IN PERSPECTIVE

Polyomavirus-driven Merkel cell carcinoma: Prospects for therapeutic vaccine development

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Abstract
Great strides have been made in cancer immunotherapy including the breakthrough successes of anti-PD-(L)1 checkpoint inhibitors. In Merkel cell carcinoma (MCC), a rare and aggressive skin cancer, PD-(L)1 blockade is highly effective. Yet, ~50% of patients either do not respond to therapy or develop PD-(L)1 refractory disease and, thus, do not experience long-term benefit. For these patients, additional or combination therapies are needed to augment immune responses that target and eliminate cancer cells. Therapeutic vaccines targeting tumor-associated antigens, mutated self-antigens, or immunogenic viral oncoproteins are currently being developed to augment T-cell responses. Approximately 80% of MCC cases in the United States are driven by the ongoing expression of viral T-antigen (T-Ag) oncoproteins from genomically integrated Merkel cell polyomavirus (MCPyV). Since T-Ag elicits specific B- and T-cell immune responses in most persons with virus-positive MCC (VP-MCC), and ongoing T-Ag expression is required to drive VP-MCC cell proliferation, therapeutic vaccination with T-Ag is a rational potential component of immunotherapy. Failure of the endogenous T-cell response to clear VP-MCC (allowing clinically evident tumors to arise) implies that therapeutic vaccination will need to be potent and synergize with other mechanisms to enhance T-cell activity against tumor cells. Here, we review the relevant underlying biology of VP-MCC, potentially effective therapeutic vaccine platforms, and antigen delivery formats. We also describe early successes in the field of therapeutic cancer vaccines and address several clinical scenarios in which VP-MCC patients could potentially benefit from a therapeutic vaccine.

KEYWORDS
cancer therapeutic vaccine, immunotherapy, MCPyV, Merkel cell carcinoma

1 | INTRODUCTION

Merkel cell carcinoma (MCC) is a rare neuroendocrine malignancy that typically occurs in the skin (recently reviewed by Harms et al.1). The MCC recurrence rate ranges from 25% to 75% depending on the stage,2 leading to a 33% to 46% disease-specific mortality.3,4 The current incidence of MCC in the United States is ~2500 cases per year. However, a recent report documented a 95% increase in MCC incidence between the years 2000 and 2013, with ~3200 cases per year expected by 2025.5 MCC has higher incidence rates among immunosuppressed populations and older persons with particularly elevated risk among Caucasian men.5 The majority of MCC cases
(~80%) are associated with clonal integration of the Merkel cell polyomavirus (MCPyV) into the host genome.6 The remaining ~20% of cases are caused by UV mutations alone and are associated with prolonged UV exposure.17.8

Primary MCPyV infection occurs during childhood. Infection is thought to be chronic, with viral DNA detected on the skin in more than 50% of healthy individuals.9.12 The cell types infected13 and possible health effects of commensal MCPyV infection require further study.

The most successful cancer vaccines to date are preventive vaccines for human papillomaviruses (HPV) and HBV. Because such a small minority of MCPyV-infected persons develop VP-MCC, a preventive vaccine for MCPyV is likely not cost-effective. In contrast, a therapeutic vaccine targeting the relevant MCPyV proteins may prevent disease recurrence and be helpful in specific populations with both limited and relapsed VP-MCC as detailed below.

2 THE BIOLOGY OF POLYOMAVIRUS-DRIVEN MERKEL CELL CARCINOMA

Seroprevalence and viral detection studies indicate that MCPyV is widespread in healthy individuals (Figure 1). At baseline, healthy people have antibodies to certain MCPyV proteins, as indicated by the high prevalence of antibodies to viral capsid proteins.15.16 In contrast, healthy individuals rarely have detectable antibody responses to MCPyV T-Ag (~1% of the population have borderline positive titers). This low immunogenicity of T-Ag may, in part, be due to limited expression of the T-Ag in the course of routine infection and also the nuclear localization signal (NLS) within the C-terminal domain of the large T-Ag (LT) oncoprotein.17 Nuclear targeting reduces antigen processing and presentation by HLA-class I, potentially accounting for low CD8 T-cell responses to T-Ag in healthy persons. In contrast to healthy individuals, patients with VP-MCC tumors often have T cells specific for MCPyV oncoproteins.18 and also robust anti-T-Ag humoral immune responses.16.19 Importantly, the magnitude of antibodies to MCPyV T-antigen oncoproteins (but not capsid proteins) correlates directly with tumor burden.19 This association is the basis for a clinical test for tumor recurrence.16.17 Such antibody responses suggest T-Ag oncoproteins are likely to be coordinated between helper CD4+ T cells and B cells, either in draining lymph nodes or potentially in organized lymphoid structures within or near tumors.20-22

Some studies demonstrate the presence of B cells in MCC tumors23,24 and tertiary lymphoid structures (TLS), typically enriched with B cells, were identified in the periphery of MCC tumors.24,25 The TLS in these MCC tumors contained CD4-, CD8-, and CD20-expressing cells (T and B cells), and correlated with recurrence-free survival.24 The presence of TLS in VP-MCC may underlie the observed direct correlation of T-Ag antibodies with tumor burden if in fact T-Ag-specific B cells are activated within or reside in or near tumors. However, the roles of B cells and TLS remain understudied and further investigation is required to understand their function in MCC.

In order for MCPyV to cause VP-MCC, two rare events are required: (a) truncation of the large T (LT) antigen proximal to the C-terminal domain involved in viral DNA replication and (b) linearization and clonal integration of MCPyV into the host genome. After genome integration, VP-MCC tumors express a truncated form of LT protein, as well as full-length small T (ST) protein. Both T-Ag isoforms are involved in MCC tumor persistence and proliferation,6,27 and drive tumorigenicity by distinct mechanisms. Truncation of the LT antigen deletes the helicase domain required for viral replication, preventing destruction of infected host cells through inappropriate DNA replication initiated within the viral sequence itself. Additionally, loss of NLS20 results in redistribution of LT to the cytoplasm,29 where it is accessible to the antigen processing machinery. Although MCPyV LT truncation mutations vary from patient to patient, the highly conserved N-terminal LXCXE motif that binds and inhibits the retinoblastoma tumor suppressor is invariably preserved.30,31 Of interest, this motif may also bind the Stimulator of Interferon genes (STING) protein, and is preserved in other pathogenic viruses such as cancer-related human papillomavirus strains.32 The ST antigen promotes cell survival and proliferation by multiple incompletely understood mechanisms. For example, the ST-stabilization domain (LSD) contributes by stabilization of truncated LT and induction of oncoproteins such as c-Myc and cyclin E.33 A different mechanism of action to stabilization of the truncated LT by ST antigen was demonstrated in a recent study.34 Additionally, ST can inhibit p53 activity in MCC cells via the canonical regulator of p53, MDM4.35

It is clear that VP-MCC survival is dependent on the continued expression of MCPyV T antigens. Indeed, many MCC patients have T-cell responses against ST and LT,36-38 but tumors also downregulate class-I HLA,39 suggesting not only dependence on oncoprotein T antigens for ongoing replication and survival but also active escape from immune pressure. This reliance on T-Ag expression and the presence of compensatory mechanisms suggests that MCPyV T-Ags provide an ideal target for a therapeutic vaccine if tumor immune evasion mechanisms can be overcome.

3 MCPyV ONCOPROTEINS AS PROMISING TARGETS FOR IMMUNOTHERAPY

One of the major limitations in tumor immunology is the identification of appropriate tumor-specific immunological targets. Tumor neoepitopes are typically discovered through whole-exome sequencing, messenger RNA quantitation, and in silico prediction using patient HLA types. This complex, customized, and the expensive process has a challenging candidate-to-hit ratio.

Virus-induced cancers express “nonself” viral antigens that are foreign to the host, potentially increasing their inherent immunogenicity compared to overexpressed, nonmutated, tumor-associated antigens, such as NYESO-1. With a combined T-Ag oncoprotein size of approximately 400 amino acids and very little variation between MCPyV strains, several groups have used standard immunologic approaches to detect T-cell responses to MCPyV T-Ag.36-38,40 Indeed, CD8 T cells appear to play a significant role in controlling MCC as patients who have brisk tumoral CD8 T-cell
infiltration and a cytotoxic T-cell profile experience markedly improved outcomes. Moreover, patients with greater intratumoral T-cell receptor diversity among their MCPyV-specific T cells also have significantly improved MCC-specific survival.

The abundance of circulating MCPyV-specific T cells as measured by peptide-HLA tetramers generally tracks with tumor burden, often being elevated at diagnosis when a larger tumor burden is present and decreasing following successful reduction of the tumor by surgery or other modalities. This fluctuation of MCPyV-specific T cells may very well be a reflection of the amount of tumor-viral antigen available and is consistent with the poor transition to long-lived memory cells. As noted above, the expansion of anti-MCPyV T-cell responses in MCC patients is supported by the detection of MCPyV-specific CD8 T cells in patients but not in healthy individuals. As observed in many cancers and infections, MCPyV-specific T cells are generally enriched at the site of disease, albeit blood is readily obtainable and thus the focus of many studies. Using an HLA-A*24:02-restricted epitope (LT92-101) a tetramer was developed which enabled recognition of MCPyV-specific T cells in the PBMC of 7 of 11 (64%) HLA-A*24:02-positive patients. Another study of

**FIGURE 1** Etiology of virus-positive MCC and potential clinical scenarios for a therapeutic vaccine. Seroprevalence of polyomavirus-specific capsid antibodies indicates that MCPyV is common in the general population starting in childhood. MCPyV then colonizes skin throughout life without causing known disease, except in rare cases when it integrates into the human genome and leads to MCC. This occurs mainly in persons aged over 65 and is likely to be driven by immune senescence and rare mutational events. Following viral integration, two oncoproteins, truncated Large T (tLT) and Small T (ST) antigens, are expressed and promote carcinogenesis. A therapeutic vaccine could potentially be tested in localized or advanced disease. MCC, Merkel cell carcinoma; MCPyV, Merkel cell polyomavirus [Color figure can be viewed at wileyonlinelibrary.com]
27 individuals used a tetramer-enrichment strategy and identified nine potential T-Ag T-cell epitopes restricted by several population-prevalent HLA alleles (HLA-A*01, HLA-A*02, HLA-A*03, HLA-A*11, or HLA-B*07), exclusively in the PBMC of MCC patients and not in healthy individuals. While studying circulating T cells is important for understanding the immunogenicity and T-cell specificity, blood-based lymphocytes do not provide an accurate picture of their roles within the "battlefield" of the tumor microenvironment. Accordingly, studies have focused on detecting specific tumor-infiltrating lymphocyte (TIL) responses by both measuring cytokine production from CD8 T cells upon recognition of tumor-associated antigens and via HLA-peptide tetramers. The apparent proportion of VP-MCC TIL that include T-Ag-specific CD8 T-cell responses appears to vary with the technology used for detection (Figure 2). When a single HLA-appropriate tetramer was used in one report, 5 of 24 patients' TIL had detectable antigen-specific responses. In another study, 6 of 21 TIL from VP-MCC subjects were positive when assayed with a limited panel of patient-matching artificial antigen-presenting cells (aAPC). Recently, we used the aAPC approach to probe patients' TIL for multiple relevant HLA-A and B allelic variants. This study detected T-Ag-specific CD8 TIL in the majority of biopsies (9 of 12) from persons with VP-MCC. While the true proportion of tumors infiltrated with T-Ag-specific CD8 T cells may vary between populations and assay methods, the failure of these cells to clear tumors by definition indicates that further augmentation of the endogenous response may be required.

CD4 T-cell responses, which are both essential for mediating humoral responses and for dendritic cell "licensing" (promoting the capacity of dendritic cells to stimulate cytotoxic T cells) and subsequent CD8 T-cell activation, have also been identified within TIL from VP-MCC. Indeed, a total of 6 distinct CD4 T-cell epitopes were detected within a limited region of LT. One epitope (LT209-228) was highly prevalent among MCC patients, as tetramer-positive cells were detected in 14 of 18 patients. These studies demonstrate that TIL from MCC tumors are often specific for MCPyV oncoproteins and that MCPyV-specific T-cells can also circulate. However, patient T-cell responses against epitope-HLA combinations predicted to be immunogenic are often not detectable. While knowledge of and assays for HLA-appropriate T-cell responses are rapidly advancing, it is likely that in some patients, certain HLA-peptide T-cell responses are not only below current detection thresholds but are actually absent. Indeed, the presence of clinically evident tumors implies that T-cell responses are frequently ineffective. A therapeutic vaccine could provide support to these cancer-specific T cells and promote antitumor immunity. Unlike preventive vaccines, a therapeutic vaccine is thought to improve immunity by supporting insufficiently primed T cells in addition to possibly stimulating naive CD8 T cells. Supporting ineffectively primed T cells is particularly important since naïve T cells are produced in low numbers in older patients and existing CD8 T cells have likely encountered cognate tumor antigen due to the large antigen burden of cancer. Improperly primed CD8 T cells have not seen their cognate antigen on dendritic cells with costimulatory signals. They exhibit decreased effector function and a state similar to exhaustion. This is supported by recent studies showing therapeutic vaccination before immunotherapy preserves T-cell function and mediates tumor regression via induction of early exhausted (stem-like) CD8 T cells that respond to immune therapy. Each of these concepts provides support for the development of a therapeutic vaccine that may enable tumor-specific T-cell responses to reach a functionally effective threshold, especially if combined with measures to enhance trafficking to tumors, overcome tumor endogenous immune escape factors and promote persistence of T cells.

4 | VACCINE DESIGN

4.1 | Target antigens

One of the first tasks when designing a therapeutic cancer vaccine is identifying an optimal antigen. In general, tumor antigens can be divided into cancer-associated antigens and cancer-specific antigens. Cancer-associated antigens such as cancer-testis
antigens (ie, MAGE-A3) and overexpressed proteins (ie, HER-2/neu) are germine-coded antigens that may be expressed at low levels in healthy tissues or in gonadal tissues. Although these antigens are attractive vaccine targets because they are shared across many patients and cancers, they have numerous drawbacks including their dispensable nature to most cancers and frequent toxicity of immune responses to healthy tissues. In contrast, tumor-specific antigens such as oncoviral and neoantigens are attractive since they are exclusively expressed in tumor tissue. Some oncoviral antigens, in particular, are ideal vaccine targets since they drive carcinogenesis and thus cannot be downregulated to avoid immune destruction. Oncoviral-antigens are also shared between patients so a vaccine would not need to be personalized for each patient. VP-MCC has two such oncoviral antigens, the MCPyV small and large T antigens (ST and LT). As noted above, there are well-documented T-cell responses to these antigens and autologous T-cell therapies targeting these antigens have induced clinical responses. However, the endogenous T-cell response is often suboptimal with many patients having undetectable T-cell responses against known ST or LT antigens (see Section 3). When present, the T-cell responses exhibit characteristics of dysfunction. These observations suggest that MCPyV ST and LT antigens are appealing candidates for a therapeutic MCC vaccine.

4.2 | Methods of antigen delivery: Protein and peptides

One of the simplest ways to deliver tumor antigens is polypeptides or whole protein. This approach is used for some preventive and therapeutic vaccines for infectious disease and cancer. A key benefit of the simple approach is that patients would not develop immunity against other vaccine components such as vector proteins. This allows multiple rounds of vaccination without limiting boosts due to the development of antivector adaptive immune responses. This is in contrast to viral-vectored vaccines where pre-existing immunity to viral-vector is associated with a weaker immune response, relating to adaptive immunity preventing or limiting vector infection and transduction. However, since peptides alone are prone to induce tolerance, a danger signal must also be administered, typically an adjuvant compound (discussed below). Overall, the simplicity and safety of this approach make it an appealing option for a therapeutic vaccine (Table 1).

Whole proteins or protein antigens conjugated to immune-enhancing molecules, are preferred to peptides as they contain all potential epitopes within individual patients and the HLA-diverse population. Whole proteins also require endogenous antigen processing which promotes the presentation of biologically relevant cleavage peptides in the proper HLA context. However, in the specific case of MCPyV T-Ag, there is a possible risk of promoting oncogenesis by delivering unmodified oncoproteins, a concern shared by some other vaccine platforms (discussed below). To avoid this, multiple synthetic long peptides (SLP) covering the oncoprotein sequence can be used. This avoids the risk of carcinogenesis, has been shown to induce both CD4 and CD8 T-cell responses and to have antitumor activity for HPV and remains an attractive strategy for VP-MCPyV. To date, SLP-based vaccines appear to be most effective in early-stage cancers or carcinoma in situ. An additional concern is the processing and presentation of HLA-binding minimal epitopes from SLP could differ from those derived from the whole protein.

Peptide or protein antigens can also be modified to increase immunogenicity or selectively promote processing and presentation to T cells. One such approach is to covalently link the antigen of interest to an adjuvant. This ensures that the same APC that take up antigen receive additional danger signals and thus are competent to provide costimulatory signals to T cells. This approach has been successful in animal models that conjugate long peptides to a TLR agonist. Peptides can also be expressed as fusions with proteins that direct them towards antigen processing machinery and HLA presentation. Overall, polypeptides of various lengths are attractive for their general lack of toxicity, clinical experience, and ease of manufacturing. Several studies have shown their ability to stimulate T-cell responses against tumor antigens.

4.3 | Methods of antigen delivery: Nucleic acids

Tumor antigens can also be encoded in nucleic acid products to express tumor antigens in vivo. This approach shares some of the design constraints and attractive options of subunit protein vaccines, with additional challenges and potential. For example, antigens, adjuvants, and targeting moieties can be included in a single construct. Nucleic acids also have auto-adjuvant activity, which can be further enhanced by the insertion of specific, stimulatory sequences. Cytoplasmic dsDNA stimulates immune responses via the cGAMP/STING pathway, and TLR9, while exogenous RNAs can signal through the RIG-I pathway and TLRs 3, 7, and 8. These and other nucleic acid sensor pathways can promote robust immune responses against target antigens. A dose-sparing effect can be obtained using self-amplifying RNA molecules such as alphavirus replicons, which encode an RNA-dependent RNA polymerase to increase the copy number of immune-stimulatory and antigen-encoding RNA in transduced cells.

A DNA vaccine has been tested in an animal model of MCC. In these studies from Hung Lab, murine melanoma cells were engineered to express MCPyV tumor antigens. Mice were then treated with a DNA vaccine expressing the ST or LT antigens, inducing tumor regression. Interestingly, initial studies using vaccination of LT antigen alone stimulated a strong CD4 response. However, a follow-up study in which the LT antigen was conjugated to calreticulin a much more robust CD8 T-cell response was induced, possibly due to increased antigen uptake by cross-presenting dendritic cells. These findings further support conjugating tumor antigens to other proteins that can modulate T-cell responses and may be particularly relevant for MCC.
<table>
<thead>
<tr>
<th>Modality</th>
<th>Type</th>
<th>Rationale</th>
<th>Benefits</th>
<th>Drawbacks</th>
<th>Relevant citations or trials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>Neoantigen</td>
<td>Cancers often have mutations in proteins that can be recognized as nonself by T cells</td>
<td>Can target proteins necessary for cancer survival</td>
<td>Patient-specific, difficult to identify neoantigens that would drive strong immune responses</td>
<td>54,58</td>
</tr>
<tr>
<td></td>
<td>Viral tumor antigen</td>
<td>Viral proteins are highly immunogenic, sometimes required for tumor growth</td>
<td>Shared across patients, truly foreign, highly immunogenic, often required for carcogenesis</td>
<td>Not applicable for most cancers</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Tumor-associated antigen</td>
<td>Many cancers express antigens which would only otherwise be expressed in embryonic or gonadal tissues or lowly expressed in healthy tissues</td>
<td>Shared across patients</td>
<td>Since these are often not required for cancer survival, tumors can downregulate these proteins to evade an immune response</td>
<td>60</td>
</tr>
<tr>
<td>Nucleic Acid</td>
<td>DNA</td>
<td>Putative tumor antigens are encoded into DNA constructs which can then be delivered to patients where the antigens are transcribed and translated</td>
<td>Stable and inexpensive mechanism of antigen delivery, intrinsic adjuvanticity as can encode protein adjuvants</td>
<td>Difficulties in transfection, degradation by DNases</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Messenger RNA (mRNA)</td>
<td>Putative tumor antigens are encoded into mRNA constructs which can then be delivered to patients where the antigens are directly translated</td>
<td>RNA is highly immunogenic, can encode protein adjuvants</td>
<td>Also, difficulties in transfection, degradation by RNases</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Self-amplifying RNA</td>
<td>Viral RNA encodes an RNA polymerase in addition to tumor antigen(s), increasing both transcription and adjuvanticity</td>
<td>Higher levels of transcription and immunogenicity</td>
<td>Less tested</td>
<td>63,64</td>
</tr>
<tr>
<td>Cellular</td>
<td>Tumor cell</td>
<td>Tumor cells are expanded ex vivo and killed cells are injected with adjuvants. This does not require identifying individual neoantigens</td>
<td>Does not require identification of tumor antigens, tumor cells can be engineered to express adjuvants or even costimulatory signals</td>
<td>Potential autoimmune side effects, low proportion of neoantigens</td>
<td>65-67</td>
</tr>
<tr>
<td></td>
<td>Dendritic cell</td>
<td>Dendritic cells are professional antigen-presenting cells primarily responsible for strong T-cell responses; can be expanded and treated with tumor antigens and infused into patients.</td>
<td>Allows direct presentation to T cells with costimulatory signals.</td>
<td>Difficult and expensive manufacturing. HLA diversity precludes an &quot;off-the-shelf&quot; product</td>
<td>68</td>
</tr>
<tr>
<td>Pathogen</td>
<td>Viral</td>
<td>Viral antigens can be encoded into viral vectors allowing efficient delivery to patients</td>
<td>More efficient delivery than nucleic acids</td>
<td>Immunity to vector complicates repeat vaccination</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Bacteria phages</td>
<td>Phages are well known to display antigens on their capsids which could be sources of antigen in a vaccine</td>
<td>Simple manufacturing (bacterial culture) contain some PAMPS (pathogen-associated molecular patterns)</td>
<td>No replication in eukaryotic cells</td>
<td>NCT04034225</td>
</tr>
<tr>
<td></td>
<td>Bacterial</td>
<td>Bacteria expressing tumor antigens can be killed and used as a vaccine</td>
<td>Bacteria encode numerous immune agonists, relatively simple manufacturing</td>
<td>Difficulty in precise control over antigen and adjuvant levels</td>
<td>70-72</td>
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It is worth noting that nucleic acid-based vaccines can also incorporate features of peptide vaccines such as fusion to proteins that influence antigen trafficking, processing, and presentation or co-expression with a protein adjuvant. One example of how this could be pursued in MCC is a DNA vaccine that conjugates MCPyV ST or LT to lysosome-associated membrane glycoprotein (LAMP). This directs these antigens towards the lysosome and HLA-II presentation, to promote a CD4 response. This approach has been shown to be safe in phase I trials to induce allergen tolerance.89 The use of LAMP to elicit functional antitumor responses to MCC is under active investigation in animal models.84

Despite their advantages, nucleic acid-based vaccines have not been reliably effective in humans. Several vaccines have had disappointing clinical trials with only a small fraction of patients developing T-cell responses against target antigens.62,90,91 These trials tested numerous tumor-associated antigens in several cancer types including renal cell carcinoma, non–small-cell lung cancer, and melanoma. However, the vaccines induced weaker responses in humans than in animal models, possibly due to low antigen expression or differences in immune nucleic acid sensing pathways. As a result, no nucleic acid-based vaccine is currently licensed in humans. Promising adjuncts such as lipid nanoparticle or electroporation-based delivery systems and dose escalation can increase immunogenicity.92 There is still substantial interest in nucleic acid-based vaccines93 and these platforms will likely continue to improve.

4.4 Methods of antigen delivery:
Microbe-vectored vaccines

Microbes can also be used to deliver tumor antigens and stimulate adaptive immune responses. This approach is attractive since microbes can naturally stimulate a strong immune response. In general, there is a correlation between the replication competence of the vector and the strength of the immune response to the tumor antigens, as vector amplification increases the stimulation of pathogen-associated molecular patterns and antigen dose. The tradeoff, however, is that replication competence may bring with it virulence, especially in immune-compromised cancer patients, such that vector attenuation and safety need to be extremely carefully addressed in animal models and early-stage clinical trials. In addition, many pathogens have evolved immune evasion mechanisms, which need to be understood and engineered out of vaccine vectors to improve responses.32,94,95 This issue, combined with microbe genome stability during manufacturing and the complexity of manufacturing add considerable time and expense to microbe-vectored vaccine workflows.

Several therapeutic cancer vaccine trials using pox or adenoviral vectors have been conducted in humans with mixed results. A trial using vaccinia and fowlpox vectors to express the NYESO-1 tumor-antigen in melanoma patients induced CD8 T-cell responses in a majority of patients (88%) by the conclusion of the trial. However, only 14% of patients had a clinical response to vaccination.69

### TABLE 1 (Continued)

<table>
<thead>
<tr>
<th>Modality</th>
<th>Type</th>
<th>Rationale</th>
<th>Benefits</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In situ Adjuvant vaccine</td>
<td>Intralesional injection of adjuvant stimulates intratumoral dendritic cells and T cells</td>
<td>Does not require identification of tumor antigens, tumor cells can be engineered to express adjuvants or even costimulatory signals</td>
<td>Less likely to develop adaptive immune responses, Intralesional environment is suboptimal site to stimulate T-cell responses due to suppressive characteristics</td>
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<td></td>
<td>Oncolytic viruses</td>
<td>Viruses attenuated to replicate in cancer cells but not healthy cells could drive a systemic immune response against tumor antigens in addition to local immune effects</td>
<td>Agnostic to tumor type, augments both innate and adaptive immune responses, replicative capacity of virus increases immunogenicity</td>
<td>Typically requires intralesional delivery which is not possible in some cases</td>
</tr>
<tr>
<td></td>
<td>Radiation</td>
<td>Radiation has well-documented in vivo and in vitro tumoricidal effects independent of immunogenicity</td>
<td>Radiation has well-documented in vivo and in vitro tumoricidal effects independent of immunogenicity</td>
<td>Abscopal effect is rare and poorly understood</td>
</tr>
</tbody>
</table>

In rare circumstances, radiation can induce systemic responses through an abscopal effect. Radiation has well-documented in vivo and in vitro tumoricidal effects independent of immunogenicity.

Radiation has well-documented in vivo and in vitro tumoricidal effects independent of immunogenicity.

Abscopal effect is rare and poorly understood.
Therapeutic vaccination vectors other than human viruses are also beginning to move into trials. The most common of these are Listeria monocytogenes engineered to express tumor antigens. An initial trial had sepsis-related adverse events but did induce T-cell responses to the vaccine target antigens.96 Subsequent trials with additional vector attenuation have proven safer97 and to improve survival in patients with refractory pancreatic cancer.71 Another such approach uses bacteriophages to deliver cancer antigens of interest.97,98 Phages have been known to be capable of promoting immunity in vivo since 1985.99 An advantage of this approach is the ease and low cost of phage manufacture in bacterial culture. While at least one phage vaccine has reached the clinical stage for cancer (NCT03120832), overall this platform remains unproven and requires more immunogenicity and safety work. If multiple doses are used, the development of binding antibodies, known to occur after administration of some phages100 could divert antigen either away or potentially even towards the antigen processing pathways required for T-cell responses, adding further complexity. In summary, microbe-based vaccines have several advantages including self-adjuvanticity and the potential for intracellular delivery of antigens. Although less developed than other vaccine formats, these platforms are likely to be of further interest in the near future (Table 1).

4.5 Methods of antigen delivery: Cell-based vaccination

While the adoptive cell transfer has recently been clinically approved for the delivery of redirected T cells, cellular-based vaccine therapies can alternatively be used to stimulate endogenous T-cell responses. Tumor cells expanded and inactivated ex vivo can be reinjected back into patients where they can act as a source of antigen or even antigen-presenting cells.101 Alternatively, autologous dendritic cells loaded with tumor antigens have the potential to present tumor peptides in the correct HLA context, together with costimulatory signals.

For inactivated tumor cells vaccines, a tumor biopsy is obtained and tumor cells are then expanded ex vivo.102,103 The tumor cells can be modified to express adjuvants or costimulatory signals and then, they are killed or inactivated before administration back into patients. Large trials using this approach have shown modest efficacy in early-stage cancers in the early 2000s, however, the substantial cost in lower-risk cancers has hindered further development.102,103

Vaccination with dendritic cells (DC) includes loading antigen into DC ex vivo, which provides control over DC phenotype and maturation. Most such vaccines that now reach the clinical stage use cross-presenting DCs, with the potential to stimulate CD8 T cells in vivo upon readministration to the patient. A noteworthy example of DC vaccination is Sipuleucel-T, a Food and Drug Administration-approved vaccine for the treatment of castration-resistant metastatic prostate cancer. Although the clinical benefit of Sipuleucel-T is relatively modest (4-mo improved survival104), these trials provide a strong rationale for future trials of dendritic cell vaccines. These agents are limited by costly, complex, and time-consuming manufacturing and individualized nature and are thus most relevant for patients with particularly high-risk disease (Table 1).

4.6 Adjuvants and in situ vaccination

Adjuvant selection is thought to be as important as choosing an antigen delivery system. Adjuvants can be broken down into two major categories: Innate immune agonists that largely target innate immune cells and cytokines that typically support T cells or APC. Innate immune adjuvants are usually small molecules that mimic molecular patterns found in bacteria or viruses such as nucleic acids or TLR agonists which are thought to act by activating relevant antigen-presenting cells.85 Adaptive immune adjuvants are usually proteins which would normally be expressed by immune cells to support APC or T-cell growth or differentiation.105

The importance of adjuvants can be illustrated by “adjuvant-only” or in situ vaccines. In contrast to the above vaccination approaches which rely on delivering tumor antigens as part of a vaccine, in situ vaccines rely on endogenous antigens in the tumor and aim to improve responses to these antigens. Indeed, it is now recognized that some irradiation and cytotoxic chemotherapy regimens may work at least in part by promoting immunogenic cell death.106,107 These “adjuvant-only” concepts have had moderate success in small MCC trials. GLA-100 is a stable emulsion of a synthetic TLR4 agonist and was tested in seven patients with metastatic MCC. This compound is thought to modulate innate immune cells such as macrophages in the tumor microenvironment so that they shift from a suppressive and towards an inflammatory phenotype. In this small trial, two patients had sustained partial responses in the target lesion.73 A separate trial tested an IL-12-expressing plasmid in MCC using DNA electroporation directly into tumors. IL-12 supports T-cell differentiation and polarizes CD4 T cells towards a Th1 phenotype. In this trial, 3 of 10 patients with multiple metastatic lesions had partial responses, including in nontarget tumors.74

Encouraged by these results, two newer trials delivering immune agonists into tumors are ongoing. One approach targets the STING (NCT03172936). The STING pathway senses intracellular DNA and activates interferon signaling, known to be critical for immune responses following radiation.108,109 STING agonists have been shown to reverse anti-PD-1 therapy resistance in animal models.110 A separate trial investigating AST-008, a nanoparticle TLR9 agonist, is being studied for PD-(L)1 refractory MCC (NCT03684785). The nanoparticle formulation is designed to increase phagocytic APC uptake and delivery to endosomal TLR9.

In situ vaccine trials in MCC support the notion that adjuvants on their own can stimulate innate and adaptive immune cells. Combining these therapies with optimally delivered tumor antigen will likely further strengthen immune responses in MCC.
5 | EARLY SUCCESSES OF THERAPEUTIC VACCINES

5.1 | Personalized neoantigens vaccines

As mentioned above, tumor-associated antigens are often divided into two groups: (a) nonmutated self-antigens that may be tissue-specific and overexpressed in various cancers and (b) neoantigenic peptides derived from somatic mutations in cancer cell genomes. Therapeutic cancer vaccines that target tumor-associated self-antigens often have low clinical efficacy and generate poor T-cell responses. Presumably, because it correlates with low-avidity T cells, as high avidity T cells targeting self-antigens are eliminated during thymic selection. In contrast, neoantigens are ideal targets for therapeutic vaccines as the T-cell repertoire, including avidity, is not reduced by thymic selection.

Because most cancer mutations are patient-specific, personalized approaches are appropriate for high tumor mutational burden (TMB) malignancies. Indeed, recent developments of in silico prediction approaches enable characterization and selection of neoantigens for personalized therapeutic vaccines. Two groups provided clinical data demonstrating the promise of personalized neoantigen vaccines. Sahin et al identified tumor-associated mutations in 13 patients with advanced melanoma. Using HLA data and epitope prediction tools, 10 peptides were selected per patient and administered as RNA vaccines. Eight patients (61%) who started the treatment without any detectable radiological lesions had a robust immune response and remained recurrence-free up to 23 months. The other patients relapsed after the initiation of the vaccine. Among these, one had a complete response after completion of the vaccine series and the second after receiving subsequent anti-PD-1 treatment.

Analyses of PBMC suggested that the majority of T-cell responses to these vaccines were de novo rather than boosts of pre-existing responses. CD4 T-cell responses dominated over CD8 recognition of vaccine epitopes, despite vaccine design targeting CD8 T cells as discussed above. In the second study, Ott et al tested a peptide-based vaccine in six patients with previously untreated advanced melanoma. Patient-specific somatic mutations were identified, and twenty neoantigens were selected with HLA prediction tools. Thirty-amino acid-long peptides were injected with poly IC:LC adjuvant. Four patients (66%) who entered after surgical resection remained free from disease recurrence for 25 months. The other two patients who entered the study with lung metastases had complete responses after subsequent anti-PD-1 treatment. The vaccine stimulated both CD8 and CD4 PBMC interferon (IFN)-γ responses, with a preponderance of CD4 responses. T-cell receptor (TCR) analyses suggested that vaccine-elicited cells included de novo T-cell clonotypes.

Another vaccine (based on 20 personalized long peptides per patient) was recently tested in glioblastoma. Glioblastoma is an immunologically "cold" tumor with a relatively low mutational burden. While this trial did not result in clinical benefit, a significant benchmark was achieved by the demonstration of specific T-cell responses to a classically nonimmunogenic tumor. In addition to generating CD4 T-cell responses, patients had an increase in tumor-infiltrating T cells.

5.2 | HPV therapeutic vaccines

HPVs are circular DNA viruses. Some genotypes are associated with cervical, anal, vulval, and oropharyngeal cancers. Similar to VP-MCC, a limited number of viral oncoproteins are persistently expressed in HPV-associated cancers and mechanistically drive cell proliferation. Prophylactic vaccination prevents HPV-associated neoplastic diseases when used before primary infection. This great success has led to an interest in therapeutic vaccination that is administered after disease presentation.

HPV16 and HPV18-driven malignant transformation are strongly dependent on the E6 and E7 oncoproteins. Many prototypes of therapeutic vaccines that induce T-cell responses to E6 and E7 have been developed. Vaccination with mixed SLP (ISA101 vaccine) from HPV E6 and E7 oncoproteins with adjuvant in women with HPV16-positive vulvar intraepithelial neoplasia resulted in regression in 94% of patients, of whom 47% experienced a complete response. This was associated with both CD8 and CD4 T-cell responses. An E6 and E7 DNA vaccine (VGX-3100) was tested in phase 2, double-blind trial in women with HPV16/18 positive precancerous conditions. Histopathological regression was observed in 49% of treated patients compared to 36% in the placebo group. CD8 T-cell and antibody responses correlated with the histopathological regression. The vaccine is currently in phase III trials (NCT03185013 and NCT03721978).

In contrast to clinical responses in precancerous conditions, E6 and E7 therapeutic cancer vaccines have yielded clinically disappointing results in patients with advanced or recurrent HPV16-related cancers, despite the enhancement of blood T-cell responses. This suggests that vaccine-activated T cells are being inhibited by immunosuppression. Therefore, synergism with approaches to overcome local and systemic immune suppression are rational to enhance the clinical activity of cancer vaccines in persons with established tumors. A phase-2 clinical trial that combined ISA101 and PD-1 blockade, in patients with recurrent and invasive HPV16-associated malignancies, resulted in a 33% overall response rate (ORR) (8/24 subjects), higher than the 16% to 22% ORR with PD-1 blockade alone. Similar trials are underway (NCT03260023).

HPV murine models support such combination therapy. A therapeutic vaccine combined with anti-PD-1 resulted in a better tumor regression when compared to anti-PD-1 treatment alone. Moreover, it was shown that vaccines alone increased tumor-infiltrating CD8 T cells but also raised PD-1 expression levels. Combination of the vaccine with anti-PD-1 preserved tumor-infiltrating CD8 cells and reduced the numbers of T cells expressing PD-1, and demonstrated synergistic antitumoral responses.
6 | BIOLOGICAL CONSIDERATIONS BEFORE CLINICAL IMPLEMENTATION

Selection of the appropriate treatment for MCC depends on many factors. Practice guidelines emphasize disease stage, with the early disease typically being managed with surgery and radiation while more advanced disease is managed with systemic immunotherapies such as anti-PD-(L)1 blockade. We envision possible roles for therapeutic vaccination at both ends of the VP-MCC clinical spectrum.

6.1 | Adjuvant therapy for early MCC

Patients diagnosed with local MCC will often undergo surgical excision. Radiation of the excised area and the adjacent lymph nodes is also often included. This combination is extremely effective in rendering patients free of detectable disease, but recurrences frequently arise near the primary tumor site, in the nodal region, and/or distantly. Vaccination after local treatment could generate or enhance a systemic immune response that recognizes early emerging microscopic tumors and eliminates them by efficient effector responses. Therefore, a safe therapeutic vaccine to augment MCPyV immunity would be useful to help prevent MCC recurrence. With regard to clinical trials, an appealing alternative to adjuvant studies is the neoadjuvant setting, in which vaccine is initiated in the short window between diagnostic biopsy and excision. This provides an opportunity for histopathologic study of the removed tumor after potential immune-boosting. The conserved nature of MCPyV T-Ag allows consideration of a neoantigen trial as the vaccine candidate product would be off-the-shelf and ready to administer.

6.2 | Systemic/advanced disease

Patients with advanced disease currently receive PD-(L)1 blockade as the preferred first-line therapy. The response rates, 50% to 65%, are higher than those for most other cancers. Unfortunately, approximately half of all treated patients do not experience prolonged benefit. The fact that some MCC patients have modest or nondetectable T-cell immune responses to MCPyV antigens suggests that a paucity of antigen-specific T cells capable of being functionally augmented by anti-PD-(L)1 may contribute to suboptimal clinical responses. Therefore, a combination of a therapeutic vaccine and PD-(L)1 inhibitor could potentially be beneficial for VP-MCC patients. Indeed, several studies in mice and humans suggest it is possible to both stimulate the T-cell function and prevent the induction of T-cell exhaustion. Such studies have demonstrated that vaccination increases the number of T cells infiltrating tumors. The increase in T-cell number occurs by generating either de novo T-cell responses or by augmenting existing T-cell responses. However, in advanced disease, an increase of T-cell numbers by a therapeutic vaccine is usually not sufficient to clear tumors completely. Furthermore, alongside the increase in T-cell responses and infiltration into tumors, an increase in exhaustion markers, such as PD-1 on TIL and PD-L1 on tumor cells, is also often observed. Importantly, mice treated simultaneously with a therapeutic vaccine and either anti-PD-1 or anti-PD-L1 demonstrated prolonged T-cell stimulation and stronger suppression of tumor growth. Additionally, a clinical trial in men and women with recurrent HPV has tested anti-PD-1 treatment combined with a synthetic long peptide vaccine, leading to promising antitumor responses compared to PD-1 blockade alone. These studies suggest that combining a cancer vaccine with PD-(L)1 blockade has significant clinical potential for VP-MCC.

The sequencing of vaccination and PD-(L)1 therapy was explored in a recent mouse study that showed how different treatment protocols result in distinct responses. Verma et al showed that simultaneous PD-1 inhibitor and vaccination was beneficial, but pretreatment with PD-1 blockade before combination therapy abrogated responses. The inhibitory effect of pretreatment with anti-PD-1 was mediated by PD-1CD38^hi CD8 T cells, the depletion of which resulted in a more robust antitumor response and improved mouse survival.

In the difficult setting of patients whose tumors are already refractory to anti-PD-(L)1, it may be beneficial to add additional therapy, such as a T-cell modulatory cytokine. A rational candidate is the immunomodulator, interleukin-12 (IL-12). IL-12 has been shown to be a potent producer of antitumor immunity, which stimulates different effector cells (NK and T cells). IL-12 has been broadly tested in the clinic; several studies have demonstrated that IL-12 treatment increases tumor-infiltrating lymphocytes and the circulating T-cell response in refractory settings. Potentially, such a cytokine could restore some of the immune functions and support further benefit from a combined therapeutic vaccine with PD-(L)1 blockade.

7 | POTENTIAL CLINICAL TRIAL DESIGNS

It is mechanistically attractive to consider an up-front combination of therapeutic vaccination with anti-PD-(L)1 therapy, but it is probably not feasible to carry out such a trial in a rare disease with a relatively high response rate to anti-PD-(L)1 monotherapy. The establishment of incremental benefit for a therapeutic vaccine would require a large and expensive trial. For example, to detect an increase in response rate from 60% to 80%, ~162 patients would be required for a randomized trial, with 80% power to detect a difference of that magnitude. A 20-percentage point improvement in the response rate is a very optimistic estimate, representing a 50% decrease in the number of nonresponders. The number of patients required for a trial is very sensitive to the assumed effect size. If the effect size is cut to 10% (60% response rate increases to 70% with vaccine), ~712 patients would be required for the trial. Since the largest trial to date in advanced MCC (88 patients) required more than a dozen international sites, it is likely impractical to explore the first-line combination of a therapeutic vaccine with PD-(L)1 blockade. The
greatest unmet need in MCC is to develop an effective therapy for patients with PD-(L)1 refractory VP-MCC. Because anti-PD-(L)1 refractory metastatic MCC has no proven effective therapy, even occasional responses in this setting could be clinically meaningful and indicative of the benefit of vaccine therapy.

For the adjuvant setting, clinical trial design is again challenging based on a relatively large number of patients required to determine efficacy. Statistically, adequate sample size depends on the number of anticipated events (recurrences) which varies significantly based on the stage at presentation. Higher-risk groups (eg, stage IIIb disease) would require fewer patients to meet statistical significance because ~70% of these patients are expected to recur after initial treatment. In this scenario, only 62 patients would need to be randomized to vaccine versus placebo to assess clinical benefit, assuming the vaccine reduced the recurrence rate by one half. In contrast, for stage I (~20% risk of recurrence), a clinical trial would require nearly 400 patients, assuming the vaccine also reduced the recurrence rate by one half (to 10%) in this setting. Since there are several other adjuvant trials being conducted (NCT03712605 and NCT03271372), and they focus on higher-risk patients, an adjuvant vaccine trial would likely need to preferentially enroll lower-risk patients, meaning target trial size may need to be ~200, based on appropriate risk stratification for the stage. Before embarking on a significant trial to assess efficacy in the adjuvant setting, a smaller feasibility and immunogenicity clinical trial would likely make sense after a candidate vaccine has been evaluated in a preclinical model. In this initial human study, the extent and persistence of anti-MCPyV immune responses to a vaccine could be assessed as outlined in the MCPyV oncoproteins section above. This analysis will be facilitated by the fact that after tumor removal, these immune responses typically fall very rapidly.

8 | CONCLUSIONS

The clinical efficacy of cancer vaccines has remained modest despite decades of effort. Different cancers pose distinct challenges that must be addressed in a customized manner. VP-MCC is an appealing candidate for a therapeutic vaccine because (a) MCC is an inherently immune-sensitive cancer, strongly associated with baseline immune status, and generally responsive to immunotherapies, (b) The small and conserved antigenic space of the relevant MCPyV-encoded oncoproteins are amenable to vaccine construction and well-defined immune monitoring tools such as tetramers and TCR sequencing, (c) MCPyV viral antigens are inherently immunogenic, leading to robust B- and T-lymphocyte responses that can be readily detected throughout the clinical course.

Several studies that have characterized MCPyV-specific T cells in MCC tumors clearly demonstrate that T-cell infiltration, TCR diversity, and T-cell frequency are associated with better patient outcomes. However, in most cases, these responses are not sufficient and T cells fail to clear tumors, indicating that augmentation of the endogenous response may be needed. Treatment with a therapeutic vaccine could potentially both enhance existing immune responses and induce de novo T-cell responses. Depending on the clinical scenario evaluated, as discussed above, vaccination could prevent late recurrences by eliminating micrometastases or could rescue patients with advanced disease who do not have initial or sustained responses to checkpoint inhibition.

Several factors suggest that VP-MCC is an interesting model of therapeutic vaccination. The viral oncoprotein is T-cell immunogenic providing a trackable biomarker to measure responses. Since both T- and B-cell responses drop after tumor removal, persistent responses from the vaccine would contrast with naturally occurring transitory responses and help to differentiate the two. The clonotypic complexity of T-cell responses to LT can readily be followed in blood, tumor biopsies, and TIL providing another biomarker for vaccine responses.

Because of the unique advantages of this disease, it should be a high priority for the field to explore therapeutic vaccination in this setting. It is likely that clinical trials of a therapeutic vaccine for VP-MCC will yield valuable insights relevant to other immunogenic cancers that may be more common.

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