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The Potential of Immune Checkpoint Blockade in Cervical Cancer: Can Combinatorial Regimens Maximize Response? A Review of the Literature

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Opinion statement

Cervical cancer (CC) is most often caused by the human papillomavirus (HPV). In principle, these ties to the virus should make HPV tumors a relatively easy target for clearance by the immune system. However, these HPV-associated tumors have evolved strategies to escape immune attack. Checkpoint inhibition immunotherapy, which has had remarkable success in cancer treatment, has the potential to overcome the immune escape in CC by harnessing the patient's own immune system and priming it to recognize and kill tumors. Recent work involving PD-1/PD-L1 inhibitors in CC lends credence to this belief, as pembrolizumab has shown evidence of clinical efficacy and consequently been granted accelerated approval by the FDA. That being said, the oncologic outcomes following monotherapy with these biologics have mostly been modest and variable, and this can be attributed to alternative resistance mechanisms to tumor response. The use of therapies that stimulate immune responses via checkpoint-independent activation will therefore augment release of T cell inhibition by checkpoint inhibitors for stronger and more sustained clinical responses. Such a combinatorial approach holds promise for weak- or non-responders to checkpoint therapies as supported by evidence from various, recent pre-clinical, and preliminary clinical studies.

Introduction

Tremendous improvements have been made in strategies designed to prevent development of CC. Prophylactic vaccines and various population-based screening strategies now offer significant protection against development of invasive disease [1–3]. However, CC remains a significant clinical challenge and the burden of the disease is high. Implementation of some of preventative measures has been met with serious challenges. Uptake rates of the vaccine has been suboptimal in resource-poor settings as well as developed markets such as the US [4–6]. Even if 100% coverage could be achieved, given that the vaccine is most suitable for young women pre-infection, there remain approximately 3 generations of women who are ineligible for vaccination. Currently, CC ranks the 4th commonest cancer by incidence and 4th largest contributor to female cancer-related deaths [7].

To a large extent, the fatality rate of CC is explained by the paucity of effective and targeted therapies. Unlike early-stage CC, which can be treated with surgery, the later stages of CC are typically treated with some combination of chemotherapy and radiation. Unfortunately, the disease often recurs or persists. For the patients in this persistence, recurrence, or metastasis setting, a cocktail of cisplatin or paclitaxel with bevacizumab (Avastin™) is the standard of care [8]. There are no other standard treatment options beyond this regimen if it fails. Several cytotoxic and targeted chemotherapy clinical trials addressing second-line therapies have been initiated, though only modest gains in survival or intolerable toxicities have been realized thus far [8, 9]. CC patients in this population therefore represent a serious and unmet clinical need.

The recent advent of immunotherapy, particularly immune checkpoint inhibition, in the treatment of cancer could help to fill this void. Immune checkpoint blockade (ICB) has shifted the paradigm in the treatment of both solid and hematological cancers. Currently, the FDA has approved checkpoint inhibitors for the treatment of a variety of disparate cancers in both a first- and second-line therapy setting. Moreover, unprecedentedly long durations of response have been observed, even in heavily previously treated patients presenting with relapsed and metastatic disease [10, 11]. Such success stories have inspired the current flurry of activity in search of immune checkpoint inhibitors for the treatment of cancers without effective first- or second-line therapies. With respect to cervical cancer, the hope for finding an effective immune checkpoint inhibitor has received a lift with the recent FDA priority review of pembrolizumab (Keytruda™) in 2018 [12]. This means that patients who experience recurrence now have pembrolizumab as an available option. Despite the priority review, the majority of patients did not respond to pembrolizumab, and further clinical validation is required. Finding answers to overcome this challenge posed by non-responders will likely involve untangling the complex immune biology of CC. In the immediate future, however, combinatorial therapies present a tantalizing alternative to standard of care. Studies reported in the literature offer ample preliminary supporting evidence to clinicians and investigators to rationally design effective combinations using checkpoint inhibitors so as to extend clinical benefit to those patients currently not responding [13–16].

In this review, we highlight and rationalize how immune response is compromised during development and progression of CC and how certain therapies can maximize the response of CC patients to checkpoint inhibitors.

Immune escape and CC development: from infection to malignancy

Evidence shows that following an infection of the mucosa by HPV, the host's immune system is often able to eliminate the virus. About 90% of all HPV infections are acute and subclinical, resolving spontaneously within the first

2 years. In cases where the infection manifests clinically, pre-cancerous lesions (cervical intraepithelial neoplasias) are observed in the cervix. In majority of these patients, both the innate and adaptive immunity will eradicate the lesions by deploying macrophages, Th1 and CD8 T cells, directed against HPV-encoded proteins [17–20].

Nonetheless, lesions sometimes fail to regress in immune compromised patients and in cases where the virus successfully evades the immune surveillance. To maintain a low profile and avoid immune attack, the virus keeps expression of viral antigenic proteins low and away from sentinel immune cells. The life cycle also does not involve a viremic phase, and host cells are not lysed when new virions are shed [19, 21]. Additionally, the HPV oncoproteins E6 and E7 are known to antagonize innate immunity viral sensors such as Toll-like receptors (TLRs) and their downstream targets IRFs, NFκB, and type I interferons (IFNs), that facilitate the secretion of pro-inflammatory cytokines (IL-1β, TNFα, IL-6, and IL-8) and chemokines (CCL20) [17, 22–24]. Without chemokines and pro-inflammatory cytokines, keratinocytes are unable to recruit and activate resident immune cells such as Langerhans, stromal dendritic cells (DCs), and macrophages. Together with the down-regulation of major histocompatibility complexes (MHC I and MHC II) by HPV [17, 21, 25], the virus impairs antigen acquisition, presentation, and the provision of a stimulus for humoral- and cytotoxic T cell-mediated immunity to create an environment of immunological ignorance.

Immunological ignorance allows the virus to persist and establish chronic infection. The more the virus lingers during chronic infection, the higher the probability of stochastic events of HPV DNA integrating into the host genome. These events are enhanced by cellular stress due to viral proteins or inflammation from factors such as co-infections and smoking. Indeed, in our lab we have shown that an isoform of E6 down-regulates expression of the anti-oxidant enzymes and elevates levels of reactive oxygen species (ROS) in HPV-positive cells. The ROS levels cause significant DNA damage and induce the integration of plasmid and episomal HPV DNA into cervical keratinocytes [22, 26–29]. Following successful integration, certain viral genes are often lost, including E2. Loss of E2 releases control of expression of the oncoproteins, E6 and E7, and the overexpression of E6 and E7 that ensues will undermine the functions of telomeres, tumor suppressors, apoptosis machinery, and genomic maintenance. This leads to transformation and sustained proliferation of infected cells [30–33].

Although integration of HPV DNA is considered a hallmark of cervical cancer, the progression to malignancy still requires additional events. One of these important changes occurs in the tumor micro-environment (TME), and reciprocal interactions between keratinocytes and stromal cells enable transformed cells to escape anti-tumor immune defenses [34]. Studies show that IL-6 is one of the initiators and among its pleitropic functions, it exerts influence on stromal monocytes and myeloid cells and activates STAT3 [35–42]. STAT3 activation increases the production of CCL2, a chemokine that promotes inflammation through attraction of additional myelomonocytic infiltrates. CCL2 also brings about architectural changes that promote progression towards neoplasia via production of MMP-9 [22, 23, 39, 40, 43, 44]. IL-1β is another key pro-inflammatory cytokine whose levels become elevated. Studies show that IL-1β activates cancer-associated fibroblasts (CAFs) into inflammatory cells that

secrete a host of carcinogenesis-promoting factors including CCL20 and Cox-2. In turn, more monocytes, macrophages, neutrophils, and Th17 cells are recruited into the stroma. It is from these cells that tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs) are derived [45–48] .

TAMs and MDSCs promote the down-regulation of IFN-γ and IL-12 and up-regulate several inhibitory cytokines and skew the Th1–Th2 balance towards Th2 cells. TAMs will also drive expression of proteolytic enzymes and angiogenic factors such as VEGF [22, 23, 34, 43, 49–52]. Another equally important tolerogenic mediator is stromal dendritic cells (DCs). E6 and E7 tend to reduce expression of the chemokine receptor, CCR7 [44]. This prevents migration of DCs to the lymph nodes for antigen presentation and traps them in the stroma. The immature stromal DCs will add to the growing number of suppressive factors by producing IDO, TGF-β, IL-10, and Foxp3$^+$ Tregs [18, 22, 23]. Due to all of these changes, the general clinical observation is that as the immunosuppressive milieu strengthens, the CIN grade also increases in parallel [18, 22]. Even though research is still building on this topic, it is becoming clear that tolerogenicity noted in CINs is not simply an inconsequential phenotype meant to help disarm antigen presentation and generation of peripheral Th1 and CD8 cytotoxic anti-tumor responses. Rather, it is a necessary step that helps to create a protective and immune privileged niche for invasive cervical carcinoma to fully develop.

Immune checkpoints and immune checkpoint blockade

The various soluble mediators, suppressive metabolites, and pro-tumor immune cells discussed above represent the many strategies used by CC to drive its progression. In addition to these immune suppressors, immune checkpoints are an additional modulator that also depress response to tumors. With the advent of immunotherapy however, these various factors also represent opportunities for targeting by immuno-therapeutic agents. Blockade of checkpoints in particular harnesses the body's own immune system to fight tumors, and this has clinically revolutionized the treatment of many cancers [10, 11].

Immune checkpoints are physiological negative regulators of the immune system that help to maintain immune homeostasis and self-tolerance. They achieve this by arresting the activation and proliferation of T cells in both lymphoid and peripheral tissues. CTLA-4 is the main checkpoint in lymph nodes that inhibits T cells after successful priming and initial activation. PD-1 is the peripheral compartment checkpoint that regulates activation of effector T cells. PD-1's primary ligand is known as PD-L1 and is expressed on various somatic cells including APCs. When the PD-L1 ligand binds to PD-1, an inhibitory signaling follows and arrests further activation of effector T cells [10, 11, 53].

Cancers can co-opt this mechanism by up-regulating the expression of these immune checkpoints on various cells in the TME, thereby disabling T cells that recognize the antigens they display [10, 11, 54]. Immune checkpoints therefore allow cancers to neutralize any anti-tumor response mounted against them despite expression of immunogenic antigens. Eventually, chronic stimulation of TCRs by antigens found on surviving tumor cells will diminish the effector

function of tumor-reactive T cells and lead to their exhaustion. As such, the use of antibodies that antagonize immune checkpoints should reverse this T cell dysfunction. In current clinical practice, the blockade of immune checkpoints not only re-boots anti-tumor immunity, but it also results in durable tumor regression and remission in some patients [10, 11].

Biological basis for ICB in cancer: is ICB likely to be effective in treating CC?

Generally, the factors that predict the response of tumors to ICB in various malignancies include the mutational load, immune profile, and status of immune checkpoint expression. High mutational load is associated with more neo-antigens, greater TCR diversity, and robust effector function of T cells. Accordingly, cancers associated with chronic mutagens or deficiency in DNA mismatch repair (dMMR) tend to display a higher clinical response to ICB [55–57]. The immune profile is also important because T cells are ultimately the entities that engage the tumor cells and mediate the effects of checkpoint inhibitors. High responses to ICB have hence been observed in tumors with an immune-inflamed phenotype. "Inflamed" tumors are usually infiltrated with tumor antigen-specific CD8 T cells, pro-inflammatory cytokines, Th1 (T-bet⁺) cells, and Th1-type chemokines. Non-inflamed tumors, on the other hand, have no or low levels of CD8 T cells [58, 59]. Ultimately, the expressions of the PD-1, CTLA-4, and PD-L1 molecules are also essential because these immune check-point molecules generally serve as a surrogate for the pre-existence of activated tumor-reactive T cells. Granted, some patients negative for PD-L1 expression still respond to anti-PD1/PDL1 therapies and vice versa, calling the predictive reliability of its expression into question, but PD-L1 is considered a reasonable biomarker to use to clinically stratify patients [55, 57, 60].

The Genetic landscape and mutational load in CC

The molecular features of CC tumors are consistent with cancers that respond to ICB. CC is a virally induced malignancy that constitutively expresses two viral oncoproteins, E6 and E7, that have no human homologs. Because of their viral origins, E6 and E7 have long been regarded as primarily responsible for the immunogenicity of HPV tumors. Indeed, this notion has been confirmed by various E6/E7 vaccines that have entered clinical trials, as well as the promising anti-tumor activity of autologous E6/E7-directed T cells reported in recent adoptive cell transfer (ACT) patient studies [61, 62].

The immunogenicity of CC also stems from non-viral sources, such as somatic mutations in the genome of host cells. E6 and E7 are notorious for driving APOBEC-signature mutations and copy number alterations, as well as impairing the DNA damage repair response [63–66]. Genetic alterations resulting from such aberrations are likely to result in neo-antigens that have sufficient immune reactivity. Some studies have already identified specific neo-epitopes in cervical cancer tumors, including those emanating from oncogenic driver genes [67•]. The other non-viral source of immunogenicity are tumor-expressed testis associated (TTA) genes. Some cancers, including cervical cancer, express these non-self germline genes [62]. Specifically, significant expression of

the NY-ESO-1 and MAGE-A family genes, TAAs with a long history of antigenicity, was detected in nearly 50% of recurrent CC tumors [68]. The importance of these neo-epitopes and TTAs in conferring T cell reactivity to CC tumors was elegantly demonstrated by a recent study published in science. Antigens of HPV origin were compared against epitopes resulting from somatic mutations as well as TTAs derived from ACT patients in remission. Interestingly, non-HPV specific TILs from these patients demonstrated higher TCR clonality and functional avidity than those directed against HPV epitopes [69••]. Even though the sample size in this study was small, this finding reveals that there potentially exist multiple strong epitopes for use in immunotherapies other than HPV proteins. In fact, studies that have looked at the total number of mutations in CC in comparison with other cancers have found the data to be quite promising. CC has a fairly diverse and high number of neo-antigens, and its median number of mutations clusters together with cancers that already have been approved for ICB therapy, such as head and neck and bladder cancers [61, 63, 69••, 70–72]. From a standpoint of mutations and antigens alone, therefore, CC appears to be well suited for ICB-based clinical interventions.

Tumor-reactive T cell recruitment in CC

Immunologists and clinicians have long observed that the accumulations of TILs generally portend good patient outcomes [13, 73, 74]. This correlation is not unique to standard therapies, however, as pre-existing tumor-specific TILs are also the active agents following treatment with immune checkpoint inhibitors. As such, their recruitment into the TME is an essential determinant of the response of tumors to ICB. In CC patients, active, polyclonal TILs are evident in both primary tumors and lymph nodes, and their presence correlates with outcomes [75]. For instance, CC tumors that have high levels of CD8$^+$ T cells with tumor-specific immune responses display a lower frequency of metastasis to lymph nodes [76]. Interestingly, TILs within the tumor epithelial cells are better predictors of good prognosis in CC patients after radiotherapy and chemotherapy than are TILs in the stroma [77]. Furthermore, these parenchymal TILs tend to be co-expressed with activation markers of tumor-lytic T cells. The evidence for the presence of T cells that infiltrate CC in response to tumor-specific antigens is also apparent in ACT studies. For example, in CC patients undergoing ACT therapy, the positive relationship between tumor-specific TILs and outcomes has held, in that only patients displaying T cell infiltrates specific for HPV proteins in the resected tumors experienced an objective clinical response [62]. That said, the fact that CC still advances and metastasizes in patients presenting these effector, tumor-specific TILs points to the existence of powerful negative regulators in the TME such as the immune checkpoints.

Expression of immune checkpoints in CC

Even though TILs express activation markers, co-inhibitory checkpoints that exhaust T cells and attenuate their responses are also expressed in CC [78–80]. Inhibitory immune receptors such as CTLA4, PD-1, and TIGIT (T cell immunoreceptor with Ig and ITIM domains) are up-regulated on CD4 and CD8 T cells of tumors. In particular, PD-1 and CTLA-4 can be found on CD103$^+$ CD8 T cells embedded in the tumor parenchyma. Not only are these CD103$^+$ CD8 T cells tumor-reactive, but they are also associated with good prognoses, making

their inactivation particularly problematic [77]. An important research study by Stevanovic et al. provided even more compelling evidence for PD-1 expression in CC and its connection to tumor specificity. This study found that the PD1$^+$ T cell compartment is also the niche where high avidity tumor-lytic T cells generated from both somatic mutations and viral epitopes reside [69••]. Other findings similarly show fairly high expression of PD-1 ranging from 46.97 to 60.82% [81, 82]. Moreover, there is evidence for existence of alternate immune checkpoints such as TIM-3 and LAG-3 on memory anti-tumor CD8 T cells [83].

Another critical component of the immune checkpoint system that plays an important role in CC is PD-L1. The expression of PD-L1 in CC is elevated on tumor cells as well as on TAMs and DCs [79•]. In about 67% of CC specimens, a copy gain mutation in the 9p24.1 locus for PD-L1 was found [84]. For reference, classical Hodgkin lymphoma (CHL) is another cancer with similarly high PD-L1 expression due to a copy gain mutation in the same locus. In CHL, virtually all patients carry this mutation, which confers a remarkable sensitivity to PD1 blockade. Specifically, the average clinical objective response rate (ORR) to PD-1 blockade in CHL is about 80%, the highest ORR to ICB measured in all cancers to date [10]. Whether the immune phenotype in CC with the copy gain mutation is similar to that seen in CHL is not yet clear.

In addition to the high expression of PD-L1 due to genetic amplification, PD-L1 expression in CC can also increase due to adaptive resistance. PD-L1, similarly to PD-1, has been found to co-localize with TIL densities. One study has found that about 60% of TILs and tumor cells expressed PD-1 and PD-L1, respectively [81, 85, 86]. Another study has found that even though normal epithelial cells express no PD-L1, about 95% of CIN and 80% of CC tissues express PD-L1 [87]. Such high expression patterns support earlier findings that local immunity is impaired early on and that impairment is used as a means of immune escape. Based on this evidence, therefore, inhibition of immune checkpoints is predicted to restore protective immunity.

Other HPV-associated cancers: HNSCC as a benchmark?

A reasonable precedent for assessing the potential of ICB in CC is head and neck squamous cell carcinoma (HNSCC), as both CC and HNSCC are often caused by HPV. HNSCC has had two ICB agents, pembrolizumab and nivolumab, approved for use by the FDA in 2016 [10]. Importantly, HPV$^+$ HNSCC displayed a better clinical response to ICB than did HPV$^-$ HNSCC. In addition, HNSCC and CC cluster in the same group in terms of total and median number of mutations and predicted response to ICB. Furthermore, it is not only the number of mutations that these two cancers share; the patterns and characteristics of the mutations found in these two cancers are also similar [70, 71]. It is a reasonable prediction, therefore, that the successful inhibition of immune checkpoints in HNSCC could inform similar endeavors in CC. Nonetheless, caution should be practiced in extrapolating these findings. For example, there are differences in the anatomical location and natural history of infection when comparing HPV$^+$ HNSCC with HPV$^+$ CC. In HNSCC, the HPV infects the oropharyngeal epithelium in the tonsils, and tonsils are a lymphoid-rich organ. On the other hand, in CC, the HPV infects the mucosal epithelia of the cervix, which is relatively shielded from the immune surveillance provided by lymphatic tissue. Also, some studies have shown that the local HPV-specific T cell

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response, Th1 levels, and IFN-γ production are relatively better in HPV⁺ HNSCC [88–90]. Together, the molecular and immune landscape in CC suggests feasibility and potential therapeutic benefit of employing ICB in the treatment of CC.

Clinical experience regarding the use of single checkpoint inhibitors in CC

The clinical success of ICB in malignant diseases was initially tested in cancers such as melanoma, NSCLC, and RCC over the past 5 years [10], while gynecological cancers such as CC have been considered only more recently. However, there have been a number of clinical trials in recent years exploring the effectiveness of single checkpoint inhibitors in CC, with the number growing every year [8, 91]. For perspective on the progress made so far, a few key studies involving the antibodies ipilimumab, nivolumab, and pembrolizumab are discussed below.

Ipilimumab

The Chicago N01 Consortia study as reported by Lheureux et al. was one of the key studies that first investigated CTLA-4 inhibitors in CC. Heavily pretreated patients were administered the CTLA-4 antibody, ipilimumab. From the standpoint of clinical benefit, the performance of ipilimumab was below par. The objective response, PFS, and OS were low and not close to the targeted end points. However, ipilimumab demonstrated safety and manageable toxicities in CC patients. Today, more clinical trials involving ipilimumab or other CTLA-4 antibodies are being conducted using this study as a reference [8, 92].

Nivolumab

Nivolumab, which targets the PD-1 immune checkpoint, is another monoclonal antibody that has been studied in CC patients quite extensively. Check-358 is one of the key early studies that assessed the efficacy and safety of nivolumab in CC [8, 92]. The study included 19 patients who had disease in the R/M setting and prior systemic therapies. The overall response rate was 26.3%, one of the highest yet found in CC ICB studies. Nivolumab also showed low toxicity and signs of a durable response in some patients. The major conclusion reached in this study was that nivolumab has some encouraging activity and a good safety profile. Future confirmatory nivolumab studies were thus warranted for validation, and one such study is the NRGGY002 trial [8]. Unfortunately, even though safety was confirmed, the findings presented at the ASCO annual meeting in 2018 concluded that the anti-tumor activity of nivolumab in CC is low. Therefore, definitive answers regarding the clinical efficacy of nivolumab in CC are not yet available.

Pembrolizumab

Considering the challenges that ipilimumab and nivolumab have faced, pembrolizumab, another anti-PD1 antibody, may represent the most promising single ICB therapy tested in CC thus far. In 2016, results from the

KEYNOTE-028 phase 1b study in 24 patients who had advanced CC disease and prior systemic treatment showed that pembrolizumab is well tolerated with relatively good responses. The median OS was approximately 11 months in these patients, with an ORR of about 17% [8, 9]. These findings were followed up by the KEYNOTE-158 study in a larger patient population. Results of the KEYNOTE-158 study were presented at the 2018 ASCO annual meeting. In 91% of the patients studied, the duration of response was at least 6 months, and the OS was 9.4 months. Despite the relatively low ORR of 14.3%, the major conclusion was that pembrolizumab had a durable anti-tumor activity and a safe profile. Based on this, the FDA accelerated the approval of pembrolizumab, with a final approval given in June 2018, making it the first approved check-point inhibitor for the treatment of CC. All patients who responded were positive for PD-L1, and none without PD-L1 responded [8, 9, 92, 93]. Even though the 14% RR of pembrolizumab may not have been sufficient for other cancers, its approval underscores the complete lack of viable, alternative second-line therapies in CC. Now patients in the relapse setting can use this antibody, giving them a second chance at survival.

Combinations in ICB: the quest for increasing ICB response in patients

Although the studies described above regarding single immune checkpoint therapy in CC are encouraging, it is clear that there is still much work to be done before the great potential of ICB, as seen in some other malignancies, can be fully realized. Ipilimumab and nivolumab, albeit safe in CC patients, have exhibited low anti-tumor activity. Pembrolizumab is the only antibody approved thus far, but its approval is still partial; full approval is contingent on future clinical studies that substantiate the initial clinical benefit observed. In addition, the accelerated approval was based on a study that showed 14% ORR, meaning only a select few patients are likely to benefit from pembrolizumab.

In this context, it should be kept in mind that cervical cancer meets most criteria regarding ICB suitability, from its robust PD1/PDL1 expression and a T cell-inflamed phenotype to the mutational load and immunogenicity of HPV[+] tumors. So how then are the impressive ICB responses observed in other cancers not seen in CC? Clearly, there are gaps in our understanding of the immune biology in CC as it pertains to ICB and the strategies to boost the paltry 14% ORR to maybe 25–30% or more. More light thus needs to be shed on the immune landscape of CC, and greater research efforts are needed in identifying biomarkers other than PD-L1 and ascertaining their robustness in predicting who benefits. Additional studies are needed on inflammatory gene signatures and the pathways to target in combination therapy as well as the identity of mutations, viral peptide, and non-viral epitope pools in CC. Furthermore, immunosuppressive mediators such as TAMs, Tregs, MDSCs, IDO, and alternate checkpoints and their impact on disease progression and the efficacy of ICB need to be elucidated [94, 95]. CC tumors are known to down-regulate expression of MHC complexes for antigen presentation. Also, a recent comprehensive multi-omic study showed that CC is more heterogeneous and molecularly complex than previously thought, which might suggest that the

effectiveness and durability of ICB depend on how patients are stratified for treatment [64]. All these features are potential escape mechanisms contributing to the attenuation of ICB efficacy (Fig. 1). While research efforts will continue to unravel the immune context of CC and these mechanisms, combination of therapies represent an alternative and viable route forward in the immediate future. Exciting findings are being reported regarding ICB combinations in other cancers [96, 97], and the data presented in the literature supports the anticipation that certain agents and modalities in combination with ICB may have synergistic effects and extend clinical benefit to many non-responders in CC. These modalities include cytotoxic chemotherapy, targeted small molecules, radiation therapy, and vaccines.
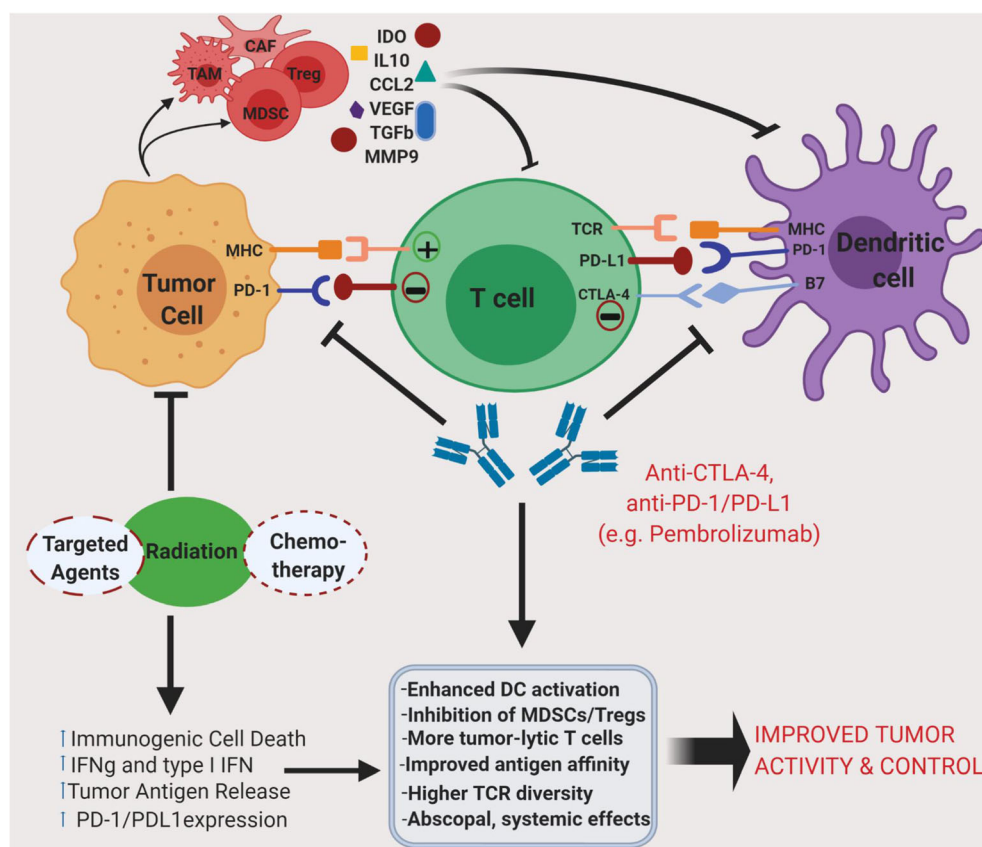

Fig. 1. Combination therapies to potentiate the efficacy of ICB monotherapy. Various putative mechanisms of resistance (innate or acquired) to ICB may arise due to tumor heterogeneity, lack of sufficiently reactive neo-antigens, inability of T cells to infiltrate tumors, impairment of antigen presentation, exhaustion of T cells and/or increased expression of immunosuppressive metabolic mediators, immune suppressive cells and alternate immune checkpoints. ICB as a monotherapy may help to re-activate anti-tumor T cell immunity and ameliorate exhaustion, while chemo, targeted, and radiation-mediated therapy may promote antigenicity, improve IFN signaling and intra-tumor infiltration, and increase expression of PD-1/PD-L1. Combinations of ICB with any of these modalities will potentially enhance function of DCs and antigen presentation, reduce depletion of immunosuppressive milieu, and enhance diversity and robustness of tumor reactivity of CD4 and CD8 T cells as well as potential systemic immunity, together leading to significantly improved immunological tumor control. **+** represents stimulating TCR-MHC interactions and **−** represent inhibitory PD1/PDL1 or B7/CTLA-4 interactions on T cells.

Chemotherapy and radiation: basis for synergy with ICB

Single immune checkpoint inhibitors in CC have failed to objectively shrink tumors or prolong survival in the majority of patients. Such observations in these patients may be attributed to (i) their tumors being immunologically cold, lacking TILs and antigens and (ii) their tumors having diminished effector T cell activity due to checkpoint-independent immunosuppression. Can chemotherapy or radiotherapy overcome these challenges?

This may indeed be the case. An accumulating body of evidence in various cancers shows that both chemotherapy (CT) and radiotherapy (RT) can have immune-stimulatory effects. With regard to cold tumors, RT and CT can induce immunogenic cell death (ICD) that increases the formation of cancer-specific peptides or DNA and exposure of damage-associated molecular patterns (DAMPs) in immune cell poor tumors [14, 98–101]. The available tumor antigens and DAMPs will augment antigen presentation, T cell priming, and activation either directly or as adjuvants and boost T cell infiltration.

PD1/PDL1-independent immunological barriers such as inhibitory immune cells, suppressive cytokines, and metabolites can also limit the efficacy of ICB. Nevertheless, certain chemotherapies and radiation regimens have been shown to deplete immune regulatory factors (MDSCs, Tregs, IDO), increase expression of class I/II MHCs, and enhance function of DCs for T cell priming. This also makes the TME more hospitable, freeing up PD1$^+$/PDL1$^+$ CTLs and poising them for ICB-mediated re-activation. Combination therapies therefore could potentiate the efficacy of ICB by acting as spark plugs that alter the tumor immune landscape and make it more supportive of ICB therapy (Fig. 1).

In many cancers such as melanoma, NSCLC, and breast and bladder cancers, combinations of ICB with cytotoxic chemotherapies, targeted small molecules, monoclonal antibodies, and radiation therapy are already being tested in clinical trials with promising results [73, 97, 99, 101]. In particular, the FDA has already approved the combination of chemotherapy (pemetrexed and cisplatin/carboplatin) with pembrolizumab for first-line treatment of metastatic NSCLC [102]. Another great case in point is the recent FDA approval of atezolizumab in combination with nanoparticle albumin-bound (nab)-paclitaxel in triple negative breast cancer (TNBC). As expected, the combination therapy was better than either therapy alone, with overall response jumping from 10% in the monotherapy trial to about 40% in the combination trial [103]. Even though chemotherapy might exacerbate toxicity of ICB, these examples of success of chemotherapy cooperating with ICB to broaden clinical response should encourage the testing of combinations in cancers such as cervical cancer where modest activity and response rates are being observed with monotherapy.

Chemotherapy: pre-clinical models and preliminary clinical evidence

Combinatorial studies of ICB with cytotoxic chemotherapy in HPV-associated cancers are still in their infancy relative to cancers such as NSCLC. However, a number of pre-clinical studies provide a rationale for conducting such trials in the clinic. Research findings reported by Spanos et al. almost a decade ago provided the first clear link between chemo-sensitivity and tumor immunogenicity in HPV-associated tumors. The study found that HNSCC tumors in mice were sensitive to cisplatin only when the immune system of the tumor-bearing

host was competent [104]. The authors concluded that the sensitization to cisplatin was primarily due to the induction of the immune response in immune-competent mice and thus challenged the previous understanding that the effectiveness of chemotherapy stems autonomously from its cytotoxic or cytostatic effects on tumor cells. This seminal study has since been corroborated by more recent studies. For example, the Schmitt group has found that not only does cisplatin induce ICD and up-regulate PD-L1, but it also enhances antigen presentation and T cell tumor reactivity. When the group combined anti-PDL1 therapy and cisplatin subsequently, synergistic anti-tumor effects were observed [105]. In line with this study, PD-L1 overexpression following cisplatin has also been observed clinically in HNSCC patients [106].

Importantly, similar findings have been found in the context of CC. In mice, the treatment of tumors with cisplatin was associated with an increase in PD-1, implying recent in vivo T cell activation. A cisplatin plus anti-PD1 cocktail was associated with better tumor regression and reconstitution of tumor-active T cells [107]. In patients with CC, cisplatin was also shown to support immune cell activation and infiltration of the tumors by T cells. Consistently,

the activation of T cells was also associated with the development of potentially deleterious adaptive resistance (PD-L1 up-regulation), providing another reason for combining ICB with chemotherapy [82]. Another standard cytotoxic regimen that has been investigated for immune-modulatory effects in the clinic is carboplatin plus paclitaxel. The immune cell subpopulation that was most affected by this chemotherapy doublet was myeloid suppressor cells. Their abrogation was followed by enhancement in DC function, T cell reactivity, and prolonged patient survival [108]. The take home from their findings was that relief of tumor-lytic T cells from myeloid-driven immunosuppression may be a critical step needed before activity of these cells can be fully restored by immunotherapies. This is line with the evidence that shows that tumor-directed TILs in CC tumors are often accompanied by MDSCs, Tregs, and a variety of suppressive cytokines [79].

As summarized above, a fairly extensive collection of pre-clinical information in the literature supports the concept of ICB in combination with cytotoxic chemotherapy. Combinatorial studies of ICB with targeted chemotherapy therapy, on the other hand, are still few in number and limited in scope. Nonetheless, the potential in some studies is evident. For example, treatment of cervical cancer cells in vitro with Smac mimetics has shown evidence of necroptosis and up-regulation of markers for ICD (HMGB1, CRT, ATP) [109]. Clinical trials for CC involving Smac mimetics in combination with checkpoint inhibitors are also already underway. Another recent study explored potential novel targeted therapy effectors for combination with immune checkpoint blockade using gene expression analysis of recurrent CC tissues. An evaluation of cancer immune genes and cancer-specific molecular pathways yielded new promising targets in the DNA damage repair pathway (e.g., PARP) and epithelial-mesenchymal transition-related genes (e.g., STAT3) [110]. These targets could be considered in future pre-clinical and clinical trial immune checkpoint combination studies. Table 1 lists ongoing ICB combinatorial clinical trials in CC.

Table 1. Ongoing clinical trials of immune checkpoint inhibitors in combination with other cancer treatment modalities for patients with cervical cancer

Category	CT identifier	Combination agents	Indication	Phase
Chemotherapy	NCT02914470	Atezolizumab, CBP, cyclophosphamide(CP)	Advanced CC	Ib
	NCT02921269	Atezolizumab, bevacizumab (Avastin™)	CC in R/M setting	II
	NCT01711515	Ipilimumab, CCDP*	Locally advanced CC	I
	NCT03104699	Atezolizumab, bevacizumab	Local, advanced CC	I/II
	NCT02587962	Pembrolizumab, birinapant	Advanced CC	I/II
	NCT03518606	Durvalumab, tremelimumab, vinorelbine	Advanced CC	I/II
	NCT03192059	Pembrolizumab, cocktail including CP	Advanced CC	II
	NCT03367871	Pembrolizumab, PTX*, CBP*, Avastin™	CC in R/M setting	II
	NCT03635567	Pembrolizumab, PTX, CBP, Avastin™	CC in R/M setting	III
	NCT03340376	Atezolizumab, doxorubicin	Recurrent CC	II
	NCT03556839	Atezolizumab, CCDP, PTX, Avastin™	CC in R/M setting	II
Radiation	NCT03144466	Pembrolizumab, chemoradiation (CRT)	Locally advanced CC	I
	NCT02635360	Pembrolizumab, CCDP, brachytherapy	Locally advanced CC	II
	NCT03298893	Nivolumab, CCDP, radiation	Locally advanced CC	I/II
	NCT01711515	Ipilimumab, CCDP, radiation	Locally advanced CC	I
	NCT03614949	Atezolizumab, radiation	CC in R/M setting	II
	NCT03277482	Durvalumab, tremelimumab, radiation	CC in R/M setting	I
	NCT03612791	Atezolizumab, CCDP, radiation	Locally advanced CC	II
	NCT03298893	Nivolumab, CCDP, radiation	Locally advanced CC	I/II
	NCT03527264	Nivolumab, CCDP, radiation	Locally advanced CC	II
	NCT03738228	Atezolizumab, CCDP, radiation	Locally advanced CC	I
Dual inhibition	NCT01975831	Durvalumab, tremelimumab	Advanced CC	I
	NCT02488759	Nivolumab, ipilimumab	Advanced CC	I/II
	NCT02834013	Nivolumab, ipilimumab	Locally advanced CC	II
	NCT02671435	Durvalumab, monalizumab (IPH2201)	Advanced CC	I/II
	NCT02658890	Nivolumab, BMS-986205	Advanced CC	I/IIa
Vaccines	NCT 02426892	Nivolumab, ISA 101	Advanced CC	II
	NCT02291055	Durvalumab, ADXS11–001	Local, advanced CC	I/II
	NCT03444376	Pembrolizumab, GX-188E	Advanced CC	Ib/II
	NCT03073525	Atezolizumab, vigil	Advanced CC	II
	NCT02725489	Durvalumab, vigil	Advanced CC	II
	NCT03439085	Durvalumab, MEDI0457/IL-12	Advanced CC	II
	NCT03618953	Atezolizumab, AD-E6E7/MG1-E6E7	Advanced CC	I/Ib

CCDP cisplatin, *CBP* carboplatin, *PTX* paclitaxel

Radiation therapy: pre-clinical models and preliminary clinical evidence

Pre-clinical and retrospective clinical evidence in HNSCC and CC involving ICB and RT combinations is consistent with that observed using chemotherapy. HNSCC preclinical models show that PD-L1 is up-regulated on tumor and myeloid cells following RT. When checkpoint blockade is applied, T cell infiltration into the tumor lesions with concomitant enhancement of tumor control and prolonged mouse survival are observed [111●]. The same phenotypes and outcomes are also seen clinically in HPV⁺ HNSCC patients who are treated with radiotherapy [112, 113].

Rodent studies modeling CC also confirm the immuno-modulatory role of RT. In the Wistar rat model, treatment with RT led to overexpression of PD-L1, as seen in other cancers [114]. In a TC-1 mouse model, RT failed to clear tumors in immune-compromised mice, again showing the interplay between host immunity and RT [104]. In addition, combinations involving antigen challenge were shown to increase the robustness of tumor-targeted T cell generation by radiotherapy in mice [77]. While these pre-clinical reports are promising, there remain hurdles in integrating RT with ICB clinically, particularly with regard to determining the optimum radiation dosage, fractions to use, and the order of combination with immunotherapy. Low radiation may not be sufficiently immunogenic, while high radiation can destroy the very immune cells that mediate ICB effects. In addition, it is not yet definitive as to whether fractionated or conventional radiation is more effective [99, 115]. More research therefore is needed to fine-tune the conditions for optimized regimens. To that end, some studies in CC are already looking into how RT affects the frequency of immune cells and their activation in the clinic. In a clinical study by Dorta-Estremera et al., patients treated with fractionated radiation initially experienced a transient decline in CD4 and CD8 T cells. Within 2 weeks, however, a proliferative phenotype of these cells rebounded, together with increased TCR diversity and enhanced local antigen presentation [116]. Similar preliminary findings of improved efficacy in the combination setting were found in another clinical study [117]. These data bolster the idea that RT and ICB together may yield more effective disease control. Ongoing combination clinical trials are shown in Table 1, and results from the early phases of some these combination clinical trials are already showing encouraging signs of tolerability, feasibility, and effectiveness of chemoradiation-immunotherapy combinations [12, 118].

Therapeutic vaccines: basis for synergy with ICB

Suboptimal T cell priming, activation, and expansion are some of the early events involved in disabling protective tumor immunity. Lack of antigens or their acquisition can be the primary cause for the below par lymphocyte activation seen in CC. Vaccination can help overcome this hurdle by artificially generating and magnifying the low population of TA-specific T cells and increase their reactivity upon tumor challenge. In this way, vaccines help to improve the presentation process of tumor antigens and subsequent activation of T cells [61, 62]. However, despite observations that therapeutic vaccines against CC are capable of inducing immunogenicity, most have failed to show meaningful clinical anti-tumor activity as a single therapy in patients. Studies in vitro as well as preclinical models suggest that the clinical potential of vaccines may be restricted by tumor-mediated immune suppression, which includes immune checkpoints that deactivate the CTLs by the vaccines [17, 61, 62]. Combining vaccines and checkpoint inhibitors, given their non-redundancy in anti-tumor response, along with the possibility that ICB could overcome limitations to vaccine efficacy, carries significant clinical potential.

Although discussion is needed as to which group of patients should be targeted by vaccine/ICB combinations, patients that make up the late-stage CC population are potential beneficiaries. Studies have shown that in contrast to early-stage CC, which abounds with tumor-reactive TILs, aggressive late-stage CC is poorly infiltrated. A study by Komedeu et al. concluded that the patients

in this group may require additional interventions such as vaccines to pre-condition their tumors with T cells and thus make them candidates for a high ICB response [77]. In addition to this group, patients who express PD-L1 as a result of tumor intrinsic signaling as opposed to an immunogenic response may also benefit tremendously from vaccines. Overall, the prospects for clinical success for all patients who will end up using CC therapeutic vaccines are improving. The recent findings that dominant tumor-reactive T cell populations are actually directed against some previously unidentified germline antigens and neo-epitopes and not necessarily against HPV onco-proteins are expected to transform the field [67, 69••]. Based on this newly available broader panel of strong and non-tolerant epitopes, we should, in theory, be able to design better vaccines, monovalent or polyvalent, that will increase the amplitude and du-ration of immune response. For now, most therapeutic vaccines are still based on various peptide or full protein forms of E6 and E7.

Therapeutic vaccines: pre-clinical models and preliminary clinical evidence

Just as with chemotherapy and radiation therapy, vaccination improves the efficacy of immune checkpoint inhibitors compared with either therapy alone. In one mouse study, immunization against E6/E7 genes using viral vectors resulted in infiltration of activated CD8$^+$ CTLs into tumors followed by im-munosuppression due to PD-L1 up-regulation. When PD-1 blockade was ap-plied, potent tumor regression was observed [119]. Similar observations were made when a DC-based vaccination strategy against E7 was used followed by the treatment of the tumors with a PD-L1 inhibitor [120]. Inhibition of check-points and activation of co-stimulators in combination with an E6/E7 peptide vaccine has also been found to generate E7-specific CD8 T cells and Th CD4 cells [121]. Another DNA vaccine with potential for clinical success in CC is the live-attenuated *Listeria monocytogenes*-based vaccine. An Lm-LOO-E7 vaccine designed to secrete the E7 protein in combination with a PD1 inhibitor was associated with improved antigen presentation, TILs frequency, and significant tumor inhibition. Moreover, the combination also depleted the inhibitory regulatory cells (MDSCs and Tregs) and resulted in complete remissions in some mice [122]. These observations have been recently corroborated by an Lm-LOO-E6 vaccine combined with a PDL1 inhibitor in SiHa and TL-1 sub-cutaneous mouse models [123]. Another study that combined 4-1BB agonist or CTLA-4 blockade with E6/E7 peptide vaccine in an orthotopic syngeneic mouse model also observed depletion of immunosuppressive cells in addition to curative efficacy [124•]. More importantly, data from a recent phase II trial appears to validate these pre-clinical studies as a combination of an HPV-peptide vaccine with nivolumab yielded an ORR of 33%, which is about double on what we have generally seen with anti-PD1 monotherapies [125••]. This emerging clinical evidence has buoyed the hope that this combinatorial ap-proach will work in patients and will encourage more studies in the future. Table 1 shows other vaccines currently being tested in combination with ICB.

Dual checkpoint inhibition

Dual immune checkpoint inhibitors are also a logical combination that may increase ICB efficacy in cervical cancer. CTLA-4 antibodies trigger T cell priming and clearance of inhibitory regulatory cells. PD-1/PDL1 inhibitors, on the other

hand, enhance activation of peripheral T cells and help counteract adaptive resistance. In HPV-associated cancers, no pre-clinical data is yet available to back the use of these checkpoints together. However, the mechanistic non-redundancy of these two types of checkpoint inhibitors, their individual over-expression and demonstrated tolerability in CC, may result in additive or synergistic effects in combination. Proof that PD-1 and CTLA-4 inhibitors can be more efficacious together has already been provided by dual inhibition studies in melanoma. The combination of nivolumab (anti-PD1) plus ipilimumab (anti-CTLA4) increased the response rate by 2-fold from about 30 to 60%. Not only was response improved, but patient survival was also higher in the dual inhibition group. The approval of the two inhibitors by the FDA has opened the door for dual ICB in many other cancers [94]. The trade off, however, that comes with this increased activity is higher toxicity. Only when the response is high enough and there are no alternatives can the toxicity be justified [10, 126]. See Table 1 for similar dual checkpoint inhibitor studies for CC in clinical trials.

Conclusions

ICB has shown a marked therapeutic success in cancers such as NSLC and CHL. In cancers such as CC and other gynecological cancers, similar prospects in the future are likely, noting that research on efficacy is still in its infancy. ICB monotherapy, particularly pembrolizumab, has offered a ray of hope for some CC patients with recurrent or metastatic disease. For the majority of patients who did not respond, efforts are already underway to try to convert the non-responders into responders. A number of additional factors in the TME outside of the PD1:PDL1 axis have been suggested to contribute to ICB escape in non-responders. Therefore, composite biomarkers and a multi-modal approach that increases tumor immunogenicity, T cell priming, and T cell intratumoral infiltration might help to circumvent intrinsic or acquired resistance to ICB. Past and ongoing clinical studies we highlighted in this review will help to inform further investigations, better design of future clinical trials, and reveal strategies on how to effectively translate or integrate ICB with other treatments for better cure rates. Even though challenges lie ahead, the accelerated approval of pembrolizumab, the follow-up studies involving combinations thereof, and other studies aimed at personalized care and better predictive biomarkers could improve the utility of ICB in the clinical management of CC in upcoming years.

Compliance with Ethical Standards

Conflict of interest
Lennox Chitsike declares that there is no conflict of interest. Penelope Duerksen-Hughes declares that there is no conflict of interest.

Human and animal rights and informed consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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