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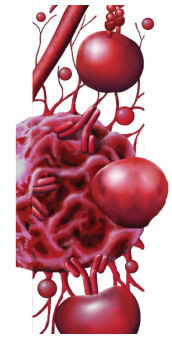

Future directions in bladder cancer immunotherapy: towards adaptive immunity

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The clinical management of bladder cancer has not changed significantly in several decades. In particular, intravesical bacillus Calmette–Guérin (BCG) immunotherapy has been a mainstay for high-risk nonmuscle invasive bladder cancer since the late 1970s/early 1980s. This is despite the fact that bladder cancer has the highest recurrence rates of any cancer and BCG immunotherapy has not been shown to induce a tumor-specific immune response. We and others have hypothesized that immunotherapies capable of inducing tumor-specific adaptive immunity are needed to impact bladder cancer morbidity and mortality. This article summarizes the preclinical and clinical development of bladder cancer immunotherapies with an emphasis on the last 5 years. Expected progress in the near future is also discussed.

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Bladder cancer overview

Bladder cancer is the 5th most common cancer in the USA with an estimated 74,000 new cases, 16,000 deaths and a total domestic prevalence of more than 570,000 in 2015 [1,2]. Most new cases (70–80%) are diagnosed as nonmuscle invasive bladder cancer (NMIBC) with an associated 15-year survival of 62–95% [3]. Low-risk NMIBC, typically represented by small, papillary tumors confined to the urothelium, is well-managed via transurethral resection of the bladder tumor (TURBT) often followed by intravesical instillation of mitomycin C. High-risk NMIBC, including high-grade carcinoma *in situ* (CIS) and tumors invading the lamina propria, is treated via TURBT followed by intravesical immunotherapy with *Mycobacterium bovis* bacillus Calmette–Guérin (BCG) [4–6]. A significant minority of patients (20–30%) initially present with one or more tumors that have invaded the muscle layer of the bladder. Because muscle invasive bladder cancer (MIBC) can rapidly progress to metastatic disease, cystectomy, or surgical removal of the bladder, is standard of care.

Despite the fact that bladder cancer is diagnosed relatively early in the course of disease, it has the highest recurrence rate of any malignancy at 50–80%. These high recurrence rates necessitate long-term maintenance therapy and regular surveillance [7,8] which in turn causes bladder cancer to have the highest lifetime treatment costs per patient of all cancers [9].

Immunologic challenges in bladder cancer

Bladder cancer, with the third highest rate of somatic mutations, is one of the most immunogenic cancers [10]. Yet bladder cancer is able to evade immune-mediated elimination even in the presence of antigen-specific immune cell infiltration. Understanding the immunoevasive strategies employed by blad-

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der tumors is key to developing therapies capable of inducing adaptive responses. This section will provide a high level overview of some of the challenges associated with generating adaptive immunity in the bladder as well as some of the means employed by bladder tumors to evade destruction.

The first challenge in inducing an adaptive response to bladder cancer is the unique immunological milieu within the organ itself. The bladder epithelium is infiltrated by mast cells, macrophages, dendritic cells and T cells, but the overall organization of the immune system within the bladder is the result of a balance between conflicting needs. On one hand, the lumen is routinely exposed to noncommensal bacteria and must implement strategies to subvert infection. On the other hand, the bladder must be able to store high concentrations of self-antigens and toxins without eliciting an immune response. The bladder's balanced strategy includes a thick mucin layer, secretion of antibacterial agents and rapid micturition to limit penetration of invading microorganisms while establishing an immunosuppressive environment to limit undesirable immune responses [11]. Because of this unique milieu, the establishment of a tumor-specific adaptive immunity in the bladder is difficult, although not impossible as will be discussed later in this review.

Another obstacle to immunotherapy is the immunosuppressive bladder tumor microenvironment. Bladder tumors can anergize tumor infiltrating lymphocytes (TILs) [12] and promote the accumulation of immunosuppressive myeloid cells. Bladder tumors have also been associated with high levels of regulatory T-cells (Tregs) and T_H1 inhibitory cytokines such as IL-10 [13]. Horn *et al.* showed that higher FOXP3:CD3 and FOXP3:CD8 ratios in bladder tumor infiltrates correspond to worse overall survival in patients who underwent radical cystectomy, suggesting that infiltration by Tregs may support tumor invasion [14]. However, a retrospective study by Winerdal *et al.* showed that a higher $FOXP3^+$ TIL density correlated with improved outcomes while $FOXP3^+$ tumor cells corresponded with poorer outcomes [15]. A recent review covers the influence of TILs in bladder cancer in greater detail [16].

Bladder cancers, like many cancers, utilize immune checkpoints to modulate immunity. Specifically, bladder tumors can promote immune tolerance by overexpressing immune checkpoint ligands capable of inhibiting activated T cells. The most commonly investigated immune checkpoint molecules are PD-1, PD-L1 and CTLA-4, but there are multiple other regulatory molecules, such as LAG-3 and TIM-3, that are potential targets for bladder cancer immunotherapy [17]. Several clinical studies have shown that bladder tumors and infiltrating immune cells exhibiting increased expression of PD-L1 and PD-1 are associated with poorer outcomes [18–21]. Investigations into the effects of CTLA-4 expression have been limited with conflicting opinions as to whether the CTLA-4 +49 A>G polymorphism increases or decreases bladder cancer risk [22–24].

An additional challenge is that bladder tumors, like many other cancers, can produce escape variants by removing their surface expression of major histocompatibility complex I (MHC I) molecules [25]. MHC I is essential for displaying tumor antigens vital for recognition by T cells. Several studies have shown that bladder tumors can be heterogeneous in their display of MHC I [26]. Some express normal levels of MHC I and are associated with better outcomes. Other, so called 'soft', tumors have low MHC I expression that can be upregulated to normal levels through IFN-mediated mechanisms. Still others, termed 'hard' tumors, have permanent alterations in MHC I expression due to a genetic mutation in the processing machinery or one of the MHC I components. There is some evidence that while both normal and soft tumors are BCG responsive, MHC I negative hard tumors may be BCG resistant leading to tumor escape [27,28]. It should be noted that any truly MHC I negative tumor should be susceptible to natural killer (NK) cell mediated cytotoxicity yet the mechanism by which these tumors might escape NK cell surveillance has not yet been described.

Limitations of BCG immunotherapy

Since the pioneering work of Morales *et al.* in 1976 [29], BCG has served as the standard intravesical immunotherapy for high-risk NMIBC. On one hand, its longevity can be used to highlight its effectiveness in the management of NMIBC. However, as mentioned previously, bladder cancer has the highest recurrence rate of all cancers and the four-decade-long lack of progress in the treatment of NMIBC underscores the need for new and more effective therapies.

Intravesically administered BCG promotes an inflammatory reaction in the bladder wall that is known to reduce recurrence rates. However, the exact mechanism(s) by which BCG exerts its antitumor effects is unknown. A suggested model of BCG immunotherapy is outlined in an excellent review by Redelman-Sidi *et al.* [30]. Briefly, BCG attaches to urothelial cells via fibronectin and integrin $\alpha5\beta1$ before internalization by bladder cancer cells through micropinocytosis. BCG infection can decrease the proliferation of bladder cancer cells and is cytotoxic only at high concentrations. Thus, it is likely that the majority of BCG's antitumor activity is accomplished indirectly. Infected bladder cancer cells secrete a number of cytokines including IL-6, IL-8, GM-CSF and TNF-α. BCG infection also causes the upregulation

Table 1. Summary of new therapeutics being investigated for bladder cancer.

Therapeutic strategy	Therapeutic form	Delivery site	Furthest stage of development	Disease targeted	Ref.
TLR agonists	TLR-9 agonist CpG	IT	Preclinical	NMIBC	[36]
	TLR-2 agonist HP-NAP	IVes, IT	Preclinical	NMIBC	[37]
	TLR-7 agonist TMX-101	IVes	Phase II	NMIBC, CIS	[38]
Cancer vaccines	DC-targeted tumor antigens	SC	Phase II	MIBC	[45,46]
	Natural adjuvants + *HER2/NEU* plasmid	SC	Preclinical	NMIBC	[39–42]
	Peptide vaccine	SC	Phase II	NMIBC	[44]
	shRNA for FOXO3 + *HER2/NEU* plasmid	SC	Preclinical	NMBIC, MIBC	[43]
	Fowlpox vector, PANVAC	SC	Phase II	NMIBC	NCT02015104
	HS-410 cell line	SC	Phase I/II	NMIBC	NCT02010203
IFN-α	Recombinant	IVes	Phase II	NMIBC	[50–55]
	rBCG	IVes	Preclinical	NMIBC	[56,58]
	Virus	IVes	Phase II	NMIBC	[57]
GM-CSF	Vaccine	IVes, SC	Preclinical	NMIBC, Met	[59–62]
	Oncolytic virus	IVes	Phase II	NMIBC	[63]
IL-2	Recombinant	IVes	Phase I/II	NMIBC	[67,70]
	Vaccine	IVes, SC	Preclinical	NMIBC, Met	[61,68,69]
	p53 TCR fusion protein	IV	Phase II	NMIBC, MIBC	NCT01326871 NCT01625260
IL-12	Recombinant	IVes	Phase I	NMIBC	[74]
	Recombinant + chitosan	IVes	Preclinical	NMIBC	[75,76]
	Plasmid	IVes, IT	Preclinical	NMIBC	[72,73,78]
	Retrovirus	SC	Preclinical	NMIBC	[77]
IL-15	Plasmid	IVes	Preclinical	NMIBC	[79]
	Fusion protein (ALT-803) + BCG	IVes	Phase I/II	NMIBC	[80]
Checkpoint inhibitors	αCTLA-4	IV	Phase II	Met	[24,81]
	αPD-L1	IV	Phase II	Met	[82]
	αCLTA-4, αPD-L1 + CpG	IT	Preclinical	NMIBC	[36]
Anti-CD40	Anti-CD40	IT	Phase I	NMIBC, Met	[83]
Anti-IL-10	Anti-IL-10	IV	Preclinical	NMIBC, Met	[84–86]

IT: Intratumoral; IV: Intravenously or systemically; Ives: Intravesically; Met: Metastatic; MIBC: Muscle-invasive bladder cancer; NMIBC: Non-muscle-invasive bladder cancer; SC: Subcutaneous.

of MHC II and ICAM-1 on bladder cancer cells, thus enhancing their antigen presenting capabilities. The initial cytokine burst leads to the recruitment of granulocytes, monocytes and macrophages and the subsequent release of additional cytokines including IL-1, IL-2, IL-5, IL-6, IL-8, IL-12, IL-18, TNF-α, IFN-γ and GM-CSF. This second cytokine burst leads to the recruitment and activation of NK cells, $CD4^+$ helper T cells (T_H) and $CD8^+$ cytotoxic T cells (CTLs). Tumor cell killing is likely primarily accomplished by NK cells, $CD8^+$ T cells, macrophages and granulocytes through the release of TRAIL. Despite the involvement of T lymphocytes, BCG immunotherapy has not been shown to induce a tumor-specific immune response.

Overall, 20–30% of patients will fail initial BCG therapy and 30–50% of BCG responders will develop recurrent tumors within 5 years [31]. An incomplete understanding of BCG's antitumor mechanisms has made it difficult: 1) to predict responses to BCG; 2) to optimize BCG immunotherapy; and 3) to understand why some patients ultimately fail BCG immunotherapy. Separately, BCG is a live pathogen and causes severe infections, including life threatening sepsis, in approximately 5% of patients [32]. At least seven

patients have died from BCG sepsis following bladder instillations [32] and 30–40% of patients discontinue intravesical BCG immunotherapy due to local and systemic toxicities [33]. Lastly, despite four decades of experience, it is still not clear which of the 6+ strains of BCG is most effective at treating NMIBC.

Novel strategies for bladder cancer immunotherapy

The aforementioned challenges associated with initiating an adaptive immune response within the bladder and bladder cancer milieu can be overcome with appropriate strategies. An effective strategy should: 1) reverse the immunosuppressive environment of the bladder tumor; 2) initiate an inflammatory response at the tumor site capable of eliminating the primary tumor; 3) activate tumor antigen-specific T-cells capable of eliminating secondary local or distant lesions; and 4) establish a reservoir of memory T-cells with polyclonal specificity to monitor for recurrent tumors. There is a long history of strategies that have attempted to overcome one or more of these challenges. The goal of this section is to present an overview of recent (published within the last 5 years) strategies being investigated in preclinical and clinical settings for the treatment of superficial and metastatic bladder cancer and to provide commentary on those strategies' ability or potential to establish an adaptive memory response. A summary of immunotherapy strategies is given in Table 1.

TLR agonists

Perhaps inspired by BCG, one of the most common strategies investigated in preclinical studies is the activation of innate immune pathways [34,35]. Toll-like receptor (TLR) pathways are of particular interest in cancer immunotherapy. TLRs are a family of receptors that bind to common components of many pathogens as well as signals released by damaged cells. TLRs are expressed on many innate immune cells, including dendritic cells. The most potent of all antigen presenting cells, dendritic cells play a pivotal role in bridging the innate and adaptive responses. Thus, targeting dendritic cell TLRs is a common strategy for enhancing adaptive immune responses. TLRs are also present on a large portion of bladder tumors where higher TLR expression is correlated with less invasive tumors [35]. With their wide expression on a variety of cells and their ability to link adaptive and innate immune responses, TLR ligands make attractive candidates for potential bladder cancer therapeutics. As such, several TLR ligands have been investigated at both the clinical and preclinical level. There are more than 15 years of studies regarding TLR ligands against bladder cancer, and for a more complete picture please see the excellent review by LaRue *et al.* [35]. The following will provide a brief update of findings within the last 5 years.

TLR9 agonistic CpG oligodeoxynucleotides were investigated preclinically in conjunction with checkpoint inhibitors against a subcutaneous (s.c.) MB49 bladder tumor model. CpG, administered peritumorally, in combination with intraperitoneal (i.p.) anti-CTLA-4, anti-PD-1 or anti-PD-L1 antibodies resulted in 6/7, 5/7 and 2/7 long-term survivors, respectively. All combinations of CpG and checkpoint inhibition were more effective than combinations of BCG plus checkpoint inhibition as well as checkpoint inhibition alone [36]. The TLR2 agonist HP-NAP, a bacterial protein produced by *helocobacter pylori*, was tested against both subcutaneous and orthotopic MB49 tumors in mice. In these studies, HP-NAP increased both the numbers and percentages of $CD4^+$ and $CD8^+$ T cells in local lymph nodes and limited tumor growth more efficiently than BCG [37].

In the clinic, the TLR7 agonist imiquimod (TMX-101) was well tolerated by 7 NMIBC patients in a Phase I study [38]. A Phase II trial with TMX-101 was recently completed against CIS (NCT01731652). No data from this study have been published at the time this review was published. Overall, TLR agonists and other stimulants of the innate immune system hold promise as adjuvants for initiating the antitumor response but will likely need to be used in conjunction with other therapeutics.

Cancer vaccines

Therapeutic cancer vaccines are under development to induce adaptive immune responses to one or more tumor-associated antigens. Several different forms of cancer vaccines have been explored recently. Vaccines comprised of tumor lysates are easy to produce and have the potential to induce polyclonal responses against both known and unknown tumor antigens. However, because tumor lysates are poorly immunogenic, the evaluation and inclusion of effective adjuvants is critical. The antimicrobial peptide shrimp anti-lipopolysaccharide factor (SALF) has been evaluated preclinically as an adjuvant for a tumor lysate vaccine. Mice were given SALF mixed with MBT-2 tumor cell lysate on days 7, 14 and 21 before subcutaneous inoculation with MBT-2 on day 28. Tumors developed in mice treated with MBT-2 lysate alone (5/7) and SALF alone (7/7), but not in those treated with the mixture (0/7). Tumor-specific memory was shown via CTL assays using splenocytes from treated mice [39]. A similar study investigated a different antimicrobial peptide, GE33. Using the same treatment schedule, tumor development was more pronounced in mice treated with MBT-2 lysate alone (5/7) and SALF alone

(7/7) than those treated with the mixture (2/7) [40]. In the last 5 years, there have been no clinical trials using tumor lysate-based vaccines against bladder cancer.

Similarly, tumor antigen encoding plasmids are a useful source of tumor antigen, however, adjuvants are needed to induce a robust immune response. Extracts from the mushroom *Clitocybe nuda* have been shown to activate dendritic cells. Mice bearing s.c. MBT-2 which express p185neu received three weekly intramuscular injections of *HER2/NEU*-encoding plasmids with or without *C. nuda* extract [41]. The addition of *C. nuda* extract increased the number of survivors at 60 days from 2/7 to 5/7. Untreated mice and mice receiving the extract alone were moribund by day 35. The spleens of treated mice were analyzed for response to HER2 peptide stimulation. There were increased percentages of activated $CD8^+$ T-cells in the combination group compared to either therapy alone. An *in vitro* stimulation of bone marrow-derived dendritic cells demonstrated that *C. nuda* was able to enhance immunomodulatory markers (CD40, CD80, CD86, MHC I and MHC II) at levels similar to LPS.

In a similar study, Ling Zhi-8 (LZ-8) isolated from *Ganoderma lucidum* (Lingzhi mushroom) was used as an adjuvant for *HER2/NEU*-encoding plasmids against s.c. MBT-2 tumors [42]. LZ-8 was found to stimulate dendritic cells through TLR4 and enhanced the ability to induce an antigen-specific immune response in an *in vitro* experiment. In antitumor studies, LZ-8 was administered i.p. while the *HER2/NEU* gene was given once a week for three weeks. LZ-8 did not improve long-term survival, however, plasmid alone or plasmid plus LZ-8 showed extended survival relative to untreated mice or LZ-8 alone.

Another strategy to enhance immune responses to plasmid-based vaccines utilized small hairpin RNA (shRNA) to silence *FOXO3*, an immunosuppressive transcription factor. Both silencing and *HER2/NEU* plasmids were administered once a week for 3 weeks to mice bearing MBT-2 tumors. The inclusion of *FOXO3* silencing increased the number of mice surviving >70 days from 1/7 to 5/7. Antitumor responses were mediated by $CD8^+$, but not $CD4^+$ cells. In an experimental pulmonary metastases model, lung weights were significantly lower in both *HER2/NEU* and *HER2/NEU* plus *FOXO3* shRNA groups when compared with untreated controls [43]. To our knowledge, no clinical trial for bladder cancer in the last 5 years has evaluated a plasmid-based cancer vaccine.

Peptide-based cancer vaccines are useful for generating immune responses against unique and well-defined epitopes. In a Phase I study in Japan, peptides from bladder cancer oncoproteins, MPHOSPH1 and DPEPDCI, most likely to bind to the most common HLA-A type in the Japanese population were identified via BIMAS HLA peptide binding predictions [44]. Six patients with unresectable bladder cancer were vaccinated with 1 mg of each peptide in incomplete Freund's adjuvant once a week for 4 weeks. The peptide vaccines were well-tolerated with no severe adverse events. There were peptide-specific $CD8^+$ responses against MPHOSPH1–278 and DEPDC1–294 peptides in 3/4 and 4/6 evaluated patients, respectively. A subsequent Phase II study is ongoing (NCT00633204).

CDX-1307 is a fusion molecule in which a mannose receptor targeted antibody is linked to a tumor antigen, β-hCG. Targeting the mannose receptor on antigen presenting cells is expected to increase the likelihood that β-hCG is taken up, processed and displayed to antigen-specific T cells. Two Phase I studies evaluating CDX-1307 in patients with advanced cancers, including bladder cancer, were recently completed, however, no published data could be found. A Phase II study (NCT01094496) to investigate the efficacy of CDX-1307 given against muscle invasive disease began recruitment in 2010, but was recently terminated due to slow enrollment [45].

A related molecule, CDX-1401, which is comprised of human monoclonal antibody specific for DEC-205, a dendritic cell receptor, fused to the full-length tumor antigen NY-ESO-1, was the subject of a recently completed Phase I study in patients with advanced malignancies, including advanced bladder cancer [46]. The vaccine was well tolerated and 2/45 patients experienced a >20% tumor regression while 13/45 patients exhibited stable disease. It could not be determined if any of these responses were in bladder cancer patients.

Two additional bladder cancer vaccines of interest are being evaluating in clinical studies with no published results at the time this review was written. An ongoing Phase I study will evaluate a fowlpox vector expressing carcinoembryonic antigen, mucin-1 and three costimulatory molecules with and without BCG in high-grade NMIBC patients who have failed BCG (NCT02015104). An ongoing Phase I/II study will assess HS-410, a vaccine comprised of irradiated bladder cancer cells engineered to secrete gp96, alone or in combination with BCG in NMIBC patients (NCT02010203).

Cytokines

Cytokines are potent nodes of communication within the immune cell network. Their potential to act as immunotherapeutics has long been recognized but translation of cytokines into the clinic has faced some significant obstacles. In particular, widespread clinical application of cytokine-based cancer immunotherapies has been prevented by inappropriate systemic deliv-

ery of proinflammatory cytokines. Nearly all clinical trials, both past and present, evaluating cytokine monotherapies utilize systemic, that is, intravenous or s.c., injections. However, cytokines function primarily through paracrine and autocrine mechanisms and thus are rarely measurable in the circulation of healthy individuals. In addition, cytokine communication networks are far more complex than initially conceived. Cytokines are pleiotropic and often redundant which can lead to off target effects. In sum, the systemic delivery of multifunctional cytokines risks unintentional signaling and dose-limiting toxicities.

Fortunately, cytokine-based bladder cancer immunotherapies have significant potential due to the bladder's accessibility and ability to sequester high concentrations of cytokines with limited systemic exposure. The following sections will cover recent studies in which cytokines in a variety of formats including recombinant protein, viral vectors, plasmids, transfected BCG and transfected tumor cells are used for the treatment of bladder cancer. Many of the cytokines investigated for intravesical therapy have been inspired by downstream molecules released after BCG treatment including IFN-α, GM-CSF, IL-2 and IL-12.

IFN-α

IFN-α is a type 1 interferon produced primarily by innate immune cells in response to invading microbes, particularly viruses [47]. IFN-α2b is approved as a monotherapy for malignant melanoma, hairy cell leukemia and Kaposi's sarcoma [48]. IFN-α binds to a heterodimeric receptor complex that is expressed on the surface of most cell types, including many cancer cells. Thus, IFN-α can act directly upon cancer cells to induce a range of effects including apoptosis and upregulation of tumor cell-surface antigens. Indirect antitumor activities of IFN-α include dendritic cell maturation.

IFN-α, as both a monotherapy and in combination with BCG, has been widely explored against bladder cancer including as the subject of a number of clinical trials [49]. Despite promising preclinical results, the consensus of clinical studies is that IFN-α alone is no more effective than current treatments at preventing recurrence of NMIBC and in some instances is less effective. One recent example showed that alternating IFN-α with BCG during maintenance therapy was less effective than BCG alone at preventing recurrence with 80% and 45% experiencing a recurrence within 15 years, respectively [50]. Another study showed that BCG was more effective than IFN-α + epirubicin with recurrence-free survival rates of 38% and 59% at a follow-up time of 5 years [51]. A retrospective study found that patients given a salvage therapy of IFN-α + BCG had reduced recurrence-free survival when compared with those patients treated with BCG alone [52].

Only a single study has directly compared IFN-α plus BCG against BCG alone in a controlled, randomized clinical trial [53]. This study found no significant difference between treatment groups in the percentage of patients that were tumor free after 2 years. However, preclinical and clinical data have shown that IFN-α can enhance the therapeutic efficacy of reduced doses of BCG, a strategy that could potentially limit the toxic side effects seen in many patients treated with BCG [54,55].

Several studies have explored the use of BCG which has been genetically modified to express IFN-α or T_H1 cytokines [56–58]. These studies have shown an enhanced ability to stimulate immune cells *in vitro* against human bladder cancer cell lines, but cytokine-expressing BCG has not progressed to clinical trials. A related strategy is the direct transfection of the tumor or the urothelium via plasmids or viruses. While there have been numerous preclinical efforts using this strategy, clinical efforts have thus far been limited to a single Phase I trial (NCT01162785) in which an adenovirus encoding IFN-α was delivered intravesically [57]. IFN-α was produced for up to 5 days after administration and was well tolerated. A Phase II trial (NCT01687244) is ongoing to further investigate the efficacy of this strategy.

Overall, IFN-α has been the most common cytokine used as either a monotherapy or a combination therapy with BCG. Despite the number of investigations, IFN-α has shown limited effectiveness. While IFN-α exhibits potent cytostatic/cytotoxic activity and can enhance tumor immunogenicity, it may not be able to effectively engage the adaptive immune system.

GM-CSF

GM-CSF is a hematopoietic growth factor and immune modulator that is secreted by a range of cells including endothelial cells, macrophages, T-cells and some tumor cells. As implied by its name, GM-CSF stimulates the production of granulocytes and monocytes from hematopoietic progenitors. In addition, GM-CSF activates macrophages and promotes dendritic cell development. GM-CSF has been widely used in cancer vaccine and immunotherapy approaches due to its ability to enhance dendritic cell function.

Preclinically in bladder cancer, the delivery of recombinant GM-CSF to the bladder tumor environment can be enhanced via streptavidin and biotin-based targeting [59–62]. For example, 1-day old orthotopic MB49 bladder tumors treated intravesically first with biotin for 30 min followed by streptavidin tagged GMCSF (SA-GMCSF) for 1 h resulted in elimination

of 6/16 tumors versus 1/10 for recombinant GM-CSF alone [59]. These results suggest that maintenance of GM-CSF at the tumor site increases its efficacy. In subsequent studies, ethanol fixed MB49 cells were labeled with SA-GMCSF *in vitro* before being applied as both therapeutic and preventative vaccines against s.c. and lung metastasized MB49 [60,62]. Mice treated with the vaccine showed extended survival while cured mice in each of these studies demonstrated tumor-specific immunity.

In recent clinical studies, CG0070, an oncolytic adenovirus virus expressing GM-CSF and programmed to replicate only in cells with retinoblastoma (Rb) pathway defects was evaluated in a Phase I trial for NMIBC (NCT00109655) [63,64]. Patients enrolled in this study had all experienced at least one failed treatment with BCG. Different doses and treatment schedules were tested intravesically on a total of 35 patients with no reports of severe adverse events. Response rates were promising with 17/35 (48.6%) of patients exhibiting a complete response. CG0070 is currently being evaluated in a Phase II trial (NCT01438112).

It should be noted that GM-CSF can be a double-edged sword. On the one hand, GM-CSF drives the proliferation of leukocytes and the development of antigen presenting cells which are needed for a robust adaptive response. On the other hand, GM-CSF has been shown to expand immature myeloid subsets, such as myeloid derived suppressor cells, that can act as immune suppressors. Knowledge of proper dosing and delivery strategies is needed to drive appropriate responses [65].

IL-2

IL-2 induces the proliferation and activation of lymphocytes and is approved for the treatment of metastatic renal cell carcinoma and metastatic melanoma. Unfortunately, IL-2 also promotes the growth of tumor-supporting Tregs. The history of IL-2 in the treatment of bladder cancer is quite long, stretching back to intralesional treatments of NMIBC in 1984 [66]. However, despite promising preclinical data, results from clinical studies have been mixed.

Clinical study of IL-2 immunotherapy for bladder cancer in the last 5 years has been limited. Most studies have been small with less than 30 participants and have not compared IL-2 against alternate therapeutics. In a recent pilot study, 36 patients with NMIBC were treated with intravesical IL-2 immunotherapy after complete or intentionally incomplete TURBT to assess the importance of residual marker lesions [67]. Marker lesions are a potentially important source of tumor-associated antigens. Intravesical IL-2 immunotherapy was found to be more effective at preventing recurrence when a marker lesion was left behind than complete TURBT with median recurrence-free survival times of 20 and 7 months, respectively [67]. One other clinical-stage investigation with IL-2 involves a fusion protein, ALT-801, composed of IL-2-linked to a p53-specific T-cell receptor. ALT-801 has shown effectiveness in other tumors and is currently being investigated in clinical trials against both MIBC (NCT01326871) and NMIBC (NCT01625260).

Preclinically, a few studies have investigated the utility of MB49 bladder cancer cells treated with biotin and streptavidin-IL-2 as a vaccine. In one study, vaccinated mice exhibited both therapeutic and preventive immune responses against MB49 lung metastases [68]. A similar study showed that sequential vaccination with GM-CSF-bound MB49 cells followed by IL-2-bound MB49 cells was more effective than either alone at prolonging survival and inducing memory [61]. Another study investigated the ability of streptavidin-IL-2 to bind to biotin-pretreated orthotopic MB49 tumors in mice. This delivery approach resulted in prolonged persistence of IL-2 on the bladder surface and led to enhanced survival over IL-2 alone. Nine of 25 mice with 2-day old tumors treated with the biotin/streptavidin-IL-2 approach survived longer than 60 days. Perhaps more importantly, 5/9 long-term survivors rejected an intravesical rechallenge with MB49 cells [69].

Another study investigated IL-2, IFN-α and gemcitabine as single agents and in combination with BCG against orthotopic AY-27 tumors in rats [70]. BCG was given at low (5×10^5 cfu/ml), medium (5×10^6 cfu/ml) and high (5×10^7 cfu/ml) doses. Recombinant IL-2 (5×10^5 units) was combined with low-dose BCG. Two out of 10, 4/10, 5/10 and 5/10 rats were alive at day 90 with 0, 3, 4 and 3 being completely tumor-free in the low, medium, high and combination treatment group, respectively. IFNα and gemcitabine combined with BCG showed no additional survival benefit over any of the agents alone. As single agents, IFN-α and gemcitabine were as effective as BCG.

Overall, IL-2 remains a promising candidate for bladder cancer immunotherapy due to its ability to induce adaptive responses and protective immunity. As with IL-2-based immunotherapies in other cancers, immune-enhancing benefits must be weighed against the potential for amplification of tumor-supporting Tregs. To assess the full potential of IL-2-based immunotherapy for bladder cancer, additional clinical studies are needed to either combine or compare IL-2 and BCG.

IL-12

IL-12 is a proinflammatory T_H1 polarizing cytokine produced primarily by antigen presenting cells

in response to danger signals. IL-12 activates NK cells, promotes proliferation of cytotoxic T-cells and increases the production of IFN-γ, a key downstream effector molecule. IFN-γ has direct cytotoxic and/or cytostatic activity on a range of cells, including cancer cells. In addition, IFN-γ promotes antitumor responses indirectly through antiangiogenic responses as well as by increasing MHC expression on tumor cells leading to enhanced immune recognition [71]. In several older preclinical studies, intravesical administration of IL-12 has shown the ability to eradicate lesions and generate local immunity when delivered against orthotopic MBT-2 tumors [72,73].

In the clinic, only one trial has investigated intravesical IL-12 immunotherapy in 15 patients with recurrent superficial bladder cancer [74]. Intravesical administration of IL-12 was well tolerated, however, a maximum tolerated dose was not found and no responses were recorded. A likely reason for the lack of response was the lack of an effective delivery strategy. The urothelium is the least permeable epithelial barrier in the body and IL-12 is a large (70 kDa) protein. It is likely that IL-12 was not able to penetrate into the tumor and surrounding urothelium to reach resident immune cells. Our group has hypothesized that enhancing the delivery of IL-12 will improve its antitumor efficacy.

Our experiments have focused on using chitosan as an intravesical delivery vehicle. Chitosan is a large polymer that forms a viscous and mucoadhesive solution capable of transiently opening the tight junctions of the urothelium. This allows for increased and sustained penetration of IL-12 into the wall of the bladder. In addition, the mucoadhesive feature of chitosan allows it to stick to bladder surface mucin. We have shown that recombinant IL-12 (5 μg) alone can eliminate 50% of established orthotopic MB49 tumors. However, when IL-12 was co-formulated with chitosan solution (chitosan/IL-12) the proportion of complete responders was increased to 88–100% [75]. Of particular interest to the bladder cancer community, 100% of cured mice rejected a subsequent intravesical tumor rechallenge for a least 18 months after the primary treatment, suggesting a local memory response.

A follow-up study investigated chitosan/IL-12, at reduced doses, against established MB49 and MBT-2 tumors [76]. Chitosan/IL-12 was able to eradicate 100% of MB49 tumors at a dose of 1 μg IL-12 and 50% of MBT-2 tumors at a dose of 2.5 μg IL-12. In this instance cured mice rejected both local (intravesical) and distant s.c. tumor rechallenges, demonstrating for the first time that chitosan/IL-12 can induce a systemic tumor-specific immune response from a localized, intravesical therapy. Another seminal finding from this study was that intravesical chitosan/IL-12 immunotherapy was capable of controlling a distant, s.c. tumor. This potentially clinically significant result suggests that intravesical chitosan/IL-12 can induce abscopal responses against metastatic lesions. Therefore, intravesical chitosan/IL-12 could represent a bladder-sparing alternative for MIBC.

Other groups have also demonstrated the potential of IL-12 in preclinical studies. One study showed that IL-12 produced in a gamma-retrovirus targeted to HER2 was able to inhibit tumor growth and enhance survival for 2/12 mice when given intratumorally against s.c. MBT-2 [77] Another study showed that IL-12 has antitumor properties even in the absence of T cells. Nude mice were given human EJ bladder cancer cells subcutaneously and then treated with chemotherapy, IL-12 plasmids, or a combination [78]. The combination therapy was the most effective at decreasing the tumor size, but was unable to eliminate the tumors.

Despite strong preclinical evidence for IL-12's ability to induce a sustained, adaptive antitumor response, there do not appear to be any IL-12-based clinical trials for bladder cancer on the horizon. It is likely that toxicity concerns remaining after two widely publicized IL-12-related deaths in a Phase II advanced renal cell carcinoma study nearly two decades ago have somewhat curbed enthusiasm for IL-12. However, IL-12's toxicity is associated with frequent systemic exposure to the cytokine. A recent focus on the development of controlled, localized delivery strategies capable of limiting systemic exposure promises to reduce toxicity while harnessing the antitumor potential of this powerful cytokine.

IL-15

IL-15 is a T-cell growth factor that shares several features with IL-2. However, among several key differences, IL-15 has been shown to specifically increase the proliferation and maintenance of $CD8^+$ memory T-cells. In bladder cancer, IL-15 has been investigated in two preclinical studies. Matsumoto *et al.* delivered six intravesical doses of liposomes containing IL-15 plasmids in mice bearing established orthotopic MBT-2 tumors [79]. Treated mice experienced reduced tumor burdens, increased $CD8^+$ T-cell infiltration and enhanced survival with 8/20 mice surviving long term. Surviving mice also showed the ability to delay, but not reject, the growth of a s.c. tumor rechallenge. These data suggest that IL-15, like IL-2 and IL-12, is capable of inducing a systemic antitumor immune response.

Another group investigated the efficacy of a mutated IL-15/IL-15Rα-FC fusion protein (termed ALT-803) in conjunction with BCG against chemically induced orthotopic tumors in rats [80]. Treatment with ALT-

803 plus BCG resulted in reduced bladder weights, increased levels of NK cells and enhanced infiltration of $CD3^+$ cells but not $CD8^+$ or $CD4^+$ cells when compared with BCG alone. IL-1α and IL-1β were the primary effector cytokines at the systemic level while RANTES was the primary local cytokine following treatment with ALT-803 plus BCG. Memory responses were not evaluated in this model, but ALT-803 had previously generated a memory response against melanoma in mice. A clinical study inspired by this data is currently recruiting NMIBC patients (NCT02138734).

In sum, IL-15-based immunotherapies are promising due to their ability to induce tumor-specific adaptive immune responses. Like IL-2 and IL-12, in order for IL-15 to reach its full potential, a well-considered delivery strategy is needed. In addition, combinations and dosing schedules with other therapeutics should also be explored. In particular, IL-15 may be most effective in sustaining or augmenting a memory response initiated by some other inductive immunotherapy.

Antibodies

Checkpoint inhibitors

Anti-CTLA-4 antibody (αCTLA-4) therapy, which is approved for the treatment of unresectable or metastatic melanoma, has been investigated in a 2010 clinical trial of 12 patients with localized bladder cancer prior to cystectomy [81]. The primary goal of this study was to identify biomarkers associated with a positive response to αCTLA-4 (ipilimumab). Patients received two doses, 3 mg/kg or 10 mg/kg, of ipilimumab 3 weeks apart with cystectomy 4 weeks after the second dose. A sustained increase in the level of $CD4^+ICOS^{high}$ lymphocytes was found to be associated with enhanced survival [81]. A Phase II clinical trial evaluating ipilimumab in patients with metastatic bladder cancer is underway and expected to be completed by June 2016 [24]. Ipilimumab is also under investigation in combination with the anti-PD-1 antibody (αPD-1), nivolumab, in three clinical studies.

Anti-PD-L1 (αPD-L1) therapies are under clinical investigation for a number of cancer indications and have demonstrated some recent successes in bladder cancer [24]. MPDL3280A was evaluated in 67 patients with metastatic bladder cancer in a Phase I trial [82]. Patients were scored for PD-L1 expression on both infiltrating immune cells and tumors. Treatment with intravenous MPDL3280A led to an objective response rate of 43% in tumors with high levels of PD-L1 expression but only 11% for low expressing tumors, indicating that PD-L1 expression can be used as a predictive biomarker for the success of PD-L1-based therapies. Based on these results, MPDL3280A received breakthrough status by the US FDA and is currently under investigation in multiple clinical trials. Regarding anti-PD-1 immunotherapy, at the time of this review, four clinical trials were recruiting bladder cancer patients to evaluate the safety and activity of nivolumab with an additional nine clinical trials exploring pembrolizumab.

Because of the clinical success of checkpoint inhibitors in other cancers, clinical studies have outpaced preclinical studies in bladder cancer. In the only preclinical study to date, Mangsbo *et al.* administered anti-CTLA-4 or anti-PD-1 antibodies i.p. in combination with peritumoral CpG or BCG in mice bearing day 7 or 8 s.c. MB49 tumors [36]. CpG plus αCTLA-4, αPD-1 or αPD-L1 induced complete regression of large tumors in 6/7, 5/7 and 2/7 mice, respectively. CpG plus check point inhibition (13/21 mice became tumor-free) was superior to BCG plus checkpoint inhibition (4/18), CpG alone (1/7), or checkpoint inhibition alone (10/33). CTLA-4 blockade was superior to PD-1 blockade and the combination was not better than αCTLA-4 alone. Mechanistically, CpG plus αCTLA-4 or αPD-1 increased levels of tumor-reactive T cells and reduced numbers of Tregs at the tumor site.

While the future of checkpoint inhibition in the treatment of bladder cancer appears promising, there is much work to be done at the preclinical level to delineate antitumor mechanisms and explore additional treatment paradigms including novel combinations. It is not clear, from the literature in bladder cancer or any cancer, if checkpoint inhibition alone is sufficient to induce durable antitumor immunity or if combination with other inductive treatments, such as CpG, radiotherapy or cytokine immunotherapy, is needed for durable antitumor immunity. An additional area of exploration is local/intravesical delivery of checkpoint inhibitors. Checkpoint inhibitors cause severe immune-related adverse events in a significant minority of patients. Similar to our IL-12 studies, intravesical delivery of checkpoint inhibitors could minimize the adverse events associated with high systemic concentrations while maintaining high local concentrations at the tumor site.

Agonistic antibodies

CD40 is a costimulatory molecule found on antigen presenting and phagocytic cells, such as macrophages and dendritic cells. The binding of CD40 to CD40L located on T_H cells results in activation of, as well as enhanced phagocytosis and cytokine production by, antigen presenting cells. CD40 is also present on some tumors where anti-CD40 antibodies can have a direct antitumor effect by inducing apoptosis or anti-

body dependent cytotoxicity. For these reasons, anti-CD40 antibodies have been investigated as a potential immunotherapeutic both clinically and preclinically, but its use against bladder cancer has been limited.

Mangsbo *et al.* showed that peritumoral administration of anti-CD40 antibodies was able to eradicate established s.c. MB49 tumors as effectively as systemic (i.p.) administration [83]. Local delivery also reduced systemic inflammation as measured by haptoglobin levels. Importantly, local delivery of anti-CD40 antibodies was able to control distant tumors while cured mice were protected from rechallenge in a tumor-specific manner. These data demonstrate that local anti-CD40 therapy is capable of inducing systemic, adaptive immunity.

A follow-up study using fully humanized anti-CD40 (ADC-1013) showed that locally administered ADC-1013 initiated an antitumor response against human s.c. EJ bladder tumors in immunodeficient mice whose immune systems were reconstituted with human dendritic cells and T-cells [52]. This study also demonstrated that ADC-1013 given peritumorally in one MB49 tumor was able to control a second distant MB49 tumor. A tumor-specific memory response mediated by $CD8^+$ and $CD4^+$ T cells was capable of rejecting MB49 rechallenge up to 5 months after treatment. A Phase I trial to assess the safety of ADC-1013 is currently recruiting patients with advanced solid tumors, including bladder tumors (NCT02379741).

While investigations into agonistic antibodies for the treatment of bladder tumors are quite new, preclinical results are promising. The demonstration of a sustained memory as well as abscopal responses via local delivery is particularly encouraging. More preclinical and clinical investigations of agonistic antibodies are warranted to assess their effectiveness in intravesical delivery as single agents and in combination with other therapies.

Anti-IL-10

IL-10 is an anti-inflammatory cytokine associated with Treg infiltration and poor tumor prognosis in bladder cancer [13]. Several preclinical studies have explored the consequences of blocking IL-10 or its receptor in the context of bladder cancer [84–86]. Blocking IL-10 function via systemically administered antibodies against the IL-10 receptor increased the IFNγ:IL-10 ratio, enhanced the efficacy of BCG against 1-day old lesions, prevented lung metastases and established systemic antitumor immunity as measured via tumor-specific cytotoxic T lymphocyte activity. Overall, the strategy of blocking the effects of IL-10 is promising especially when paired with an inductive treatment such as BCG, TLR agonists, or inflammatory cytokines.

Future perspective

After decades of research and unfulfilled promises, the era of cancer immunotherapy has finally arrived. Within the last 6 years, there have been a number of breakthrough approvals including the first therapeutic cancer vaccine, sipuleucel-T, three monoclonal antibodies targeting immune checkpoints, ipilimumab, nivolumab and pembrolizumab, and an oncolytic virus encoding GM-CSF, talimogene laherparepvec. In particular, the approvals of CTLA-4 (ipilimumab) and PD-1 (nivolumab and pembrolizumab) blockade for advanced melanoma and metastatic lung cancer have created substantial momentum. Currently, there are more than 470 clinical studies evaluating one or more of these checkpoint inhibitors, with and without additional therapies, in various cancers including bladder cancer. As mentioned previously, checkpoint blockade may induce stronger, more durable antitumor responses when combined with an inductive immunotherapy that can initiate a robust adaptive immune response.

With regard to bladder cancer, over the next 5–10 years, the expanding indications of CTLA-4 and PD-1 blockade are likely to include muscle invasive and/or metastatic bladder carcinomas. There are currently 12 clinical studies investigating CTLA-4 or PD-1 checkpoint inhibition in advanced, MIBC or high-risk bladder cancer. The 5-year survival rate for MIBC is 30 to 50% depending on tumor status and lymph node involvement. Given that advanced melanoma has similar survival rates and is similarly immunogenic as MIBC in terms of mutational load, it would not be surprising if clinical studies demonstrate similar clinical benefits. However, like in melanoma, checkpoint blockade is not expected to induce complete responses in the majority of patients with MIBC. As such, it does not appear likely that checkpoint blockade alone could prevent life altering cystectomy in a majority of patients. Perhaps combining checkpoint blockade with another inductive immunotherapy could be bladder sparing.

Another combination approach worthy of future consideration is chemotherapy plus immunotherapy. Bladder cancer is responsive to cytotoxic agents. Intravesical mitomycin C is routine for low grade NMIBC following TURBT while systemic cisplatin is standard for metastatic bladder cancer. In addition, a growing literature in diverse cancers demonstrates that cytotoxic agents can enhance tumor recognition by the immune system, in some cases revealing neoantigens. Furthermore, certain chemotherapy agents can eliminate or inhibit tumor-supporting immunosuppressive cells. Examples include cyclophosphamide for inhibition of Tregs and oxaliplatin for inhibition of regulatory B cells.

In addition to combination immunotherapies, there are two additional approaches for bladder cancer that should be considered. First, it may be more effective and safer to administer checkpoint inhibitors intravesically instead of intravenously. As described in this review, a number of preclinical studies have demonstrated that local immunomodulation of the bladder through intravesical delivery of cytokines and agonists can result in durable local and systemic adaptive immune responses. The intravesical route provides direct access to bladder tumors and a unique opportunity to reduce adverse events associated with systemic delivery of immunotherapy. Our group is currently investigating the efficacy of localized checkpoint blockade in preclinical models. Preliminary data indicate that αCTLA-4 exhibits greater orthotopic bladder tumor inhibition than αPD-1 when administered intravesically.

Secondly, although the vast majority of clinical studies investigating checkpoint blockade are in advanced cancers, it is expected that checkpoint blockade will be most effective against smaller tumor burdens such as NMIBC. As discussed below, overcoming BCG entrenchment is a significant challenge. However, combining checkpoint blockade with BCG is a logical intermediate step. Preclinical studies have shown that checkpoint blockade plus BCG is superior to BCG alone, however, combinations of checkpoint blockade with other immunotherapies are more effective.

Beyond the inevitable evaluation of checkpoint inhibition, there are a number of other immunotherapies, including IL-12, IL-15 and anti-CD40 that are deserving of clinical consideration in bladder cancer. Each of these immunotherapies, unlike BCG, have demonstrated the ability to induce tumor-specific immunity. Like most novel cancer treatments, development and clinical translation of these immunotherapies will likely require investment from the biopharmaceutical industry.

Finally, we speculate on the future of BCG immunotherapy in the treatment of high-risk NMIBC. While BCG has been touted by some as the most successful cancer immunotherapy to date, it is not without significant concerns. The immunological limitations of BCG were presented in a previous section and can be summarized by the dogma that BCG is incapable of inducing tumor-specific adaptive immunity. In order to significantly impact recurrence rates, we believe that a tumor-specific adaptive response is essential.

It has been frustrating to many, researchers, physicians and patients alike, that the use of intravesical BCG for high-risk or high-grade NMIBC is largely unchanged since Morales' study nearly four decades ago [29]. The lack of progress in the management of NMIBC is disconcerting and causes us to ask why have we not been able to find a better immunotherapy than BCG for bladder cancer? The answer to this question is likely multifactorial. First, funding for bladder cancer research is dismal. The National Cancer Institute

Executive summary

Bladder cancer overview
- The clinical management of bladder cancer has not changed significantly in several decades.
- Immunotherapy for bladder cancer is dominated by intravesical BCG for high-risk NMIBC.
- Bladder cancer, due to high recurrence rates, requires continuous and costly follow-up.

Immunologic challenges in bladder cancer
- The immunologic milieu of the bladder is uniquely suited to prevent chronic inflammation in the face of constant antigen exposure.
- Immunosuppressive strategies used by the normal bladder are likely obstacles to effective bladder cancer immunotherapy.

Limitations of BCG immunotherapy
- Intravesical BCG immunotherapy for NMIBC has remained unchanged for nearly four decades.
- BCG has not been shown to establish tumor-specific adaptive immunity.
- The exact mechanisms by which BCG exerts antitumor responses remain elusive.

Novel strategies for bladder cancer
- IFN-α has been widely explored, however, preclinical and clinical results show limited effectiveness.
- Several promising immunotherapies, including IL-12, IL-15 and anti-CD40, have demonstrated the ability to induce tumor-specific immunity.
- Checkpoint blockade has skipped preclinical evaluation and is the subject of intense clinical investigation.

Future perspective
- Checkpoint blockade will continue to be evaluated, with and without additional therapies, in advanced bladder cancer.
- Local delivery of checkpoint inhibitors may be as effective as systemic delivery but with less toxicity.
- The dominant stance held by BCG will have to be softened in order for other immunotherapies to breakthrough for the treatment of NMIBC.

(NCI) invests only about $20M per year in bladder cancer research [87]. This translates to about $280 per newly diagnosed bladder cancer patient. For comparison, breast cancer averages about $2700 in NCI-sponsored research support per newly diagnosed patient. Not to mention, breast cancer receives considerable support from a number of high profile foundations. Second, bladder cancer clinical studies are notoriously difficult to accrue to and comparative studies require large numbers of patients. Third, while BCG is not perfect, there may be a sense that it is 'good enough', and that new therapies are too risky. Such a conservative and risk-averse outlook discourages innovation. Furthermore, clinical studies investigating novel immunotherapies are typically limited to enrolling BCG refractory patients. However, the oft-cited mantra that bladder cancer treatment has not changed in 40 years may be encouraging change. Over the next 5 years, we expect the bladder cancer community's appetite for novel immunotherapies capable of replacing BCG will grow. We expect to see a greater tolerance for clinical studies in BCG naïve patients where immunotherapies have a greater chance for both accrual and better clinical outcomes. Indeed, the next 5 years can be transformational and the hope is that by focusing on immunotherapies capable of establishing adaptive tumor-specific memory, we can significantly impact bladder cancer morbidity and mortality.

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