN-I response. A synergy between ADAR1 and DDX3X knockdown in inducing ISGs was identified, indicating additional benefits of targeting multiple post-transcriptional mechanisms at once (Choi et al., 2021). Other post-transcriptional mechanisms that mediate TE immunogenicity likely exist and could serve as future therapeutic targets.

TEs as a source of adjuvants in CAR T cells

Finally, another emerging strategy that harnesses TEs for immunotherapy consists of engineering CAR T cells to express immunostimulatory RNA and exploiting their adjuvant activity. While not a TE itself, RN7SL1 is a noncoding RNA considered to be the ancestor of the SINE family Alu and can activate RIG-I and MDA5 when unshielded from its regular protein interactors. In a recent study, Johnson et al. engineered CAR T cells to express RN7SL1 and observed improved tumor control in a murine model of human pancreatic tumors. The authors found that RN7SL1 CAR T cells showed increased persistence or proliferation and displayed fewer exhaustion markers compared with control cells (Johnson et al., 2021). Using a syngeneic model of melanoma tumor, they further found that RN7SL1 was preferentially delivered to immune cells in the TME through exosomes secreted by CAR T cells, activating RNA sensors that reprogramed antigen-presenting cells such as myeloid cells, which in turn enhanced overall anti-tumoral immune response (Johnson et al., 2021).

Challenges and outstanding questions

Despite recent progress toward our understanding of the interactions between TEs and the immune system, key challenges remain to target TEs in cancer immunotherapy. The first truly complete sequence of a human genome was published this year and includes repetitive portions of the genome ignored until now. This highlights a gap in the development of genomic technologies, which were initially designed to resolve the coding portion of genomes and are not adapted to study TEs. For example, the cell-type specificity of TE expression is largely unknown, and new approaches to quantify TE transcripts at the single-cell level will enable studying their immunogenicity in complex tissue environments such as TMEs. The field is further lacking a systematic assessment of the functional significance of the different classes of TEs. However, recent advances in editing and manipulating the genome en masse offer the promise of reductionist models to interrogate the role of TEs, family by family rather than from specific loci. Additionally, many mechanistic studies have been performed so far in mice, which do not express several TE classes recently evolved in humans, such as the SINE-VNTR-Alus (SVA), limiting the ability to generalize and translate these findings. Altogether, future advances in technology and models might reconcile findings of seemingly contradictory results, with studies indicating that a given innate immune sensor, or a specific TE class, is critical to a viral mimicry response in one setting but disposable in another. We believe that addressing these challenges will move forward a field that is poised to revolutionize cancer treatment by identifying a new pool of therapeutics targets.

Conclusion

TEs represent a unique opportunity in cancer immunotherapy by simultaneously acting as innate immune ligands and tumor-specific targets of adaptive immune responses. Current and future strategies aiming at modulating the expression and immunogenicity of TEs represent an exciting approach in cancer immunotherapy.

DECLARATION OF INTERESTS

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