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Review

Parallels Between the Antiviral State and the Irradiated State

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Abstract

Improved understanding of host antiviral defense and antitumor immunity have elucidated molecular pathways important to both processes. During viral infection, RNA or DNA in the host cell serves as a danger signal that initiates the antiviral response. Recent studies have elucidated similarities in the signaling pathways activated by viruses and the signaling pathways induced by tumor DNA that is released into the cytoplasm of irradiated tumor cells. Both the host defense to viral infection and the sterile inflammation provoked by radiotherapy induce a type I interferon response that is necessary for pathogen control and immune-mediated tumor control, respectively. These findings have led to the hypothesis that radiotherapy employs a form of viral mimicry.

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The immunobiology of chronic viral infection and cancer are closely intertwined, and our mechanistic understanding of virology and host antiviral immunity has substantially contributed to the field of oncology. To this end, programmed cell death protein-1 (PD-1) was initially described as a marker of exhaustion on $CD8^+$ T cells during chronic viral infection and subsequently found to play a fundamental role in tumor immunology. Since the original discovery of PD-1 in the context of viral immunology, therapeutic antibodies targeting PD-1 and its ligands have revolutionized the field of oncology.

The molecular pathways that activate innate immunity and lead to antigen-specific T-cell responses against viral or tumor antigens also share considerable overlap. The presence of viral RNA and DNA nucleic acid species function as pathogen-associated molecular patterns (PAMPs) that are sensed by the host. These signals initiate an antiviral response characterized by the induction of type I interferons (IFNs) and upregulation of interferon-stimulated genes (ISGs). Similarly, the presence of cytosolic tumor DNA resulting from radiation-induced DNA damage can function as a damage-associated molecular pattern, which triggers cellular mechanisms similar to those elicited by viral PAMPs. This review will discuss the convergence of these signaling pathways to highlight connections between the antiviral state and the irradiated state. By drawing parallels between the immune response to viral infection and the antitumor immune response to radiation, we aim to bridge a conceptual gap between the fields of virology, cancer immunology, and radiation oncology.

Parallels Between Chronic Viral Infection and Cancer

The immune system evolved both innate and adaptive arms to eliminate pathogens and subsequently generate long-term protective immunity against a specific pathogen. This protective immunity allows the host to generate a more efficient and expedient memory response when reencountering the same pathogen. The innate immune system discriminates self (host) and non-self (pathogen) by using pattern recognition receptors (PRRs) that sense and bind conserved elements of bacteria, viruses, fungi, and protozoan parasites. Collectively, these conserved elements are termed PAMPs and include proteins and lectins expressed by pathogens, as well as DNA and RNA in the cytoplasm or endosomes during viral replication (1,2). PAMPs bind cytosolic or membrane-bound PRRs on antigen-presenting cells (APCs) such as dendritic cells (DCs) or macrophages, leading to downstream transcriptional changes that upregulate

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major histocompatibility complex (MHC) molecules and antigen-processing machinery, as well as cytokines and chemokines. APCs load antigen onto MHC molecules and migrate to the draining lymph nodes where they prime antigen-specific T cells.

The initial goal of the immune response is to clear the pathogen and resolve acute infection. In acute viral infection, viral clearance involves the eradication of infected cells by activated $CD8^+$ cytotoxic T lymphocytes (CTLs) and the eventual generation of virus-specific antibodies (3). CTLs engage death receptors on virally infected target cells via the interaction between Fas and Fas ligand. They also secrete cytotoxic granules that contain pore-forming perforins and granzymes which mediate apoptosis. In addition, there is evidence that a small number of viral antigen-specific T cells secrete cytokines that interfere with viral replication without direct lysis of infected cells. For example, hepatitis B virus (HBV)-specific $CD8^+$ T cells secrete IFN gamma (IFN-γ) and tumor necrosis factor α (TNF-α) to eliminate HBV nucleocapsid particles and destabilize viral RNA with the goal of interfering with viral replication (4).

During viral infection, PD-1 is upregulated as an early activation marker. However, repeated engagement of PD-1 can lead to T-cell exhaustion when $CD8^+$ T cells are continuously exposed to viral antigen (5). The expression of immune-inhibitory checkpoint molecules with modulation of hallmark transcriptional programs and epigenetic modifications serves as a marker for exhausted $CD8^+$ T cells that are no longer able to perform cytotoxic effector functions. Since chronic antigen stimulation drives a state of T-cell dysfunction, there is a positive correlation between viral load and level of T-cell exhaustion (6). Antigens present at lower levels *in vivo* induce functional exhaustion of T cells, whereas antigens present at higher levels may lead to the deletion of T cells (7).

In cancer immunology, tumor-associated antigens or tumor-specific neoantigens (generated via nonsynonymous somatic mutation) can prime antigen-specific $CD8^+$ T-cell responses. Malignant tumors that have escaped immune surveillance are also a chronic source of antigen and thus can induce a state of T-cell exhaustion akin to chronic viral infection. The coexistence of mixed clinical responses, where simultaneous immune-mediated tumor regression and disease progression occur, emphasizes the complexity and dynamic interplay between host immunity and tumor evolution. The evasion mechanisms co-opted in chronic viral infection have provided valuable insight for understanding T-cell dysfunction in cancer as well as the transcriptional and epigenetic programs in T cells that allow them to persist in this hypofunctional state. The reversal of T-cell exhaustion via PD-1 axis blockade to reinvigorate antitumor immunity has been a transformative advance in cancer immunotherapy.

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Innate Sensing of Viral Infection and Radiotherapy

Viral Replication Co-Opts Host Replication Machinery

When a virus enters a host cell, the virus replicates and generates the components necessary for progeny viruses to assemble. Viruses are intrinsically parasitic to the host and are dependent on host cell machinery for viral replication. Once assembled, progeny viruses exit the host cell and infect nearby cells.

The mode of viral replication is generally dependent on the genetic material packaged by the infective virus. The viral genome ranges from single-stranded (ss) RNA or DNA to double-stranded (ds) RNA or DNA, and ssRNA viruses can be further subclassified into positive- or negative-sense RNA viruses. These differences in viral genome are relevant because they engage the host antiviral response through different signaling pathways. Radiotherapy predominantly induces genotoxic stress via dsDNA breaks and subsequent cytosolic micronuclei rupture. The presence of these nucleic acid species in the cytoplasm after radiation activates an immune response similar to the antiviral host response to dsDNA viruses.

Viral dsDNA integrates into the host genome in the cytoplasm or the nucleus. In the nucleus, many dsDNA viruses such as HBV require host cell polymerases to replicate, whereas others, such as adenoviruses or herpesviruses, encode their own polymerases (8). Replication of dsDNA viral genomes requires that cells are in a state that allows DNA replication and therefore this replication is dependent on the cell cycle. In certain contexts, viruses integrate into the host genome and initiate aberrant cell division that can lead to malignant transformation due to dysregulation of host cell–intrinsic division and growth. An example of the oncogenic nature of viral infection is human papillomavirus (HPV), which has a dsDNA genome and is associated with virally induced cancers of the head and neck, cervix, and anogenital region.

In contrast to DNA viruses, which generally replicate within the nucleus, RNA viruses replicate in the cytosol. Because the presence of dsRNA in the cytoplasm triggers host defense mechanisms, dsRNA viruses encapsulate their genome in a core particle or capsid. In addition, dsRNA viruses do not require host polymerases to replicate because these capsids contain all of the enzymes required for replication. Whereas replication of many DNA viruses is dependent on the phase of the cell cycle, many RNA viruses replicate independently of the cell cycle (9).

Recognition of Nucleic Acids by Pattern Recognition Receptors and the Inflammasome

PRRs are able to recognize certain DNA- or RNA-based nucleic species that are located outside their normal subcellular locations. PRRs include toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)–like receptors (NLRs), and retinoic acid-inducible gene I (RIG-I)–like receptors (RLRs) that respond to PAMPs located within different subcellular compartments, and there is considerable crosstalk between these innate viral sensing pathways (Figure 1).

TLRs are transmembrane proteins located on the plasma membrane or within endosomes. The TLR family consists of 13 different transmembrane proteins that recognize conserved patterns of microbial components, or PAMPs, including nucleic acids, bacterial glycolipid lipopolysaccharides, and peptidoglycans (10-12). The unique subcellular localization of TLRs allows them to sense different pathogens with unique routes of entry and modes of replication. TLR2 and TLR4 are located on the cell surface where they bind to lipopolysaccharide, and TLR3, TLR7, TLR8, and TLR9 are located inside endosomes where they bind to viruses or nucleic acids that have been endocytosed or phagocytosed. PAMP recognition by TLRs can activate the Myeloid differentiation primary response 88 (MyD88)-dependent pathway or the TRIF (Toll/Interleukin-1 receptor domain containing adapter-inducing interferon-β)–dependent

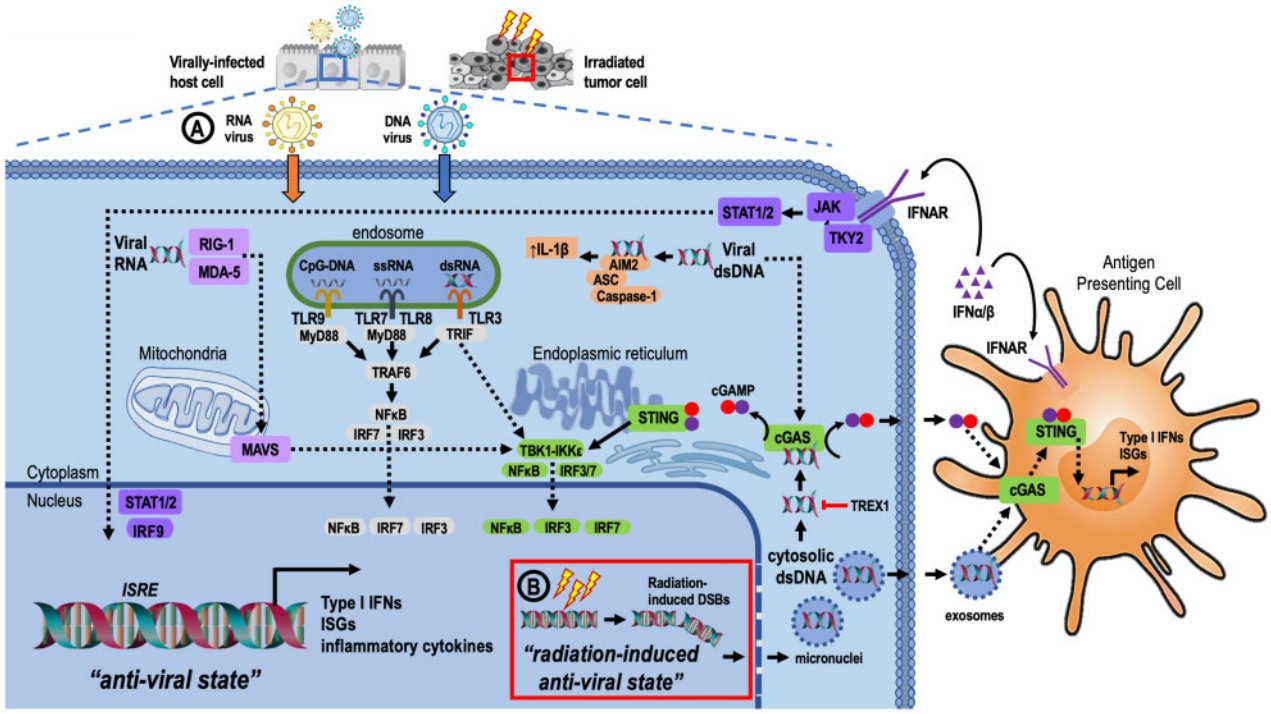

Figure 1. Innate viral sensing and cellular response to viral infection and host response to radiation-induced inflammation. **A)** The presence of viral nucleic acids within different cellular compartments activates PRRs. RIG-I and MDA-5 are cytosolic PRRs that sense viral dsRNA in association with the MAVS complex that interacts with TBK1-IKKε. IRF3 and IRF7 are phosphorylated and translocate to the nucleus to initiate transcription of type I IFNs and ISGs. A subset of endosomal TLRs detect nucleic acid species. TLR9 senses unmethylated CpG-DNA, and TLR7 and TLR8 recognize ssRNA. TLR7, 8, and 9 signal through a pathway dependent on the adapter, MyD88. TLR3 senses dsRNA and triggers the TRIF-dependent pathway. Both MyD88 and TRIF-dependent signaling converge to activate TRAF6, whereas TRIF-dependent signaling also involves TBK1-IKKε. Collectively, endosomal TLR signaling leads to Nuclear factor kappa B (NFκB) activation and transcriptional activation of type I IFN via IRF7 and IRF3. Cytosolic dsDNA can activate the AIM2 inflammasome to induce pro-inflammatory cytokines and caspase-1–mediated pyroptosis. Cytosolic viral dsDNA is also recognized by cGAS, which catalyzes production of cGAMP. cGAMP activates the endoplasmic reticulum-localized adapter, STING, causing STING to dimerize and translocate to nucleus where it activates TBK1, leading to IRF3 phosphorylation and nuclear translocation. NF-κB signaling is also activated downstream of STING via IKKε. **B)** Radiation-induced DNA damage results in double-stranded DNA breaks (DSBs) and micronuclei carrying fragmented dsDNA rupture in the cytoplasm, triggering cGAS-STING signaling and a type I IFN response. Exosomes loaded with radiation-induced dsDNA fragments as well as extracellular cGAMP activate the cGAS-STING pathway and type I IFN signaling. Type I IFNs bind cognate IFNAR complexes leading to phosphorylation of JAK1 and TYK2. This promotes the recruitment of STAT1 and STAT2 and IRF9 to form the ISGF3 complex, which translocates to the nucleus and binds ISREs, leading to transcription of ISGs. AIM2 = absent in melanoma 2; cGAMP = 2'3'-cyclic CMP-AMP; cGAS = cGAMP synthase; dsDNA = double-stranded DNA; dsRNA = double-stranded RNA; IFN = interferon; IFNAR = interferon-alpha/beta receptor; IKK = IκB kinase; IRF = interferon regulatory factor; ISG = interferon-stimulated genes; ISREs = interferon-stimulated response elements; JAK1 = Janus kinase 1; MAVS = mitochondrial antiviral signaling; MDA-5 = melanoma differentiation-associated protein 5; PRR = pattern recognition receptor; RIG-I = retinoic acid-inducible gene-I; ssRNA = singe-stranded RNA; STAT = signal transducers and activators of transcription; STING = stimulator of interferon genes; TBK1 = TANK binding kinase 1; TLR = toll-like receptor; TRIF = TIR domian containing adapter-inducing interferon-β; TYK2 = tyrosine kinase 2.

pathway (13,14). For example, TLR9 senses CpG-DNA and signals through the MyD88 adaptor protein complex in conjunction with IRAK-4 (Interleukin-1 receptor associated kinase 4) to activate MAP (Mitogen activated protein)-kinase, NF-κB and interferon regulatory factor (IRF) signaling (15). These TLR-initiated signaling pathways ultimately induce the expression of downstream genes that are critical for the innate immune response.

RLRs are a family of PRRs that detect viral replication within the cytosol and/or the nucleus and include RIG-I (retinoic acid-inducible gene I) and melanoma differentiation–associated protein 5 (2). RLR recognition of RNA in the cytosol leads to the aggregation of the mitochondrial antiviral signaling (MAVS) and activation of TANK-binding kinase 1 IκB kinase-ε (TBK1-IKKε), which activates IRF3, IRF7, and NF-κB.

NLRs are cytosolic receptors that enter the cell via phagocytosis or membrane pores to form protein complexes called inflammasomes, which induce caspase-1–mediated activation of pro-IL-1β. The NLR family of inflammasomes includes NOD (nucleotide-binding oligomerization) and NLRPs (NOD-like leucine rich repeat and pyrin domains) that recognize bacterial, fungal, and parasitic PAMPs, as well as the AIM2 (absent in melanoma 2) receptor that detects bacterial and viral DNA. The NLRP3 inflammasome is activated by efflux of K^+ from damaged cells, and the AIM2 inflammasome is activated by cytosolic DNA (16). Specifically, cytosolic dsDNA binding to AIM2 results in recruitment of an adaptor protein, ASC, apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain), which recruits and activates caspase-1 to generate the AIM2 inflammasome.

Innate immune activation by the cytosolic DNA-sensing, cyclic GMP-AMP (cyclic guanosine monophosphate-adenosine monophosphate) synthase/stimulator of interferon genes (cGAS-STING) pathway has been a subject of recent investigation. During infection, cGAS senses viral dsDNA and catalyzes the generation of cGAMP (17). cGAMP then binds STING dimers in the endoplasmic reticulum, where a conformational change allows for binding and activation of TBK1-IKKε. Akin to RLR signaling, cGAS-STING signaling results in the activation of IRF3/NF-κB and type I IFN production. Because IFN-α and -β signaling

is important for activation and maturation of DCs, which are required for effective priming of $CD8^+$ T cells (18), the activation of the cGAS-STING IFN-β pathway serves as a critical bridge between innate and adaptive immunity.

Many pathogens have evolved mechanisms to escape host recognition of foreign nucleic acids. For example, cytomegalovirus (CMV) inhibits DNA sensing by cGAS to abrogate the antiviral immune response (19). Some organisms, such as bats, have adapted to chronic viral infection by self-limiting their ability to mount a host antiviral response via downregulation of cGAS-STING signaling (20).

Innate Viral Sensing as a Framework to Understand the Host Response to Radiotherapy

In response to radiotherapy, tumor cells release nucleic acids into the cytoplasm to activate similar innate signaling pathways as activated by viral infection (13). Radiation generates DNA damage, produces dsDNA breaks, and disrupts the nuclear envelope, allowing small DNA fragments to leak into the cytoplasm. Further, radiation can induce chromosomal aberrations and the formation of abnormal DNA structures such as micronuclei (21). Radiation can also de-repress certain noncoding RNAs that have viral origins, including endogenous retroviruses or retroelements, which can bind to cytoplasmic PRRs, including RIG-I. Of note, radiation also induces molecular hallmarks of immunogenic cell death, which serve as damage-associated molecular patterns, including translocation of calreticulin from the endoplasmic reticulum to cell surface, extracellular release of high mobility group box 1 (HMGB1), and extracellular secretion of ATP (22).

Seminal work by Deng et al. (23) has demonstrated that radiation-mediated antitumor immunity requires cGAS-STING to sense cytosolic DNA and induce IFN-β. Exogenous IFN-β treatment is sufficient to rescue a defect in cross-priming DCs observed in $Tmem173^{-/-}$ mice (23), suggesting that the engulfment of irradiated tumor cells in the context of immunogenic cell death can activate $Baft3^+$ DCs via IFN-β (24). $Batf3^+$ conventional type I DCs (cDC1) play a key role in cross-presentation of tumor-derived antigens, leading to direct priming of $CD8^+$ T cells (18).

Although modulation of cGAS-STING pathway appears to be a promising therapeutic avenue to boost antitumor immunity (25), it is important to remember that this conserved biology largely evolved as a form of antiviral host defense and is exquisitely complex. Initial efforts utilizing STING agonists for tumor control were underwhelming, partly because natural cyclic dinucleotide ligands are metabolically unstable. Recently, multiple research groups have developed synthetic non-nucleotide STING agonists, including linked aminobenzimidazole-based compounds (26), MSA-2 (27), and a cGAMP (cyclic guanosine monophosphate-adenosine monophosphate) mimetic (28) to induce IFN-β and antitumor immunity. However, other evidence suggests that chronic activation of the cGAS-STING axis may yield deleterious effects on viral infection and tumor control. Bakhoum et al. (29) demonstrated that cancer cells with chromosomal instability co-opt cGAS-STING signaling to promote metastatic dissemination. Therefore, compensatory and/or regulatory mechanisms may have evolved to limit deleterious chronic cGAS-STING signaling and prevent excessive host tissue damage.

A relevant example is 3-prime repair exonuclease-1 (TREX1), which was initially discovered for its role in HIV-1 infection, because it degrades HIV-1 DNA to subvert recognition by PRRs and prevent type I IFN production (30). Preclinical work by Vanpouille-Box et al. (31) demonstrated that cytosolic DNA extruded into the cytoplasm following radiation is degraded by the DNA exonuclease activity of TREX1. Importantly, radiation induces *TREX1* expression in a dose-dependent manner to dampen cGAS-STING IFN-β signaling. This data has led to the hypothesis that higher fractional doses of radiation may be less immunogenic because of their ability to induce *TREX1* expression—a potential mechanism for dose-dependent activation of the immune response.

In addition, the complement system is a system of serum proteins that evolved 3 pathways: the classical pathway, the mannose-binding lectin pathway, and the alternative pathway. The system opsonizes pathogens, recruits effector cells, and forms a "membrane attack complex" to lyse infected cells. Because there is cross talk between TLRs and the complement system, the complement system has been implicated as a key system linking innate and adaptive immunity (32,33). Therefore, it is not surprising that many viruses (such as West Nile virus) have evolved mechanisms to evade or alter the complement system in order to interfere with host defense to viral infections (34-36). In addition, recent work has highlighted a role for components of the complement cascade in the radiation-induced immune response (37). Pro-inflammatory anaphylatoxins C3a and C5a play a role in radiotherapy-induced antitumor immunity, and various forms of radiation lead to an increase in serum levels of complement C1q and C3 (38). Just as the complement system plays a critical role in antiviral immunity, this work suggests that radiation-induced complement activation may boost antitumor immunity.

The Antiviral State

The antiviral state describes the collective changes induced in host cells resulting from activation of type I IFNs (IFN-α and -β) and type III IFN (IFN-λ). This review will focus on type I IFNs, which are secreted by a variety of cells in response to infection, including epithelial cells, natural killer cells (NK cells), B cells, T cells, macrophages, fibroblasts, and endothelial cells. All nucleated cells express the type I IFN receptor (IFNAR) complex, allowing IFN-α and -β to act on a broad range of infected cells. Binding of IFN-α and -β leads to dimerization of IFNAR1 and IFNAR2 subunits, and the resulting phosphorylation activates the receptor-associated kinases, tyrosine kinase 2 and Janus kinase 1 (JAK1). These kinases recruit STAT 1 (signal transducer and activator of transcription 1) and STAT 2. STAT homodimers and heterodimers translocate to the nucleus to initiate transcription of more than 300 ISGs. Some ISGs are nucleic acid-binding proteins that suppress viral replication, whereas other ISGs bind to PRRs to activate innate immunity.

Type I IFN signaling upregulates MHC class I (MHC-I) that is required for antigen presentation and T-cell–mediated elimination of virus-infected cells. Autocrine signaling loops involving IFN-β on DCs promote their activation and maturation, leading to upregulation of antigen presentation machinery and co-stimulatory molecules (CD80 and CD86) (39). Type I IFNs also directly promote effector functions of $CD8^+$ T cells and natural killer cells and modulate the hematopoietic compartment by promoting differentiation of bone marrow progenitors into monocytic DCs (40).

Whereas type I IFN signaling stimulates innate and adaptive immunity to promote the resolution of viral infection (41), various regulatory mechanisms determine whether pathogens are cleared or chronic infection develops (42). Just as viruses encode viral proteins that inhibit cGAS-STING signaling, other viral

proteins inhibit IFN-α/-β signaling, highlighting the evolutionary pressure exerted by type I IFN in the control of viral infection. Moreover, during lymphocytic choriomeningitis virus (LCMV) clone-13 infection, elevated type I IFN upregulates negative immune-regulatory molecules like PD-L1 and IL-10, promoting T-cell exhaustion and viral persistence (43,44).

Harnessing Adaptive Immunity and Overcoming T-Cell Exhaustion

Antigen Presentation Propels Adaptive Immunity

The foundation of adaptive immunity is the development of antigen-specific T-cell and B-cell responses. The uptake, processing, and presentation of antigens are the first steps required for antigen-specific immunity against a viral infection or an irradiated tumor (Figure 2). The intracellular antigen-presenting pathway predominates in virally infected cells. Viral proteins undergo proteasomal degradation in the cytosol, and these antigens are trafficked to the endoplasmic reticulum where they are loaded on MHC-I molecules. The newly formed MHC-I–antigen complex is presented on the cell surface and recognized by $CD8^+$ T cells with cognate T-cell receptors (TCRs). Alternatively, the exogenous (extracellular) pathway processes extracellular proteins that are internalized (via endocytosis or phagocytosis) by APCs. These peptides undergo processing within endosomes where they associate with MHC-II and are presented on the surface of APCs and recognized by $CD4^+$ T cells. Importantly, there is cross talk between these pathways in the form of cross-presentation where extracellularly derived antigens are initially processed via the exogenous pathway but subsequently loaded on MHC-I to generate $CD8^+$ T-cell responses. This distinction carries relevance because cross-presentation is an important mechanism by which radiation elicits adaptive immunity (45). This occurs through conventional dendritic cell 1 (cDC1) engulfment of irradiated dying tumor cells and processing of tumor-derived peptides onto MHC-I machinery (46).

Additionally, there is emerging data that radiation may drive a cellular program in tumor cells that enhances transcription and translation of mutant peptides, which are recognized as neoantigens (47). Because immunogenic tumor neoantigens derived from somatic mutations appear to be important targets of T-cell–directed immunotherapy, the ability of radiation to "expose" immunogenic neoantigens to the immune system may prove to be an important mechanism of radiation-mediated adaptive immunity (48,49).

Vaccination and Memory $CD8^+$ T-Cell Formation

The first successful vaccine against smallpox, developed by Dr Edward Jenner in 1796, was based on the demonstration that prior inoculation with cowpox could prevent infection upon

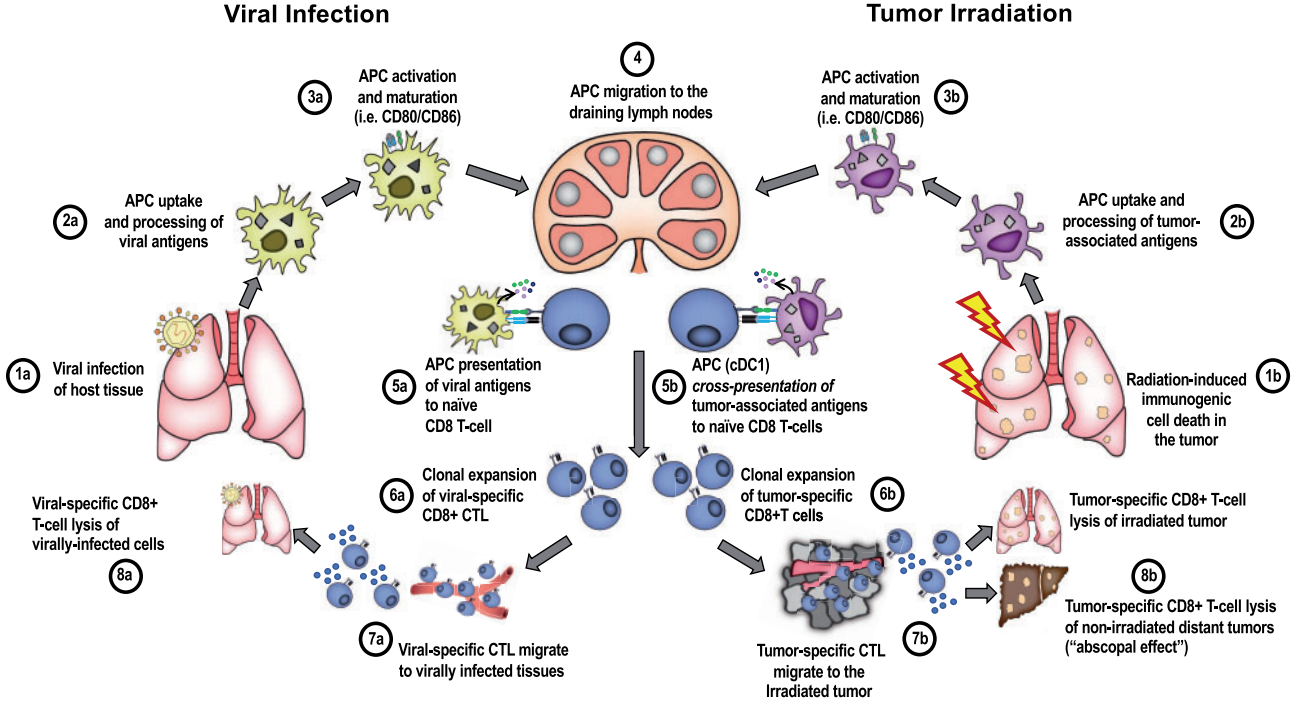

Figure 2. Parallels between the adaptive immune response to viral infection and radiation-driven antitumor immunity. **A)** In the context of viral infection, (**1a**) viral entry into host cells leads to detection of PAMPs by PRRs; (**2a**) IFN-α and -β production, which recruits APCs that engulf viral antigens and process via the MHC-I antigen presentation pathway; (**3a**) APC activation and maturation induces expression of costimulatory molecules (4). Activated APCs loaded with viral antigen migrate to draining lymph nodes where they (**5a**) prime naïve T cells via 3 signals: (**6a**) signal 1 (TCR-antigen–MHC-I), signal 2 (co-stimulation; CD28-CD80/CD86), and signal 3 (cytokines), which result in clonal expansion of viral-antigen–specific $CD8^+$ T cells, which then (**7a**) migrate to virally infected tissues. **8a**) Antigen-specific $CD8^+$ T cells lyse infected cells. **B)** In the context of radiation-induced antitumor immunity, (**1b**) tumor irradiation induces DNA damage and activates the cGAS-STING pathway leading to IFN-I signaling. (**2b**) cDC1 are recruited to the irradiated TME where they engulf and process antigen for cross-presentation on MHC-I. **3b**) DC activation, maturation, and (4) trafficking to tumor-associated draining lymph nodes. **5b**) DCs cross-present antigens to $CD8^+$ T cells via TCR binding to antigen–MHC-I. **6b**) Clonal expansion occurs as described in 6a. **7b**) Tumor antigen-specific effector $CD8^+$ T cells migrate to the irradiated tumor and (**8b**) infiltrate the irradiated TME where they recognize tumor cells expressing cognate antigen and lyse tumor cells. They also migrate to nonirradiated tumor sites and recognize shared epitopes that drive T-cell mediated tumor lysis. APC = antigen-presenting cell; cDC1 = conventional dendritic cell 1; cGAS-STING = cyclic GMP-AMP synthase; CTL = cytotoxic T lymphocyte; DC = dendritic cell; IFN-1 = type I interferon; ISG = interferon-stimulated gene; MHC-I = MHC class I; PAMPs = pathogen-associated molecular patterns; PRR = pattern recognition receptor; STING = stimulator of interferon genes; TCR = T-cell receptor; TME = tumor microenvironment;.

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subsequent challenge with smallpox. Ultimately, all vaccines are designed to educate the host immune system to confer antigen-specific immunity against a pathogen using an antigen and an adjuvant. Vaccines trigger host adaptive immunity by providing an antigen that cross-primes T cells via MHC-antigen binding to the TCR (signal 1), and an adjuvant that functions as a PAMP to activate PRRs and induce co-stimulation (signal 2) and pro-inflammatory cytokines (signal 3).

A hallmark of vaccination is the generation of long-lasting, protective immunologic memory. LCMV is a useful preclinical model to study memory T-cell responses because different strains can induce acute infection (Armstrong) or chronic infection (clone-13) *in vivo*, with similarities in memory T-cell formation observed in both viral infection models (50,51). Mechanistic studies on memory T-cell formation have illustrated that the T-cell response to viral infection is a highly orchestrated process defined by 3 phases: expansion, contraction, and memory formation (52,53). Seminal studies of adoptively transferred LCMV-specific, TCR-transgenic $CD8^+$ T cells have shown that a population of viral antigen-specific T cells initially undergoes massive clonal expansion in response to acute infection before migrating throughout the body to kill virally infected cells and eradicate systemic infection (Figure 2) (45,55). After the acute viral infection resolves, a contraction phase leads to rapid reduction in the number of virus-specific T cells, allowing the host to return to a more energetically efficient state. Following this contraction phase, a subpopulation of effector $CD8^+$ T cells (approximately 5%-10%) become memory T cells, which persist at low levels to defend against rechallenge by the same pathogen (3,48,56).

Memory $CD8^+$ T cells are a heterogeneous population that exists along a spectrum of differentiation states dictated by the integration of intrinsic transcriptional programs, epigenetic modulation, and environmental cues (58,59). There are 3 broad classes of memory T cells—effector memory (T_{EM}), central memory (T_{CM}), and tissue-resident memory (T_{RM}) cells—which serve complementary functions to protect the host from re-infection. T_{EM} circulate between tissues and secondary lymphoid organs to provide baseline effector function, and T_{CM} reside in secondary lymphoid organs and rapidly expand in the context of re-exposure to antigen. T_{RM} cells are located in peripheral tissues and provide rapid protection at sites of infection. Recent work has highlighted the growing complexity in defining various T_{RM} populations because differences in organ site and tissue specificity also shape T-cell fate (60). Further, using single-cell RNA sequencing, Milner et al. (61) dissected the T_{RM} landscape by identifying distinct subsets defined by B lymphocyte-induced maturation protein-1 and Id3 expression with contrasting effector-like and memory-like properties. Importantly, the distinct subsets of T_{RM} that were identified in response to LCMV infection were also observed in the tumor microenvironment. Jansen et al. (62) recently reported the presence of distinct tumor-infiltrating $CD8^+$ T-cell populations in human kidney tumors that recapitulate the terminally differentiated state or the CXCR5-enriched "stem-like state" that has been described in preclinical LCMV models. Taken together, these findings highlight the strong parallels between T cell biology in cancer and viral infection.

Evolving Understanding of T-Cell Exhaustion

Persistent antigenic stimulation drives functional impairment of $CD8^+$ T cells. This dysfunctional state of T-cell exhaustion is a dynamic process characterized by progressive loss of effector functions and increased expression of immune-inhibitory checkpoints (ie, PD-1, LAG-3, TIM3) in a hierarchical fashion. PD-1 is the prototypical immune-inhibitory receptor, and its role in T-cell exhaustion was originally established in the context of LCMV infection (63). Mice with chronic viral infection upregulate PD-1 on virus-specific T cells, and these PD-1^+ virus-specific T cells are functionally exhausted T cells (T_{EX}). T_{EX} demonstrate alterations in TCR and cytokine signaling, differential expression of genes involved in T-cell migration and homing, as well as distinct transcriptional and metabolic programs (64). Interestingly, blockade of PD-1 is able to restore T_{EX} function and promote viral clearance (65).

Over the past few decades, a working model of T-cell exhaustion has been continually refined in an effort to better characterize the different subsets of T_{EX} and their underlying biology. These T_{EX} subsets exist along a spectrum of differentiation states and display considerable plasticity. However, T_{EX} can cross a threshold into a state of terminal differentiation where exhaustion becomes irreversible (66). This underlies the rationale for PD-1 axis blockade in cancer and, to a lesser extent, chronic viral infection, since blocking the PD-1 axis can reverse T-cell exhaustion and restore T-cell effector functions before irreversible exhaustion occurs. Both transcriptional programs and epigenetic mechanisms regulate T_{EX} fate and the transition between different exhaustion states. For example, the transcription factor, T-cell factor 1 (TCF1), is expressed on TCF1^+ T_{EX} progenitors, and this expression is lost as T_{EX} move toward a more terminally exhausted state. Recently, thymocyte selection-associated high mobility group box (TOX) has been identified as a critical transcription factor that enforces T-cell exhaustion in both chronic viral infection and cancer (67,68). TOX expression is induced by persistent antigen presentation and results in the upregulation of immune-inhibitory receptors and decrease in cytokine production via chromatin remodeling and transcriptomic modulation. A developmental framework has recently been proposed to describe these different T_{EX} subsets by integrating molecular, transcriptomic, and epigenetic information pertinent to chronic viral infection and cancer. These subsets are distinguished by Ly108 (*Slamf6*) and CD69 expression, and the transition of T_{EX} subsets is regulated by transcriptional and epigenetic checkpoints that involve TCF1, Tbet, and TOX. TCF1 predominates in less terminally differentiated subsets, whereas TOX predominates in the terminally exhausted T_{EX} irreversible state (66).

The Irradiated State: How Does Radiotherapy Boost Antitumor Immunity?

Radiotherapy has emerged as an ideal partner for immunotherapy given its direct immunomodulatory effects on the tumor microenvironment and its ability to augment both innate and adaptive immunity. The notion that focal tumor irradiation can incite systemic regression of nonirradiated tumors via immune-based mechanisms (abscopal response) has gained substantial interest, yet it is rarely observed in the clinic. Using the principles of the innate and adaptive host antiviral response as a conceptual framework, we discuss potential mechanisms and review clinical evidence of radiation-mediated immunogenicity (Figure 3).

Can Radiotherapy Convert the Irradiated Tumor Into a Personalized in Situ Vaccine?

The concept of in situ vaccination with radiotherapy was introduced at the turn of the 21st century. Early studies

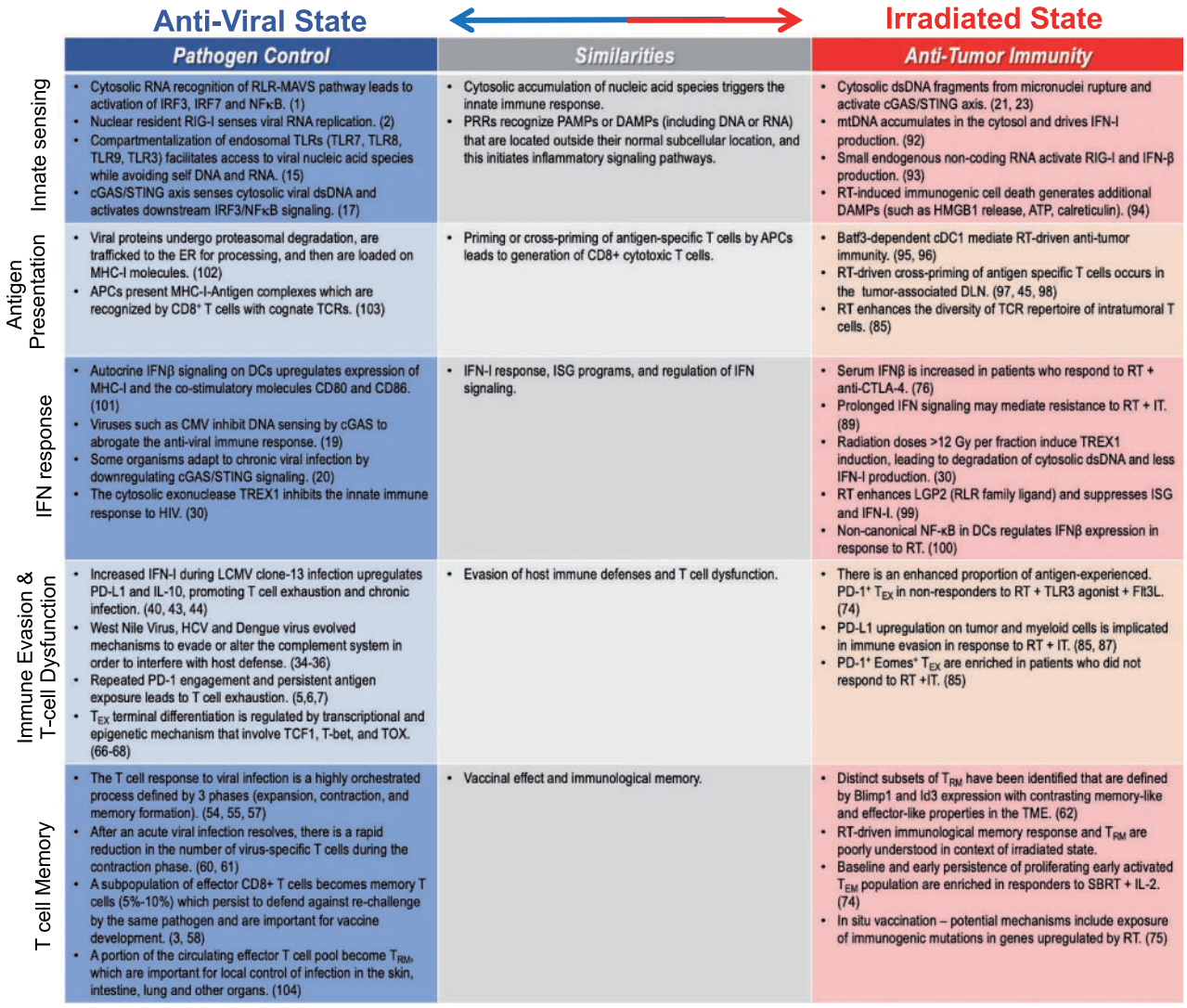

Figure 3. Similarities between the antiviral state and the irradiated state. 1) Cytosolic accumulation of nucleic acids triggers the innate immune response. PRRs recognize PAMPs or DAMPs (including DNA or RNA) that are located outside their normal subcellular location, and this initiates inflammatory signaling pathways; 2) Priming or cross-priming of antigen-specific T cells by APCs leads to generation of cytotoxic CD8+ T cells; 3) IFN-I response, ISG programs, and regulation of IFN signaling; 4) Evasion of host immune defenses and T-cell dysfunction; 5) Vaccine effect and immunologic memory. APC = antigen-presenting cell; ATP = adenosine triphosphate; Batf3 = basic leucine zipper transcription factor ATF-like 3; Blimp1 = B lymphocyte-induced maturation protein–1; cDC1 = conventional type 1 dendritic cells; cGAS/STING = cyclic GMP-AMP synthase/stimulator of interferon genes; CMV = cytomegalovirus; CTLA-4 = cytotoxic T-lymphocyte associated protein 4; DAMP = damage-associated molecular pattern; DLN = draining lymph node; dsDNA = double-stranded DNA; Eomes = Eomesodermin; ER = endoplasmic reticulum; Flt3L = FMS-like tyrosine kinase 3 ligand; HCV = hepatitis C virus; HMBG1 = high mobility box group protein 1; ICD = immunogenic cell death; Id3= inhibitor of DNA binding 3; IFN- = interferon beta; IFN-I = type I interferon; IL-2 = interleukin-2; IRF = interferon regulatory factor; ISG = interferon-stimulated genes; IT = immunotherapy; LCMV = lymphocytic choriomeningitis virus; LGP2 = laboratory of genetics and physiology 2; MAVS = mitochondrial antiviral signaling; MHC-I = major histocompatibility complex class I; mtDNA = mitochondrial DNA; NFkB = nuclear factor kappa light chain enhancer of activated B cells; PAMP = pathogen-associated molecular pattern; PD-1 = programmed cell death protein 1; PD-L1 = programmed death ligand 1; PRR = pattern recognition receptor; RIG-I = retinoic acid inducible gene-I; RLR = RIG-I–like receptor; RT = radiotherapy; SBRT = stereotactic body radiation therapy; TCR = T-cell receptor; TCF1 = T-cell factor 1; T-bet = T-box transcription factor; T_{EM} = effector memory T cells; T_{EX} = exhausted T cells; TLR = toll-like receptor; TME = tumor microenvironment; TOX = thymocyte selection-associated high mobility group box gene; TREX = 3-prime repair exonuclease 1; T_{RM} = resident memory T cells.

demonstrated that the combination of radiotherapy and Flt3 (FMS-like tyrosine kinase 3) ligand achieved local and abscopal tumor control in a tumor-specific, T cell dependent manner (69,70). These preclinical investigations led to the hypothesis that focal tumor irradiation can generate an *in vivo* vaccination based on the release of tumor antigens in response to radiation—thus, each tumor represents an opportunity to generate a personalized tumor-specific vaccine. Further, immunogenic modulation of both intratumoral myeloid and T-cell compartments by radiation can promote antigen presentation and T-cell activation. Radiotherapy alone generates a very modest vaccine effect as evidenced by extremely rare occurrences of abscopal responses in the clinic, however, occasional systemic responses have been observed when combining radiotherapy with various immunomodulatory agents.

Effective vaccination to generate antigen-specific immunity and memory formation generally requires both antigen and adjuvant. To enhance the adjuvanticity of radiation-driven

antigen release, several investigators have attempted to stimulate innate immune signaling with PAMPs in combination with focal irradiation. Proof-of-concept clinical studies of intratumoral TLR9 agonist administration with low-dose radiotherapy in advanced lymphoma have demonstrated systemic disease regression and formation of tumor-specific memory T-cell responses, as evidenced by post-treatment enrichment of $CD137^+$ $CD45RO^+$ $CD8^+$ T cells in the blood (71,72).

Other studies have attempted to optimize *in situ* vaccination with radiotherapy by providing stimuli that enhance DC maturation as an immune adjuvant. A proof-of-principle trial combining radiotherapy with granulocyte-macrophage colony-stimulating factor met its prespecified primary endpoint of observed abscopal responses in 26.8% of patients, defined as an out-of-field response in at least one nonirradiated lesion (73). Alternatively, cytokine therapy with IL-2 and IFN-α have demonstrated activity in patients with both melanoma and renal cell carcinoma. Seung et al. (74) combined IL-2 with stereotactic body radiotherapy in a pilot study of renal cell carcinoma and melanoma patients, reporting an objective response rate in nonirradiated lesions of 71.4% and 60%, respectively. These preliminary findings compare favorably with response rates observed with IL-2 alone, suggesting that cytokines that activate T cells may also enhance radiation-mediated *in situ* vaccination. Interestingly, in this study, a population of proliferating T_{EM} ($Ki67^+CD45RA^+CD8^+$ T cells) were enriched in the peripheral blood of responders at baseline and early time points, suggesting these patients may have pre-existing immunity that was enhanced by radiation and IL-2.

Emerging evidence that radiation exposes neoantigens is an intriguing opportunity to harness adaptive antitumor immunity. Reits et al. (75) provided the first evidence that radiation enhances the MHC-I immunopeptidome to increase antigen presentation. These investigators demonstrated that radiation increases the antigen pool via degrading existing peptides as well as enhanced translation. Intriguingly, several uniquely expressed radiation-induced peptides were identified by mass spectrometry but were potentially nonimmunogenic. Nearly 15 years later, Formenti et al. (76) provided clinical evidence that radiation increases exposure of immunogenic neoantigens that drive antitumor immunity. Immunogenomic profiling of an exceptional responder to radiotherapy and anti–cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) identified rapid *in vivo* expansion of $CD8^+$ T-cell clones recognizing an immunogenic neoantigen encoded by a somatic mutation in Karyopherin alpha 2 (KPNA2)—a gene that is directly upregulated by radiotherapy. The potential to develop a vaccination strategy incorporating radiation-induced neoantigens opens a novel line of investigation that could form the basis for a truly personalized *in situ* vaccine.

However, recent data highlight the complexity and challenges of radiation-induced *in situ* vaccination. Hammerich et al. (77) conducted a trial combining a radiotherapy with a TLR3 agonist [poly(I: C), a dsRNA analogue] with the addition of Flt3 ligand to mobilize cross-priming cDC1s. This multimodal approach achieved systemic regression of bulky disease in a subset of patients, with 2 complete responses out of 11 treated patients. Among the nonresponders, *in situ* vaccination appeared to enhance the proportion of antigen-experienced T_{EX} ($PD\text{-}1^+TIGIT^+HLA\text{-}DR^+CD45A^-$), implicating T-cell exhaustion as an important escape mechanism. This was validated in a preclinical model where the addition of PD-1 blockade to *in situ* vaccination extended median survival.

Combining Radiotherapy With Immune Checkpoint Inhibition to Overcome T-Cell Exhaustion

Although the potential of radiation-driven *in situ* vaccination is yet to be realized, radiotherapy can promote antitumor immunity by enhancing preexisting immunity to overcome T-cell exhaustion and other immune evasion programs. Radiotherapy modifies the tumor microenvironment and induces production of chemokines and cytokines that recruit CTLs and other immune cells into the tumor microenvironment (78-80). Furthermore, radiation promotes immunogenic modulation of tumor cells, so that the surviving fraction of irradiated tumor cells undergoes phenotypic and transcriptional modifications that enhance immune recognition and susceptibility to CTL-mediated lysis (81). Several studies have demonstrated that radiation upregulates MHC-I, cell death receptors, tumor-associated antigens, and immune checkpoint molecules (82,83).

There is increasing interest in combining radiotherapy and immune checkpoint inhibitors to re-invigorate preexisting antitumor immunity (84), and multiple preclinical studies suggest that combining these therapies activates synergistic $CD8^+$ T-cell–dependent mechanisms. Twyman-Saint Victor et al. (85) studied resistance mechanisms of melanoma patients undergoing combination treatment with stereotactic body radiotherapy and anti–CTLA-4 and noted that a T-cell exhaustion signature ($PD\text{-}1^+Eomes^+$ T_{EX}) was enriched in nonresponders, whereas PD-L1 expression was upregulated on both tumor cells and myeloid cells as a dominant immune evasion mechanism. These researchers extended their clinical observations in a preclinical model and found that the addition of PD-L1 blockade significantly improved survival. Mechanistically, PD-L1 blockade reinvigorated T_{EX} cells, and radiotherapy enhanced the diversity of the intratumoral TCR repertoire. Other groups have reported similar adaptive resistance mechanisms in response to radiotherapy and a similar capacity of radiation to expand relevant tumor-specific $CD8^+$ clonotypes within the tumor and the periphery (86,87).

On the other hand, radiotherapy may be able to restore responsiveness to immunotherapy. Using an anti-PD-1-refractory lung cancer model, Wang et al. (88) demonstrated that radiation-induced type I IFN and upregulation of MHC-I on tumor cells was sufficient to overcome defects in antigen presentation. These findings support a potential role for radiotherapy to reignite antitumor immunity in tumors resistant to immune checkpoint inhibition. However, caution is warranted because Benci et al. (89) have demonstrated that chronic IFN signaling in tumor cells drives resistance to immune checkpoint blockade through upregulation of T-cell inhibitory receptors and epigenetic reprogramming of T cells. This is further supported by Chen et al. (90), who reported that radiation-induced type I IFN may also have a tumor-protective role that limits CTL-mediated cytotoxicity. Taken together, these data highlight the complexity and context-dependence of radiation-driven IFN signaling, which has a dual role in promoting and restraining antitumor immunity.

The direct cytotoxic effects of radiotherapy are often overlooked as immunomodulatory. As discussed previously, high tumor burden is a source of chronic antigen exposure that promotes T_{EX} dysfunction. Therefore, cytotoxic therapies that re-

duce tumor burden and antigen load may facilitate antitumor immunity and reverse T-cell exhaustion. Huang et al. (6) described a correlation between tumor burden and reinvigoration of T_{EX} (Ki-67$^+$PD-1$^+$CD8$^+$) in a cohort of advanced melanoma patients treated with anti–PD-1 therapy. These investigators reported that a lower ratio of reinvigoration-to-tumor burden was associated with inferior survival suggesting that T_{EX} reinvigoration may be insufficient in the context of high tumor burden. Integrating radiotherapy as a form of cytoreduction to potentiate responses to immunotherapy is being tested in clinical studies, and early evidence suggests this may be a promising approach (91).

Combining immune checkpoint inhibition and radiotherapy remains an intriguing strategy. However, it is necessary to understand mechanisms of resistance and determinants of response to design rational combination studies. Moreover, it is likely that multimodal approaches are needed to achieve clinical benefit in tumors that lack preexisting endogenous immunity. Although PD-1 axis blockade and CD8$^+$ T-cell exhaustion have been the focus of this discussion, additional resistance mechanisms intrinsic to the tumor microenvironment as well as radiation-induced immune suppression must be addressed to optimize patient outcomes in the future.

Conclusion

Our understanding of radiation-induced immunogenicity remains in its nascency. Decades of work in viral immunology deciphering immunological mechanisms of the host antiviral response have provided valuable insight for the field of oncology to understand how radiotherapy can activate innate and adaptive immunity to improve local control as well as distant tumor control. It has become clear that radiotherapy can employ a form of viral mimicry by coopting innate sensing pathways to drive a type I IFN program that parallels the antiviral state. Additional similarities exist between ways that viral antigens and radiation-induced antigens activate T cells to direct cellular immunity. Vaccine development has outlined the elements required to augment both antigenicity and adjuvanticity of radiotherapy as well as the immunological barriers such as T-cell exhaustion that must be overcome to successfully convert the irradiated tumor into a personalized *in situ* vaccine.

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Data Availability

Given that this article is a review paper, all of the data mentioned in the paper has already been published and all references have been cited appropriately. Although some of the concepts are new, no new data were generated or analyzed in support of this review paper.

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