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Review

The Perfect Storm: Viral Mimicry Meets Cancer Dark Matter

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Abstract: The central mechanism for immune recognition of cancer remains a subject of debate. Some theories emphasize the importance of cancer antigen specificity and immunogenicity while others focus on innate chemo attractive and immune-modulatory properties of cancer cells. The immune system evolved under strong selection to protect individuals during their reproductive years from infectious epidemics, which helped to preserve species. Since common cancers often occur after the reproductive stage of life, there is less evolutionary pressure to eliminate them as they do not pose a direct threat to the survival of the species. So, why does the immune system care about cancer? The answer may be simpler than what has been conjectured in the past: the neoplastic process deviates sufficiently from normal tissue physiology to slowly align its phenotype to that of pathogen-infected cells. Simply put, cancer cells look infected to the immune system. Epigenetic alterations germane to cancer cell biology lead to aberrant production of nucleic acids and peptides recapitulating those produced by pathogen-infected cells. This results in two phenomena: (a) production of double stranded nucleic acids that trigger cancer cell intracellular sensors that consequently activate innate immunity; and (b) aberrant production of peptides from genomic regions silenced in normal cells or cancer-specific alteration of cellular processes. The former phenomenon is referred to as "viral mimicry" while the latter is referred to as "dark matter". The symbiotic interplay between the two phenomena and their causality is the subject of this review.

Keywords: cancer microenvironment; cancer immunotherapy; viral mimicry; cancer dark matter

1. Introduction

For decades, immunologists have been trying to explain why cancers can be rejected by the immune system. While the mechanism leading to immune-mediated cancer rejection is now unmistakably clear, its root cause remains controversial. Although this review focuses on the latter question, it is important to understand what happens when immune activation reaches the threshold required to cause cancer rejection [1,2]. This is because cancer immune surveillance spans a continuum from the coexistence of immune and cancer cells within the tumor microenvironment (TME), to the full activation of immune effector mechanisms leading to cancer clearance [2]. Immune infiltrated tumors display a better prognosis and are more likely to respond to immunotherapy because the immunological substrate is there and only its amplification or release from suppression is required to complete the task [3,4], while immune desert tumors lack such basis.

Cancer rejection is a facet of a highly conserved phenomenon defined as immune-mediated, tissue-specific destruction observable across all mammalian species, affecting tissues beyond just cancer. This includes other

phenomena such as clearance of pathogen-infected cells during acute viral infections, acute allograft rejection, graft-versus-host disease, and destructive flares of autoimmunity. This phenomenon called the "immunologic constant of rejection" (ICR) occurs when a chronic inflammatory process turns into an acute one capable of total clearance of the target cells [1]. The ICR encompasses the activation of the interferon (IFN)-g pathway which, in turn, results in the production of T cell-attracting chemokines (such as C-X-C motif chemokine ligands (CXCL)-9, 10, and 11), the induction of cytotoxic effector mechanisms (such as granzymes and perforins), and the release of powerful homeostatic cytokines by activated immune cells.

It remains unclear, however, why immune cells are in the TME in the first place, whether they are sustaining a lingering inflammatory process that may even foster tumor growth [5], or they are actively clearing the disease [2]. In other words, what attracts T cells in the TME and sustains their persistence?

In this review, we dissect this question by describing (1) distinct phenotypes of TMEs and their prognostic and predictive relevance, (2) the epigenetic dysregulation of cancer cells that shapes these TMEs, (3) the production of double-stranded nucleic acids that, upon activation of intracellular sensors, spark a signaling cascade resulting in the attraction and activation of immune cells (viral mimicry), and (4) the aberrant production of peptides (dark matter) derived by non-coding genomic regions or other alterations of cancer-specific cellular processes. Since these peptides are not expressed by normal cells, they act as potent "neoepitopes" capable of inducing powerful T cell responses.

2. Immune Phenotypes of Cancer and Their Raison D'être

Independent of ontogeny, cancers segregate according to three landscapes: immune active, immune silent, and, in between, immune excluded [6]. Immunohistochemical analysis of the pattern of immune infiltration of about 1000 histological tissue slides from 177 patients bearing cancers of different histology revealed that in several cases there was a strong correlation between the infiltration of the tumor core and the invasive margins: these patterns are defined as "immune active" or "hot" tumors. Tumors with high immune cell density in the outer invasive margin, but low density in the core were defined as "immune excluded" [6]. Low immune cell density in all compartments are characterized as "immune desert" or "cold" tumors. This phenomenology has practical implications because the level on intratumoral T cell infiltration is a strong predictor of immune responsiveness to therapy and the strongest favorable prognostic indicator in multivariate analyses [2,3,7].

The distinction of immune active from immune excluded tumors has functional implications and aligns with the "immune score" proposed by Jerome Galon, whereby the intra-tumoral presence of CD8+ lymphocytes distinguished lesions with better prognosis and most likely to respond to anti-cancer immunotherapy, while immune excluded tumors where more likely to behave clinically as cold tumors [2,8,9]. While the clinical implications are different, we propose, however, that the biology of immune excluded tumors is partially similar to that of the immune active compared to the immune silent phenotype [10]. This hypothesis is based on the premise that chemo-attraction can recruit the T cells to the tumor periphery in either case and that some stimulus can preserve their persistence. However, additional mechanisms are required to stimulate infiltration within the core of hot tumors or, conversely, immune exclusion could be determined by chemo-repulsive mechanisms that are not present in hot tumors. The various theories raised to explain cancer immune exclusion have been described extensively by Pai S et al. [10]. For the purpose of the current discussion, we suggest that hot and excluded tumors share general chemo attractive properties, and therefore, can be categorized mechanistically together, while cold tumors are defined primarily by the absence of chemoattraction, rousing the question of what brings the T cells within or around a tumor deposit.

To explain the distinct TME phenotypes several hypotheses have been raised encompassing patients' germline background, somatic mutational pattern of tumors, and exterior elements such as environmental, behavioral and anamnestic factors [11]. The list of hypotheses brought up to describe the phenomenon of cancer immunogenicity is extensive, yet unlikely to pinpoint the primary mechanism and has been recently summarized by Cesano A et al. [11] For instance, an analysis of common and rare germline variants in ~9000 patients with cancers of different ontologies from the "The Cancer Genome Atlas" (TCGA) demonstrated that, indeed, germline factors can influence the functional orientation of the TME, determining immune infiltration by NK and T cells, as well as activation of IFN signaling. However, their role is contributory rather than pivotal [12,13]. Environmental factors play an important role shaping immune phenotypes of cancer, influencing its prognosis and responsiveness to immunotherapy—for example, through the presence of specific microbes in the gut [14] or within the cancer tissue [11,15].

Nevertheless, the stronger determinants of cancer immunogenicity remain those related to the intrinsic mutational pattern of cancer cells and/or their epigenetic dysregulation [16–20]. This "cancer-centric" hypotheses

can be in turn parted into two main categories; on one hand, emphasis is put on the pro-inflammatory and chemo-attractive/chemo-repulsive properties of cancer cells, and on the other, a central role is believed to be played by the antigenic properties of individual cancers [21]. Likely, only the combination of both can lead to the "perfect storm" required to reject cancer.

3. The Inflammation-Driven Chemo Attractive and Pro-Inflammatory Hypothesis

The inflammation-driven hypothesis emphasizes the chemo attractive properties of cancer cells leading to immune cell, in particular T cell, infiltration and their opportunity to be exposed to cancer antigens [21,22]. Likely, the combination of chemoattraction through the release of CXCL chemokines and the activation of immunity by IFNs and other pro-inflammatory factors creates the substrate for the activation, proliferation, and differentiation of T cells. This concept follows the basic notion that full activation of T cells requires at least three signals: signal-1, which is antigen recognition through the T cell receptor, signal-2, which is responsible for T cell co-stimulation to amplify signal-1, and signal-3, which boosts proliferation and shapes T cell differentiation via cytokines [23]. In addition, immune cells need to be within the affected tissue for the three signals to act and this can only be achieved by the production of potent chemo attractive factors [22]. Thus, successful activation of anti-cancer immune responses is a multifactorial event that recapitulates the natural phenomenon of recognition of pathogen-infected cells [24]. In this review we will discuss how, in the first place, viral mimicry leads to the recruitment and persistence of immune cell in the TME by providing chemoattraction and activation of macrophages, dendritic cells, NK and T cells (signal-2 and -3), and the role that dark matter plays in the provision of signal-1 through the release by dying cancer cells of their bioproducts for antigen uptake, processing and presentation [22].

4. The Role of Cancer-Specific Antigens

Cancers produce peptides, referred to as "neoepitopes", not expressed by normal cells because they are the product of the mutational pattern inherent to cancer cell biology [25,26] These antigens, referred to as "canonical neoepitopes", include cancer germline antigens, or peptides resulting from oncogenic missense or frameshift mutations, splice-site variants, or oncogenic viruses [25]. Most emphasis has been previously assigned to the concept that peptides not expressed by normal cells avoid thymic selection during development, are not susceptible to immune tolerance and, therefore, are promptly recognized by naïve circulating lymphocytes [27]. In theory, tumors with high mutational burden are more likely to express neoepitopes responsible to sustain a resident adaptive immune response in the TME [25,28]. Although the presence of canonical neo-epitopes represent a compelling reason to explain T cell-mediated immune responses, most studies with large collections of cancer tissue assessing the relationship between elevated mutational rates and T cell infiltration in the TME, demonstrate at best a weak association with TME phenotypes [15–17,29], or responsiveness to immunotherapy [15,16,30,31]. Moreover, the efficacy of canonical neoepitope-based vaccines in eliciting CD8+ T cells is relatively low compared with the effectiveness of anti-viral epitopes, due to various reasons including low T cell receptor affinity and suboptimal activation of adjuvancy [32].

The limited success of canonical epitope-based vaccines has prompted interest in expanding antigen discovery beyond the current canonical exon-driven boundaries, hence the list of neoepitopes is rapidly expanding, encompassing the new generation of "noncanonical" neoepitopes referred to as the "cancer dark matter". These that are peptide products not encoded by the normal cell genome or other processes belonging to normal cell physiology [25]. Noncanonical neoepitopes are not mutated and are broadly expressed by most cancers compared to canonical neoepitopes [33–40]. Noncanonical neoepitopes include alternative splicing-derived, post-translational modification-derived, RNA editing-derived, and aberrant mRNA translation-derived epitopes.

5. Epigenetic Dysregulation of Cancer

Although exonic regions account for just 2% of the genome of normal cells and is considered responsible for protein coding [41], analyses based on bulk and single cell transcriptomics, ribosome profiling, and tandem mass spectrometry identified hundreds of shared and tumor-specific noncanonical epitopes that largely outnumber predictions based on exome sequencing [42–44].

Moreover, the observation that DNA-demethylating agents and other epigenetic modifiers can drive cancer immunogenicity offers a novel explanation for the remodelling of the TMEs [45,46]. This epigenetic dysregulation depends mostly, but not exclusively, upon DNA hypomethylation observable to various degrees in most cancers [47]. It was originally thought that these drugs enhanced anti-cancer immunity by lifting the epigenetic inhibition of developmentally silenced cancer/testis antigens [48]. However, it is becoming evident that they can also induce in cancer cells a state similar to antiviral response through aberrant activation of short or long DNA sequences

scattered in multiple copies throughout the human genome, referred to as repetitive elements (RE), that can be transcribed into double stranded nucleic acids perceived by immune sensors as viral byproducts [21,49,50]. Moreover, translational activation of these leads to excessive amounts of nucleic acids that can result in the aberrant production of peptide sequences not expressed by normal cells that act as noncanonical neoepitopes.

The most represented category of RE includes transposable elements (TEs) [41,51], which are interspersed repetitive DNAs distributed throughout mammalian chromosomes. TEs, with their ability to mobilize portions of DNA, can significantly influence the organization and function of genomic architecture, being at the same time the cause and consequence of cancer development. Retrotransposition can modify the structure of chromosomes, disrupt gene structures, and dysregulate gene expression. Moreover, architectural changes in *cis*-regulatory elements where enhancers and promoters play a key role in regulating gene expression without modifying the primary DNA sequence, can stimulate cancer development [52].

There are two categories of TEs [53]. Type I TEs, known as retrotransposons, transpose across the genome following a copy-and-paste replication strategy dependent upon reverse transcription. Type II TEs, instead, are DNA transposons that use a cut-and-paste excision-reinsertion mechanism [51]. Type I TEs encode their own reverse transcriptase and integrase enzymes that produce an RNA intermediate. Type I TEs are differentiated according to the presence or absence of long terminal repeats (LTR). An example of Type I TEs with LTRs are endogenous retroviruses (ERVs), dormant remnants of ancient viral infections that were permanently integrated into host genome. Human endogenous retroviruses that contain LTRs are mostly inactive [54], whereas non-LTR TEs include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) that represent the active retrotransposons of the human "mobilome" [55–57]. LINE-1-derived peptides are significant tumor-specific antigens as they are detected in half of human tumors, while remaining undetectable in most normal tissues [58].

6. Viral Mimicry, the Adjuvancy Effect, and the Recruitment of Immune Cells

TEs can generate diverse molecular patterns recognized as pathogen-associated molecular patterns (PAMPs) by innate immune receptors upon epigenetic reactivation [45,59]. Their activation generates double-stranded RNA (dsRNA) and/or DNA (dsDNA). ERVs contain long terminal repeats (LTRs) that, when activated, act as promoters for transcription and in some cases, ERV loci are transcribed in both directions (sense and antisense) simultaneously, producing complementary sequences that allow the formation of double-stranded nucleic acids. [45,46]. In addition, due to the special structure of ERV transcripts, loops of RNA can form through intra-molecular pairing [60]. Finally, reverse transcription can result in cytoplasmic RNA-DNA hybrids. The presence of dsRNA and/or dsDNA results in the aberrant increase in cytosolic genetic material [61,62] recognized by cytoplasmic and endosomal sensors adapted through evolution to detect viral infections [63,64]. Toll-like receptors (TLR) such as TLR3, cytosolic sensors such as melanoma differentiation-associated gene 5 (MDA5), and retinoic acid inducible gene-I (RIG-I) belonging to the RIG-I-like receptors (RLRs) [65] are dsRNA-sensing surveillance systems. Sterile alpha motif domain containing 9 (SAMD9) [66] is a pattern-recognition receptor (PRR) that senses both cytosolic dsDNA and dsRNA, whereas inflammation-related gene absent in melanoma 2 (AIM2) [67] and cyclic GMP-AMP synthase (cGAS) [68] are dsDNA-sensors [67,69–71].

Endogenous dsRNAs can be derived from all type I TEs, i.e., LTR, LINEs, and SINEs, [45,61,72], whereas LINEs can also reverse transcribe complementary DNA (cDNA) [73]. In particular, dsRNAs are recognized by RLRs that in turn interact with the outer membrane mitochondrial adaptor mitochondrial antiviral-signalling protein (MAVS) [74] through its caspase recruitment domain (CARD) [75,76] adaptor. The homotypic interaction between similar CARD domains of RIG-I or MDA5 with MAVS CARD promotes the recruitment of other MAVS on the outer mitochondrial membrane. MAVS-CARD aggregation stimulates the prion-like conversion of MAVS into larger functional assemblies [77] responsible for the amplification of RLR signalling, downstream IFN signalling, and cytokine production through interferon regulatory factor (IRF) 3, IRF7, and NF-κB [78].

The cGAS-STING pathway is the major dsDNA sensor. The cytosolic detection of DNA (nuclear, mitochondrial, or viral) activates cGAS-STING. cGAS is a predominantly cytoplasmic protein that can also be found in the nucleus and the plasma membrane, a localization that facilitates DNA sensing [79–81]. Dimerized cGAS binds along the entire length of dsDNA to generate a ladder network that allows its activation only when it senses long stretches of dsDNA, requiring a minimum of 18 base pairs [82]. cGAS exploits the bilobal fold of the nucleotidyltransferase in the C-terminal domain to house a conserved zinc-ion-binding module that mediates DNA binding and cGAS dimerization. The cleft between the bilobal structure binds ATP and GTP to synthesize cGAMP [83]. Upon binding to cGAMP, STING undergoes conformational changes, oligomerizes [84], and translocates to the Golgi apparatus to become active. In the Golgi, STING recruits TBK1, generating a docking site for IRF3 [85].

Subsequent phosphorylation of IRF3 induces the expression of type I and type III IFNs [75,86] and other IFN-stimulated genes (ISGs), including chemokines such as CXCL10, inflammatory cytokines and type I IFNs [87].

Another contributor to viral mimicry is mitochondrial DNA (mtDNA) recognized by the cell as a DAMP when released into the cytosol. Mitochondrial oxidative stress or apoptotic cell death [88] cause the release of mtDNA to be recognized by cytoplasmic cGAS, activating the cGAS-STING pathway [89] and stimulating type I IFN production [90,91].

The interaction of double stranded nucleic acids with their respective receptors triggers the expression of type I and III IFNs [62–64,66,68,92–96], which draws immune effector cells to the TME to initiate the anticancer immune response [22]. This is why cancer-specific modulation of REs may offer new therapeutic promises, predominantly contributing co-stimulation (signal-2) and the production of homeostatic cytokines (signal-3) [19–21,33,97,98]. Indeed, IFNs released by cancer cells functions through autocrine and paracrine signalling by binding to IFN receptors and triggering phosphorylation of signal transducer and activator of transcription (STAT) [99]. Subsequently, conserved Tyr/Ser residues of STATs are phosphorylated and the pSTAT is released in the cytosol promoting the homo- and hetero-dimerization of phosphorylated STATs [99,100] that interact with IRF9 to form a new complex named IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates into the nucleus and binds the IFN-stimulated regulatory elements (ISREs), leading to increased transcription of ISGs [99,101]. The resulting presence of proinflammatory cytokines and chemokines amplifies the immune response, facilitating macrophage activation and neutrophil recruitment [22]. Among them, CXCL chemokines and C-C chemokine ligand (CCL) 2, CCL5 and CCL8 are powerful chemoattractant of monocytes, dendritic cells, natural killer cells and T cells, resulting (among other effects) in the secretion of interleukin (IL)-12 and IL-15, which have powerful homeostatic and differentiating effects on resident T cells [102]. Moreover, the secreted form of ubiquitin ISG15 has multiple modulating functions in the TME [103]. The constitutive activation of ISG is responsible primarily for determining the TME phenotype, with cold tumors completely lacking the viral mimicry effect.

In summary, viral mimicry represents the true alarm signal that brings immune cells to the TME and provides the substrate for cancer antigen-specific recognition by T cells (signal 1).

From a therapeutic perspective, agents already are available that can trigger viral mimicry through known mechanisms and can be leveraged for therapeutic benefit. Examples include TLR ligands such as polyinosinic:polycytidylic acid (Poly(I:C)), a double stranded synthetic RNA ligand for TLR3 [104]; lipopolysaccharide (LPS), a TLR4 agonist [105], imiquimod, a small molecule agonist of TLR7 and TLR8 [106]; motolimod, a TLR8 agonist [107]; and CpG oligodeoxynuleotides (CpG ODNs), synthetic single stranded DNA with unmethylated CpG motifs that agonize TLR9 [108].

Similarly, cGAS-STING pathway activation through agents, such as manganese (MN2+), which directly agonizes cGAS [109]; 2′3′-cGAMP, a natural STING agonist [110]; and many small molecule STING agonists [111] can modulate the cGAS-STING pathway to increase production of IFN-I/III with potential therapeutic effect. Additionally, epigenetic modulators that derepress transposable elements can lead to generation of cytosolic dsDNA or dsRNA and activate the MAVS or STING pathway. These agents include DNA methyltransferase (DNMT) inhibitors such as Decitabine (5-AZA-CdR) [46] and HDAC inhibitors such as Vorinostat, which when used in combination, have been shown to induce viral mimicry in cancer [112].

Such therapies and novel approaches are likely to have a broad effect across different cancer types because viral mimicry itself results from a common biological dysregulation specific for cancer biology [21,64]. Therefore, viral mimicry offers promising prospects for therapeutic application across most solid tumors, including but not limited to, non-small cell lung cancer and ovarian [92], breast cancer [113], and colorectal cancers [46].

7. Cancer Dark Matter and the Expansion of the Immunopeptidome to Promote Signal 1

Cancer dark matter refers to a newly discovered level of transcriptional, translational or post-translational activity not related to DNA mutations rather dependent upon epigenetic alterations or other derangements of cancer cell biology [38,40]. These derangements result in the aberrant production of peptide sequences not expressed by normal tissues called, as previously mentioned, noncanonical epitopes [25]. In contrast with canonical neoepitopes, noncanonical neoepitopes are widely expressed across cancer types, because they depend upon communal cancer-driving processes and are not restricted to patient-specific mutations [25,33–40]. While canonical neoepitopes derive from exonic nonsynonymous single nucleotide cancer-specific mutations or short insertion/deletions, noncanonical neoepitopes arise from genomic elements, such as translation of non-coding RNAs, intron retention, out-of-frame translation, alternative translational initiation, gene fusions, mRNA mis-splicing, and ERV activation [40,114,115]. In particular, translation from start sites other than canonical start codons can produce derivatives from lncRNAs, circRNA, antisense RNAs and pseudogenes [116,117].

Once considered biologically irrelevant, noncanonical peptide sequences have been shown to be recognized by T cells and dominate the immune repertoire of the TME [33,118]. Proteogenomic approaches aimed at high-throughput discovery of tumor antigens recognized by T cells suggests that up to 90% of them were derived from noncoding regions that would have been missed by standard exome-based technologies [119–121]. These neoepitopes offer new opportunities since they are recognized as foreign by the patient's immune system and they are widely expressed among cancers [120], constituting a significant area of ongoing development [122] and providing optimal targets for immunotherapy [123].

7.1. ERV and Transposable Elements-Derived Dark Matter

Several epitopes derived from ERV reactivation have been shown to be presented with high efficiency, to elicit strong immune responses [124,125] and to improve survival of patients receiving checkpoint inhibitor therapy [126]. Among TE-derived non canonical neoepitopes transcribed, ultra-conserved regions (T-UCRs) and non-coding open reading frames (ncORFs) deserve further mention.

7.2. T-UCRs

T-UCRs are transcribed long non-coding RNAs (lncRNAs) of 200 nucleotides or more, consisting of totally conserved sequences across the human, mouse and rat genomes. T-UCRs are ubiquitously transcribed, but in normal tissues this does not result in translated peptide products. Although their tissue- and disease-specific expression is frequent in benign tissues, they are mostly located in unstable genomic sites and become easily dysregulated during the neoplastic process, being differentially expressed and translated into peptide products in cancer cells [127]. While T-UCRs in normal cells regulate gene expression by inhibiting or activating signaling pathways that influence the cell cycle and apoptosis [128], in cancer, they are dysregulated, causing disruption of cellular homeostasis [129] and affecting the survival and migration of tumor cells [130], cell proliferation, resistance to therapy, and other oncogenic processes [131,132].

7.3. ncORFs

ncORFs are translated genomic regions that do not follow the conventional behavior of coding genes and erratically produce noncanonical proteins or peptides specifically in tumor tissues [120,133]. They can be in various positions, including (1) upstream of canonical coding sequences (nORFs), (2) within the 5′ or 3′ untranslated regions (UTRs) of mRNAs, (3) in lncRNAs and (4) in circular RNAs (circRNAs) modulating cellular responses to stress, influencing resistance to chemotherapeutic agents [134], and regulating angiogenesis and cell cycle [36].

Their role in shaping cancer cell biology, as well as their importance as non-canonical neoepitopes in inducing cancer-specific T cell activation, are well established. Still, much needs to be done to better understand their biology. For instance, how many novel protein-coding regions exist in the human genome and what fraction of them are functionally important remains unclear. Several factors contribute to the difficulty of annotating these noncanonical peptides. First, their typically short length makes detection challenging. Second, ncORF translation often follows unconventional mechanisms, sometimes initiating from alternative start codons. Identifying peptide-coding ncORFs requires a combination of computational predictions, ribosome profiling, and mass spectrometry; therefore, this is likely only the beginning and more discovery is still ahead [133].

Non-genomic sources of noncanonical neoepitopes include those derived from cancer-specific abnormal RNA editing, aberrant mRNA production, alternative splicing, and post-translational modification.

7.4. RNA Editing and Aberrant mRNA

RNA editing is often dysregulated in cancer, although the degree in which it occurs in normal cells needs to be better documented [135]. It mostly occurs as a post-transcriptional exchange of nucleotides by deaminases acting on RNA. The most common is the adenosine-to-inosine conversion, resulting in altered amino acid sequences [136,137]. Their immunologic relevance is reflected by the presence of T cells recognizing such epitopes in the TME of multiple cancer types [138,139]. Moreover, scarcity of specific amino acids may result in ribosomal stalling and, consequently, translational frameshift-derived neoepitopes, a phenomenon that seems to be limited to the neoplastic process [140,141]. Due to the circumstantial cause of their production depending on specific amino acid depletion, it needs to be clarified how consistent the expression of these potential neoepitopes may be in a specific tumor and, therefore, their clinical relevance.

7.5. Alternative Splicing

Alternative splicing, also referred to as neo-junction, is quite common in most cancers, resulting from dysregulated control of the splicing programs that shape most cellular processes [142,143]. This can produce novel epitope sequences amenable to antigen presentation by cancer cells and can induce strong immune responses with the expansion of cognate tumor infiltrating lymphocytes [144].

7.6. Post-Translational Modifications

Cancer-specific post-translationally modified peptides are relatively uncommon and may be of limited significance for therapeutic purposes. Nevertheless, examples of cancer-specific phosphorylated peptides recognized by T cells have been described [145], as well as recognition of peptides resulting from proteasome catalyzed splicing [146].

8. Converging into the Perfect Storm

We propose here that effective anti-cancer immune responses require the convergence of several fundamental vectors into a meaningful and therapeutically impactful result (Figure 1). First innate and subsequently adaptive immune cells need to be recruited to the tumor site. Second, adaptive immune cells need to recognize specifically cancer cells though direct interaction with the cancer-specific antigen (signal-1). Finally, the activation initiated by T cell -receptor antigen presentation complex needs to be boosted by co-stimulatory cell-to-cell interaction (signals-2) and sustained by the production of homeopathic cytokines (signal-3) [22] (Figure 1).

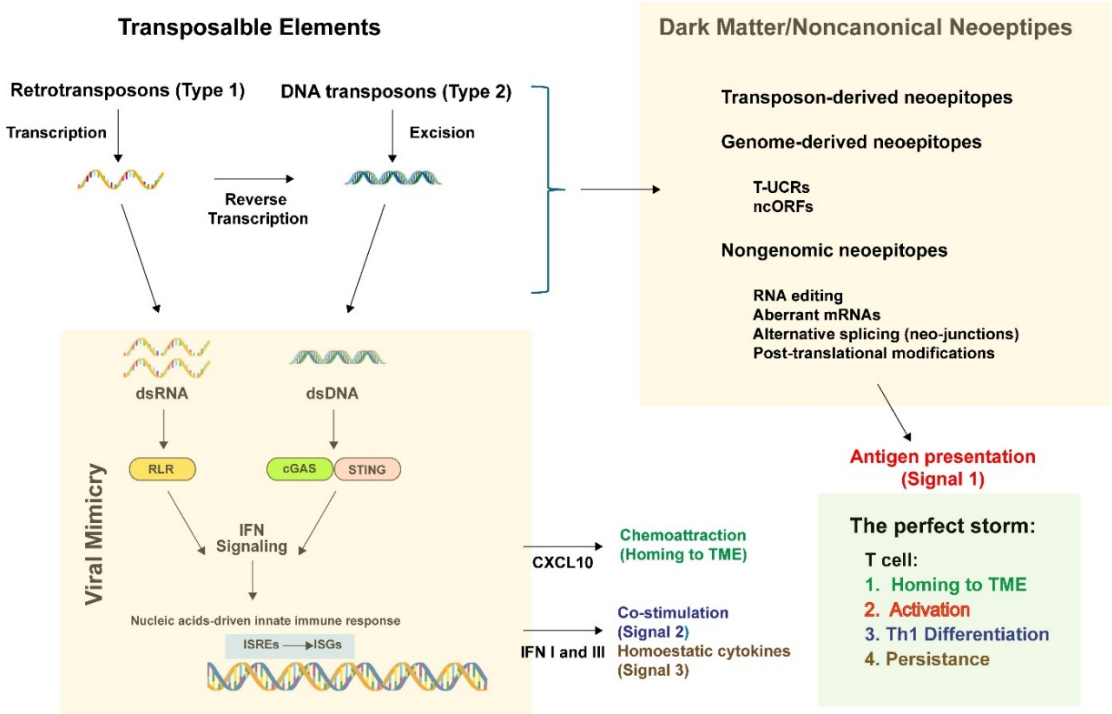

Figure 1. Building the perfect anti-cancer immunotherapy storm. Several factors need to converge to initiate and complete the anti-cancer immune response. First T cells need to be recruited within the TM. Second, the need to be activated by the interaction with the tumor-specific antigens, whether expressed by cancer cells or by professional antigen presenting cells (signal-1). Third, their activation needs to be boosted and directed toward an effector Th1 polarization (signal-2). Finally, the need to persist in the TME and maintain the correct level of differentiation (signal-3). Viral mimicry depending upon the excess production of dsRNA or dsDNA by the reactivation of TE either type I (retrotransposons) or type II (DNA transposons) activates the IFN cascade either with dsRNA interaction with RLRs or dsDNA interaction with cGAS/STING. This leads to the activation of ISGs which have both chemo-attractive properties (CXCL10) as well as pro-inflammatory properties with the production of type I and III IFNs and other soluble factors that can induced activation of co-stimulatory signals and the production of homeostatic cytokines by resident innate immunity cells such as macrophages polarized toward and M1 phenotype and mature dendritic cells. On the other hand, cancer dark matter, whether derived from normally silent but reactivated in cancer genomic regions like TEs, T-UCRs, and ncORF or derived from non-genomic processes such as RNA editing, aberrant mRNA, alternative RNA splicing (neo-junctions) or post-translational modification, provide an array of noncanonical epitopes that are strong inducers of signal-1. When all these factors converge efficiently the perfect storm is created and cancer is rejected.

Migration of T cells to normal peripheral tissues is not normal and the definition itself of inflammation is presence of them in an affected organ. The recruitment of the immune network that successfully can eliminate pathogens during acute infections is the key initiator of anti-cancer immune responses. Analysis of localization of ^{111}Indium-labeled tumor infiltrating lymphocytes (TILs) adoptively transferred into patient with metastatic melanoma demonstrated that in a significant number of cases they did not reach the tumor sites; subsequently, none of these patients responded to therapy. Localization of TILs to the TME in the other patients was associated with clinical regression [147]. This is a good example of the importance of chemoattraction of T cells to the tumor for effective anti-cancer immune responses [147]. This is probably why immune active tumors, whether hot or excluded, get off to a good start and are more likely to respond to immunotherapy than cold tumors.

In nature, viral infections induce chemoattraction and immune activation. It is sensible to expect that the same mechanism is responsible for tumor recognition. We propose, therefore that viral mimicry is responsible for attracting the immune multicellular network that predisposes cancers to be rejected [22]. The chemoattraction of T cells must be complemented by a conducive TME prepared to sustain their activation, persistence, and differentiation into an effector Th1 phenotype through the activation of antigen presenting cells and other immune regulatory cells that can provide signal-2 and signal-3 to boost the interaction of T cells with the cognate cancer-specific antigen (signal-1).

The importance of the adjuvancy effect provided by viral mimicry to boost direct antigen stimulation of T cells has been shown recently by Blass et al., who combined Montanide, poly(I:C) (which is a synthetic analog of dsRNA), local low-dose ipilimumab, and systemic nivolumab with a cancer vaccine containing 12–20 peptides. This combination demonstrated improved immunogenic effects boosting T cell responses [148].

In summary, the combination of strong dark matter antigenicity and viral mimicry adjuvancy is the secret recipe that conditions the TME immunophenotype towards the threshold of immune activation required for clearance of pathogen-infected cells and similarly cancer cells. It is only then that the perfect storm is created and the ultimate goal of cancer rejection is achieved.

Author Contributions

All authors contribute equally in the conception and writing of this article. All authors have read and agreed to the published version of the manuscript.

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Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

Abbreviations

AIM2, inflammation-related gene absent in melanoma 2; **5-AZA-CdR**, Decitabine; **CARD**, amino-terminal caspase recruitment domain; **CCL**, C-C chemokine ligand; **cDNA**, complementary DNA; **cGAS**, cyclic GAM-AMP synthetase; **circRNA**, circular RNA; **CpG ODN**, CpG oligodeoxynuleotide; **CXCL**, C-X-C motif chemokine ligand; DNMT (DNA methyltransferase); **dsDNA**, double-stranded DNA; **dsRNA**, double-stranded RNA; **ERV**, endogenous retrovirus; **ICR**, immunologic constant or rejection; **IFN**, interferon; **IL**, interleukin; **IRF**, interferon regulatory factor; **ISG**, interferon-stimulated gene; **ISGF3**, IFN-stimulated gene factor 3; **LINE**,

long interspersed nuclear element; **lncRNA**, long non-coding RNA; **LPS**, lipopolysaccharide; **LTR**, long terminal repeat; **MAVS**, mitochondrial antiviral-signaling protein; **MDA5**, melanoma differentiation-associated gene 5; **mtDNA**, mitochondrial DNA; **ncORF**, noncanonical open reading frame; **NLRP12**, nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 12; **PAMP**, pathogen-associated molecular pattern; **Poly(I:C)**, polyinosinic:polycytidylic acid; **PRR**, pattern recognition receptor; **RE**, repetitive elements; **RIG-I**, retinoic acid inducible gene-I; **RLR**, RIG-like receptor; **SAMD9**, sterile alpha motif domain containing 9; **SINE**, short interspersed nuclear element; **STAT**, signal transducer and activator of transduction; **TCGA**, The Cancer Genome Atlas; **TE**, transposable element; **TIL**, tumor infiltrating lymphocyte; **TLR**, toll-like receptor, **TME**, tumor micro-environment; **T-UCR**, transcribed ultra-conserved region; **uORF**, upstream or canonical coding sequences; **UTR**, 5′ or 3′ untranslated regions of mRNAs.

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