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MICROBIAL ANTI-CANCER THERAPY AND PREVENTION (PJF RIDER, L SWEENY, AND KG KOUSOULAS, SECTION EDITORS)

Utilizing Microbes to Treat Naturally Occurring Cancer in Veterinary Species

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Abstract

Purpose of Review Therapeutics that harness the immune system to exert their effect may be more critically tested in immunocompetent pet animals than mice or other model systems. This is because pet animals share their environment with humans and spontaneously develop complex, heterogeneous cancers that exhibit similar immunosuppressive microenvironment features. Furthermore, owners of companion animals are increasingly seeking more effective therapeutic options for their pets that go beyond traditional chemotherapy. Microbial-based anticancer therapeutics exploit evolutionarily acquired host-pathogen interactions to break host immune tolerance and/or induce tumor cell death. Therefore, this review summarizes recent studies evaluating microbial-based therapeutics for naturally occurring cancers in veterinary species.

Recent Findings Adenovirus and poxvirus vectors and genetically modified bacteria expressing tumor-associated antigens are the basis of promising therapeutics targeting an array of canine and feline cancers.

Summary Several well-funded multi-institutional clinical trials are currently underway evaluating microbial-based therapeutics for naturally occurring veterinary cancers. Recent advancements in our ability to monitor immune responses in these species and a growing appreciation for the similarities and differences in host-pathogen interactions between humans and animals will assist in future comparative studies.

Keywords Canine cancer · Feline cancer · Comparative · Oncolytic virus · Oncolytic bacteria · Immunotherapy

Introduction

Despite significant advancements beyond traditional chemotherapy regimens for systemic cancer treatment, several roadblocks still remain on the path toward prolonging long term survival for the majority of patients. The clinical efficacy of cytotoxic agents such as carboplatin and doxorubicin and targeted therapeutics such as toceranib (Palladia), which are commonly used in veterinary medicine, is often hindered by their lack of tumor specificity and consequent normal tissue toxicity. Meanwhile clinical applications of gene therapies are limited by inefficiency of gene delivery and suboptimal tissue selectivity. Additionally, peripheral and central immune tolerance, insufficient innate inflammatory signals required for immune activation and induction of tolerance in primed tumor-targeting effector T cells particularly within the tumor microenvironment are key hurdles for cancer immunotherapeutics to overcome. Oncolytic, genetically modified and immunogenic microbes exploit naturally occurring host microbe interactions that break peripheral tolerance and promote tissue specific immunity. Therefore, use of microbial-based agents represents an attractive strategy for increasing the efficacy of cancer immunotherapeutics.

While murine preclinical models have played a central role in our understanding of the mechanisms and implications of

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specific host–tumor interactions and therapeutic interventions, these models generally fail to predict safety and efficacy of novel therapies, which is reflected in the unimpressive 3.4% success rate of oncology clinical trials [90]. Furthermore, seminal work by Beura et al. revealed the immune system of specific pathogen free laboratory mice to be equivalent to that of a child and therefore not reminiscent of most patients with cancer [6]. More clinically relevant, immunocompetent "models" are needed to bridge the gap between mice and humans and to identify safe therapeutics with a greater likelihood of efficacy in human clinical trials. Such models will play a significant role in informing human clinical trial design. To more accurately recapitulate human host–tumor interactions, cancer researchers are endorsing companion animals with naturally occurring cancers [57•, 68]. Companion animals are outbred, have intact immune systems, and importantly share their environment and, to some extent, their microbiota with humans [81]. Furthermore, the human immune system more closely resembles that of dogs than mice, supporting the likelihood that dog and human immune responses to immunomodulating therapeutics are likely to be more similar than mouse and human immune responses. Furthermore, naturally occurring cancers in companion animals undergo similar microenvironmental pressures to human cancers [13, 14, 72, 89]. This selection pressure during spontaneous tumor development in companion animals and humans results in genetically and immunologically heterogeneous tumors, which are difficult to fully replicate experimentally.

In this review, we summarize results of studies evaluating microbial anticancer therapies in companion animals. We will focus on pivotal studies, particularly promising recent reports, the utility of naturally occurring cancers in companion animals to investigate treatment safety and efficacy and also clinically relevant therapeutic biomarkers. A summary of the studies discussed can be found in Table 1.

Bacteria as Immunomodulators

Reports dating back to the 1970s have evaluated the therapeutic activity of attenuated bacteria and bacterial components in companion animals with naturally occurring cancers, with successes often paralleling those in human cancers. More recently, genetic engineering of bacteria has enabled target antigen and cytokine delivery, allowing for antigen-specific T cell priming and orchestration of the incited immune response. Pathogen-associated molecular patterns naturally expressed by many bacteria stimulate pattern recognition receptors (PRRs) on innate immune cells to mediate a myriad of NF-kB mediated downstream effects that serve to initiate a coordinated immune response. Thus PRR stimulation provides an attractive strategy to break immune tolerance to cancer.

Of note, even nonspecific innate immune stimulation appears to have an anticancer effect for some canine tumor types such as osteosarcoma (OSA), as evidenced by the survival benefit associated with postoperative wound infections [47, 51]. Bacillus Calmette-Guerin (BCG), one of the most successful nonspecific immunotherapeutics to date, improved survival in dogs with osteosarcoma in the setting of microscopic disease [4]. BCG, in combination with chemotherapy, induced responses in dogs with mast cell tumors [26] and transmissible venereal tumor (TVT) [61]. To enhance immunologically potent effects of heat-killed mycobacteria in Freund's adjuvant, synthetic modifications led to the development of liposomal muramyl tripeptide phosphatidylethanolamine (L-MTP-PE) [65]. Systemic administration of L-MTP-PE increased proinflammatory cytokines including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in cancer-bearing dogs. Furthermore, adherent mononuclear cells exposed to L-MTP-PE in vitro and ex vivo displayed increases in antitumor cytostatic activity [43, 45, 80, 86]. Consequently, repetitive dosing of L-MTP-PE has shown antitumor efficacy against both canine and human OSA when administered in the setting of microscopic disease, along with a possible schedule-dependent synergism detected between L-MTP-PE and cisplatin in canine OSA [42, 44, 52, 60]. A stage-dependent benefit of adjuvant L-MTP-PE after surgical resection was also reported in prospective randomized clinical trials of canine hemangiosarcoma and oral melanoma [53, 86].

Subsequently, a genetically engineered avirulent strain of *Salmonella enterica* serovar *typhimurium* encoding IL-2 (SalpIL2) was evaluated in a phase I clinical trial in 19 dogs with OSA [18•]. *Salmonella* species have natural selectivity for the tumor microenvironment due to their inherent affinity for hypoxic environments [18•, 75]. Furthermore, induced expression of IL-2 stimulates T cell activation and enhances natural killer cell cytotoxicity [25]. Dogs were treated with the first dose of SalpIL2 10 days prior to amputation, and received 5 doses of adjuvant doxorubicin concurrently with 5 additional doses of SalpIL2 from 2 weeks post-surgery. SalpIL2 was well tolerated at all doses, and the study bacteria were not cultured from the feces of any dog. An elevated total white blood cell count was consistently observed after the first dose of SalpIL2 and was not associated with disease-free interval. SalpIL2-treated dogs exhibited a significantly longer median disease-free interval compared with historical controls receiving adjuvant doxorubicin only [18•]. The lack of apparent toxicity and potential therapeutic utility suggest that this therapy may be clinically beneficial.

Most recently, genetically modified, attenuated *Listeria monocytogenes* has shown promise as an antimetastatic treatment for canine OSA. *L. monocytogenes* is a facultative, anaerobic, intracellular bacteria that is a potent stimulator of innate and adaptive immunity [17]. Following intravenous administration, bacteria are phagocytosed by mononuclear

Table 1 Summary of the studies discussed in this review

Microbe	Rationale	Tumor type	Summary of findings	Reference
Bacteria as immunomodulators				
BCG	Pulmonary alveolar macrophage activation and induction of cytotoxic responses [5, 22].	Microscopic pulmonary metastasis of canine OSA	Prolonged median survival time in dogs receiving amputation of OSA from 13 to 40 weeks.	[4, 67]
	Combined with hCG, which exerts effect via a direct pro-apoptotic mechanism [15].	Canine mast cell tumors	LDI-100 (BCG + hCG) induced a response in 28.5% of canine mast cell tumors, which was not statistically significant to standard of care vinblastine.	[26]
	Regression of TVT is thought to be due at least in part due to immune rejection [19].	Canine TVT	The combination of intratumoral BCG with standard-of-care vincristine chemotherapy improved the time to regression compared to either treatment alone.	[61]
L-MTP-PE	Activation of mononuclear cells and increased cytostatic effects [43, 45, 80].	Canine OSA	The median survival time was significantly longer for dogs treated with amputation plus liposome/MTP-PE, compared to dogs treated with amputation and empty liposomes. Dogs that received L-MTP-PE after amputation and completion of cisplatin chemotherapy lived longer than dogs receiving empty liposomes after amputation and cisplatin. In contrast, dogs receiving concurrent L-MTP-PE with cisplatin chemotherapy after amputation did not live longer than those receiving amputation, cisplatin, and empty liposomes.	[44, 52]
		Canine melanoma	After surgical removal of stage I (<2 cm and nonmetastatic) oral melanoma, treatment with L-MTP-PE prolonged survival over treatment with empty liposomes.	[53]
		Canine splenic hemangiosarcoma	Dogs with stage II (ruptured, nonmetastatic) splenic hemangiosarcoma receiving splenectomy and chemotherapy plus L-MTP-PE, lived longer than dogs receiving splenectomy, chemotherapy and empty liposomes.	[86]
Salmonella enterica serovar *typhimurium*	Genetically modified to express IL-2 to enhance immune effector cell activity. *Salmonella* species naturally home to the tumor due to the hypoxic microenvironment.	Canine OSA	Dogs received one dose of IL-2-expressing *Salmonella* (SalpIL2) 10 days before amputation, and 5 doses each of SalpIL2 and doxorubicin after amputation. SalpIL2-treated dogs displayed a longer median disease-free interval compared to historical controls receiving doxorubicin alone.	[18]
Listeria monocytogens	Genetically modified to express HER2 fused to a truncated form of LLO and consequentially prime HER2-specific CD4+ and CD8+ T cells [91].	Canine OSA	Dogs received HER2-targeting *L. monocytogenes* (ADXS31–164) after amputation and carboplatin chemotherapy. The magnitude of treatment-induced increases in total white blood cells, neutrophils and monocytes was greater for dogs that survived beyond 18 months. HER2-specific immune responses occurred in 15 of 18 dogs within 6 months of treatment. Treatment significantly increased survival compared historical controls.	[57•]
Viral vectors for gene therapy and immunomodulation				
Adenoviridae	Ad-6 vector expressing canine TERT (dTERT). TERT is upregulated in the majority of canine tumor cells and is	Canine lymphoma	Dogs received a vaccination regimen consisting of Ad-6-dTERT and dTERT DNA plasmid electroporation. dTERT-specific immune responses were detected in treated dogs and improved survival was detected in dogs treated with this vaccination strategy in combination with COP chemotherapy, compared to dogs	[20, 36•, 69]

Table 1 (continued)

Microbe	Rationale	Tumor type	Summary of findings	Reference
	therefore targeted as a tumor-associated antigen [2, 94].		receiving COP alone. Prolonged survival was also detected in dogs with DLBCL treated with CHOP and this dTERT vaccination schedule.	
	Human IFN-γ-expressing adenovirus given intraparenchymally after surgical debulking to induce immune attack of residual tumor cells.	Brain tumors	Surgical debulking, Ad-IFNγ into the tumor bed, and postoperative tumor lysate/CpG vaccinations induced immunological responses suggestive of the development of antitumor immunity. No additional benefit of Ad-IFNγ was detected in a subsequent study.	[66, 71]
Poxviridae	Feline IL-2-expressing recombinant canarypox virus (ALVAC-fIL2 (vCP1338)), or human IL-2-expressing recombinant vaccinia virus (NYVAC-hIL2 (vP1241)) injected into the tumor bed in an attempt to recruit immune effector cells to the site of residual tumor.	Feline injection site sarcoma	Cats underwent surgical excision and postoperative iridium-based brachytherapy and were then randomized to receive either no further therapy, or ALVAC-fIL2 or NYVAC-hIL2. Cats treated with either ALVAC-fIL2 or NYVAC-hIL2 had a significantly lower recurrence rate compared to cats that did not receive adjuvant localized immunotherapy. In a subsequent study, ALVAC-fIL2-treated cats experienced a significantly prolonged median disease-free interval compared to cats not receiving immunotherapy.	[37, 39]
Oncolytic viruses				
Paramyxoviridae	CDV	Canine lymphoma	CDV infected the majority of malignant canine lymphocytes and induced their apoptosis.	[82]
		Canine histiocytic sarcoma	CDV infection decreased MMP-2 expression and induced RECK expression, illustrating potential inhibitory effects on invasion and metastasis. CDV infection also decreased expression of angiogenic factors.	[70, 73]
		Canine mammary carcinoma	CDV induces apoptosis, which may be dependent on NFκB signaling.	[50]
	Measles virus: In rMV-SLAMblind, virus entry is facilitated through nectin-4 and not SLAM to reduce pathogenicity.	Canine mammary carcinoma	rMV-SLAMblind infected 4/9 carcinomas via cell entry through nectin-4. Cytotoxicity was observed for 3/4 nectin-4 positive tumors. Overall about 45% of canine mammary carcinomas are nectin-4 positive.	[79]
	NDV: In rLAS-uPA, rLAS is genetically engineered to express uPA, enabling targeting of cells that overexpress uPAR such primary brain tumors [74].	Canine lymphoma	In vitro cytotoxicity of an attenuated NDV (NDV-MLS) was demonstrated in canine and human lymphoma cells, while no significant effects were seen in normal PBMCs.	[76]
		Canine primary brain tumors	rLAS-uPA was found to be safe. Induction of anti-rNDV antibodies and a cytokine response was observed, although no overt responses in dogs with meningioma were detected.	[41]
	Sendai virus	Canine mast cell tumor	3/4 dogs cleared recurrent or residual tumor after surgical debulking, and 2/2 dogs that received virus only achieved a CR.	[35•]
Adenoviridae	Genetic modifications to CAV-2 makes ICOCAV17 more selective for tumor cells, and enhances viral infectivity, intratumoral spread and oncolytic activity. Systemic administration of ICOCAV17-infected MSCs	Various canine malignancies	Intralesional ICOCAV17 suppressed canine OSA and melanoma murine xenografts, and 2/6 dogs with naturally occurring tumors experienced a PR. 27 dogs were treated with intravenous dCelyvir. Stromal degeneration and lymphocyte infiltration were significantly more pronounced in posttreatment compared to pretreatment biopsies. 12/16 dogs receiving dCelyvir alone experienced clinical benefit (2 CR, 3 PR, and 7 SD).	[8••, 46]

Table 1 (continued)

Microbe	Rationale	Tumor type	Summary of findings	Reference
	(dCelyvir) enables the virus to "hide" from neutralizing antibodies while MSCs home to the tumor.			
Rhabdoviridae	In VSV-hIFNβ-NIS, enforced expression of IFN-β protects healthy tissue from infection, while NIS enables noninvasive imaging.	Various canine malignancies	VSV-hIFNβ-NIS was administered to 9 dogs with cancer. Liver enzyme elevation correlated with virus copy number in PBMCs and clinical response.	[62••]
	Maraba virus	NA	In a dose-finding study in healthy cats, oncolytic Maraba virus based booster vaccinations induced mild and transient adverse effects indicative of immune activation, and no shedding of infectious virus.	[28]
Reoviridae	Dearing strain of reovirus serotype 3 (Reolysin®)	Canine mast cell, mammary, and histiocytic tumors, melanoma, and lymphoma.	Mast cell tumor cell lines appear to be highly sensitive to reovirus, while variable activity is seen in canine mammary gland tumor, histiocytic, and melanoma cell lines, and canine lymphoma and OSA cell lines appear to be more resistant.	[29, 30, 32, 34]
		Canine mammary gland tumors	Synergistic antitumor activity was detected when reovirus combined with paclitaxel, carboplatin, or gemcitabine.	[33]
		Various canine tumors	Administered intralesionally in 10 dogs, or intravenously in 9 dogs. Treatment was well tolerated, no dogs shed any infectious viral particles, and a reduction in tumor size was observed in 5 dogs.	[31•]
Poxviridae	Myxoma virus: Natural pathogenicity only in European rabbits. Myxoma virus lacking the antiapoptotic gene serp2 (MXYVΔserp2), is toxic to canine cancer cell lines while normal fibroblasts appeared to inhibit infection [85].	Canine soft tissue sarcoma	MXYVΔserp2 was administered intralesionally to ten dogs, including 5 dogs as the sole treatment, and 5 dogs undergoing subsequent surgical resection. No clinical adverse events were detected. Inflammatory infiltrates were infrequent in posttreatment tumor biopsies and no significant reductions in target lesion size were noted.	[55]

Ad adenovirus, *BCG* Bacillus Calmette-Guerin, *CAV-2* canine adenovirus-2, *CDV* canine distemper virus, *CHOP* cyclophosphamide, doxorubicin, vincristine, prednisone, *COP* cyclophosphamide, vincristine, prednisone, *CR* compelte response, *DLBCL* diffuse large B cell lymphoma, *hCG* human chorionic gonadotropin, *IFN* interferon, *IL* interleukin, *LLO* listeriolysin, *L-MTP-PE* liposomal muramyl tripeptide phosphatidylethanolamine, *MMP* matrix metalloproteinase, *NDV* Newcastle's disease virus, *NIS* sodium iodide symporter, *OSA* osteosarcoma, *PBMCs* peripheral blood mononuclear cells, *PR* partial response, *RECK* reversion-inducing cysteine-rich protein with Kazal motifs, *rLAS* avirulent La Sota strain of NDV, *SD* stable disease, *SLAM* signaling leukocyte activation molecule, *TERT* telomerase reverse transcriptase, *uPA* urokinase plasminogen activator, *uPAR* urokinase plasminogen activator receptor, *VSV* vesicular stomatitis virus

cells and processed in the phagolysosome where they secrete the pore-forming lysin listeriolysin O (LLO), which enables escape into the cytosol. Therefore, *L. monocytogenes* is subject to both cytosolic and endosomal antigen processing pathways, leading to antigen presentation by MHCI and MHCII pathways, respectively. Accordingly, genetically modified *Listeria* expressing tumor-associated antigens (TAA) is fused to a truncated form of LLO, prime tumor antigen-specific CD8+ and CD4+ T cells. This unusual biology coupled with potent innate immune stimulation can break immune tolerance and stimulate an antigen-specific cell-mediated attack on cancer [91]. A phase I clinical trial in dogs with HER2 positive OSA recently evaluated the toxicity and immunological effects of HER2-targeting *L. monocytogenes* (ADXS31–164) after primary tumor removal and adjuvant carboplatin chemotherapy [57•]. Interestingly, the magnitude of treatment-induced increases in total white blood cells, neutrophils and monocytes was greater for dogs that survived beyond 18 months post diagnosis suggesting that an early innate immune response to *L. monocytogenes* might serve as a biomarker for immune fitness and therapeutic response. HER2-specific interferon-γ (IFN-γ) responses occurred in 15 of 18 dogs within 6 months of treatment. Furthermore, ADXS31–164 significantly increased the median survival time to 956 days compared with 423 days reported for historical controls with HER2 positive OSA treated with amputation and carboplatin alone [57•]. These results have prompted a large, multi-institutional clinical trial to confirm the efficacy of ADXS31–164 in preventing metastatic disease and identify correlative biomarkers of clinical response in dogs with appendicular OSA.

Viral Vectors for Gene Therapy and Immunomodulation

Many viruses have evolved to efficiently and often selectively enter host cells and co-opt cellular machinery to replicate viral components and alter gene expression. These characteristics have been exploited to control gene expression for therapeutic gain in certain disease states. However, highly publicized life-threatening or fatal outcomes associated with oncogenic insertions or potent immune stimulation were reported for clinical trials with early generations of viral vectors [24, 49]. Therefore, considerable effort has been exerted into viral vector optimization and investigation of vector safety.

Gene therapy with recombinant poxviruses expressing IL-2 has been investigated as a strategy to reduce the high rate of recurrence of feline injection-site sarcomas. In an initial study, 54 pet cats with injection-site fibrosarcoma treated with surgical excision and postoperative iridium-based brachytherapy were randomized to receive either no further therapy, feline IL-2-expressing recombinant canarypox virus (ALVAC-fIL2 (vCP1338)), or human IL-2-expressing recombinant vaccinia virus (NYVAC-hIL2 (vP1241)) injected subcutaneously into the tumor bed [39]. Cats treated with either ALVAC-fIL2 or NYVAC-hIL2 demonstrated a significantly lower recurrence rate (28% and 39%, respectively) compared with cats that did not receive adjuvant localized immunotherapy (61% recurrence rate) [39]. Furthermore, biodistribution of both viruses in tumor-baring dogs revealed that viral particles remained optimally localized along needle tracts [39]. This finding indicates minimal vector dissemination and therefore reduced risk of patient toxicity and environmental contamination with these recombinant products. In a follow up study, 71 cats were enrolled, and, again, all cats underwent surgical excision and iridium-based brachytherapy [37]. Although a decreased frequency of tumor relapses in ALVAC-fIL2-treated cats compared with control cats did not quite reach significance, a significant difference in median disease-free interval was detected [37]. ALVAC-fIL2 is now marketed as Oncept IL-2 by Merial and was approved by the European Medicines Agency in 2013 and conditionally licensed by the USDA in 2015 [38].

Adenoviral vectors have also been used to deliver tumor-associated antigens either with or without immune adjuvants to promote tumor-specific T cell priming and activation. This approach was used clinically by Peruzzi et al. to induce cellular immunity against telomerase reverse transcriptase (TERT) in dogs with lymphoma. TERT is a universal tumor antigen that is upregulated in most canine tumor cells where it acts to prevent telomere shortening and thereby facilitates evasion of cellular senescence [2, 94]. Peruzzi et al. utilized a vaccination protocol that consisted of two components: two injections of an adenovirus 6 (Ad6) vector expressing dog TERT (dTERT; Ad6-dTERT) 2 weeks apart, followed by five DNA plasmid electroporations at 2-week intervals [69]. The DNA plasmid encoded catalytically inactive dTERT fused to human tissue plasminogen activator (TPA) and the β subunit of *Escherichia coli* heat labile enterotoxin (LTB). This two-hit strategy was found to be particularly immunogenic based on preliminary studies in mice [59]. Fourteen dogs with high-grade, multicentric lymphoma received combination COP (cyclophosphamide, vincristine, and prednisone) chemotherapy to induce clinical remission. Patients in remission then received the vaccination regimen concurrently with maintenance chemotherapy [69]. Induction of a dTERT-specific immune response was detected by IFN-γ enzyme-linked immunosorbent spot assay (ELISpot) in 13 out of 14 dogs that received the vaccination protocol but was not detected in the eight control dogs treated with chemotherapy alone [69]. No side effects were reported, and a significant increase in overall survival was detected in the 14 dogs receiving chemotherapy and dTERT vaccination compared with the eight control dogs [69]. Similar immunological and clinical results were obtained in a larger study consisting of 21 dogs treated with COP chemotherapy alone and 21 dogs treated with COP plus a similar

dTERT vaccination schedule [20]. Additionally, a correlation between lymphoma dTERT expression (mRNA) and survival time was detected in six of the vaccinated dogs that were evaluated [20]. Subsequently, this strategy also prolonged survival in dogs with diffuse large-B cell lymphoma treated concurrently treated with CHOP (COP with doxorubicin) compared with survival times previously reported for CHOP alone [36•, 88]. dTERT-specific antibodies were developed in treated dogs [36•]. These results not only highlight the potential utility of adenoviral vectors to break tolerance to tumor-associated antigens but also suggest that tumor antigen expression may serve as a predictive factor for clinical response to this type of immunotherapy.

Similar strategies have been used for therapeutic gene transfer in canine intracranial tumors where therapeutic options are often limited, and tumor selectivity is imperative for maintenance of normal brain functions [66]. A group from the University of Minnesota described the treatment of a dog with naturally occurring grade II diffuse astrocytoma treated with surgical debulking, human-IFN-γ-expressing adenovirus (Ad-IFNγ) injected into the tumor bed immediately post resection, and postoperative tumor lysate combined with CpG vaccinations [71]. Interestingly, this dog developed periodic transient neurological signs that occurred within 3–4 days of tumor lysate-CpG vaccinations. Vaccination also induced tumor-reactive IgG antibodies and augmented preexisting antigen-specific cytotoxic T cell responses, suggesting this approach can induce and promote antitumor immune responses [71]. The results of this case study justified pursuing a clinical trial testing surgical debulking plus tumor lysate-CpG vaccinations alone or in combination with intraparenchymal Ad-IFNγ [66]. In this small study of 23 dogs, no additional benefit of Ad-IFNγ injections was detected [66]. This group has conducted additional clinical trials evaluating the safety and efficacy of intratumoral delivery of genetically modified adenovirus conditionally expressing Flt3L and Herpes simplex virus type I thymidine kinase (HSV-tk), although details of this study are not published [66]. These investigations have provided a foundation for ongoing clinical trials evaluating microbial-based therapeutics as much-needed treatment options for canine intra-cranial tumors, which represent spontaneous and immunocompetent models of analogous human tumors [1].

Oncolytic Viruses

Many inherent properties of cancer cells, such as overexpression of Ras pathways components, aberrant miRNA expression, upregulation of cell surface receptors, and downregulation of type I interferon responses, facilitate viral entry and replication in tumor cells compared with normal host cells [27]. These and other differences in transformed cells can be further exploited by genetically modified oncolytic viruses to increase selectivity for tumor cells. Oncolytic viruses induce immunologic cell death of tumor cells, which promotes antigen presentation and tumor-specific T cell priming to induce and augment antitumor immunity. A thorough review of oncolytic virus strategies studied in dogs was recently published by Sanchez et al. [77]; therefore, we will focus on the most prevalent strategies and recent publications in dogs and other veterinary species.

Viruses in the single-stranded RNA virus family *Paramyxoviridae*, including Morbilliviruses canine distemper virus (CDV) and measles virus (MV), Newcastle's disease virus (NDV), and Sendai virus, have been popular choices for oncolytic virotherapy due in part to their simple two-protein entry system. Here, the proteins responsible for receptor attachment and cell fusion are separated, which has simplified retargeting strategies [7]. Certain paramyxoviruses selectively infect cancer cells based on defects in the cells' type-1 IFN response [16], a propensity for apoptosis-resistant cells [56], and a preference for dividing cells [11]. For example, CDV demonstrated infectivity and apoptotic activity in malignant canine lymphocyte, histiocytic, and mammary cancer cell lines [50, 70, 73, 82]. CDV's oncolytic effects in canine histiocytic sarcoma could involve decreased matrix metalloproteinase expression and downregulation of genes involved in angiogenesis, while selectivity for canine mammary tumor cells coincided with activation of NFκB and apoptosis pathways [50, 70, 73]. Although MV does not infect canine cells [82], one study reported the ability of a recombinant MV (rMV-SLAMblind) to infect canine mammary cancer cell lines by selectively using the polio virus receptor related 4 receptor (PVRL4/Netin-4), which is expressed in approximately 45% of canine mammary carcinomas [79]. rMV-SLAMblind elicited antitumor effects in vitro in murine xenographs of canine mammary cancer and also in ex vivo experiments [79]. An attenuated strain of NDV (NDV-MLS) also demonstrated in vitro cytotoxic activity against canine and human lymphoma cells but did not affect the viability of normal peripheral blood mononuclear cells (PBMCs) [76]. A phase I clinical trial evaluating the safety of the avirulent LaSota strain of NDV (rLAS) genetically engineered to express urokinase plasminogen activator (rLAS-uPA) was recently completed [41]. Virus expressing uPA was able to target cells, including meningioma cells that overexpress the uPA receptor (uPAR) [74]. In four dogs with presumptive meningioma based on MRI findings, rLAS-uPA was administered intravenously and found to be safe, although no overt tumor responses were detected [41]. All dogs developed antiviral antibodies and increased concentrations of circulating TNF-α, IFN-γ, and TRAIL, which support previous studies showing that NDV-infected macrophages induce apoptosis in target cells mediated by TNF/TRAIL [87]. Finally, in a small pilot study of six dogs with mast cell tumors treated with an

attenuated Sendai virus showing a selective cytotoxicity for malignant cells, three out of four dogs completely cleared recurrent or residual tumor after surgical debulking. Furthermore, two out of two dogs that received virus only without debulking surgery achieved a complete response [35•]. Together, these data suggest that oncolytic paramyxoviruses are safe and deserve further investigation into their utility in the treatment of canine cancers.

In addition to the utility of adenoviruses in gene delivery and vaccination, various strategies for increasing tissue selectivity of oncolytic adenoviruses have been evaluated in pets. Most recently, a genetically modified canine adenovirus-2 (CAV-2) called ICOCAV17 was created [46]. These modifications made ICOCAV17 selective for retinoblastoma (Rb)-deficient cells by placing viral E1A under the control of host E2F and deleting the phospho-Rb (pRb) binding site of E1A [23, 46]. Furthermore, insertion of an RGD integrin-binding motif improved viral infectivity, and hyaluronidase improved intratumoral spread and oncolytic activity [23, 46]. In Laborda et al. intralesional ICOCAV17 displayed activity against canine OSA and melanoma in murine xenografts, and two of six pet dogs with various tumor types experienced a partial response [46]. Subsequently, ICOCAV17-infected canine mesenchymal stem cells (MSCs; dCelyvir) were investigated as a "Trojan horse"-like strategy to covertly deliver oncolytic ICOCAV17 systemically while avoiding neutralization by preexisting antibodies [8••]. Adenovirus-positive tumor cells were detected in four of 15 dogs treated with intravenous dCelyvir, and stromal degeneration and lymphocyte infiltration were significantly more pronounced in posttreatment compared with pretreatment biopsies. Twelve of 16 dogs receiving dCelyvir alone for a variety of tumor types (predominantly sarcomas) displayed a clinical benefit, including two complete responses, three partial responses, and seven stable diseases. No significant adverse effects were observed. Importantly, the presence of preexisting anti-adenovirus antibodies due to routine vaccination did not affect response to treatment [8••].

Wild type vesicular stomatitis virus (VSV) can cause significant disease, predominantly in cattle, horses, and pigs, and is zoonotic, being associated with flu-like symptoms in humans. Like the paramyxoviruses, the rhabdovirus VSV has natural tumor-selective oncolytic activity afforded by its preferential infection and replication within type-1 IFN-deficient malignant cells [3]. Another attractive property of VSV is its genetic simplicity and ease with which it can be modified to attain desirable characteristics. For example, VSV has been modified to increase its therapeutic index by enforced expression of both human IFN-β to protect healthy tissue from infection and the sodium-iodide symporter (NIS) to enable non-invasive nuclear medicine imaging (VSV-hIFNβ-NIS) [48]. The toxicity and efficacy of VSV expressing either human IFN-β or canine IFN-β and NIS were evaluated in nine client-owned dogs and one research dog at the MTD established in a prior study [48, 62••]. No dogs experienced clinical signs of toxicity, although one dog each developed transient alanine aminotransferase (ALT) elevations of two-fold and tenfold the upper limit of normal, and all dogs developed mild fevers that resolved within 24 h of treatment. Dogs experiencing the most severe liver enzyme (ALT) elevations also demonstrated the highest circulating virus copy load and were the only two dogs to experience an objective response to treatment [62••]. Both dogs were diagnosed with T cell lymphoma. Another oncolytic vesiculovirus, Maraba virus, with a marked tropism for cells with defective IFN signaling, was recently evaluated for its safety in a group of healthy cats [28]. In this study, cats were primed with an adenovirus expressing a human melanoma antigen and boosted with Maraba virus engineered to express the same melanoma antigen. Treatments were typically well tolerated, although side effects such as transient pyrexia, weight loss, and leukopenia were observed [28]. While Maraba virus genomes were evident in some bodily fluids and post mortem tissues, no infectious viral particle could be isolated from these samples [28].

Reoviridae represent a family of segmented double-stranded RNA viruses that are not consistently pathogenic in mammalian species. Their selective lytic activity in cancer cells is at least in part due to the propensity of transformed cells to overexpress Ras pathway constituents [21, 40]. In dogs, only the Dearing strain of reovirus serotype 3 (Reolysin®) has been evaluated. Canine mast cell tumor cell lines appear to be highly permissive to reovirus replication and consequential cell death, while canine mammary gland tumor, histiocytic, and melanoma cell lines display variable sensitivity, and canine lymphoma and OSA cell lines appear to be more resistant [29, 30, 32, 34]. Furthermore, synergism between several chemotherapeutics and reovirus exposure was detected for a canine mammary tumor cell line in vitro [33]. Most recently, Reolysin® was administered to 19 client-owned dogs with a variety of different naturally occurring cancers [31•]. Delivery of drug was either intralesional (10 dogs) or intravenous (9 dogs) for inaccessible tumors. Treatment was well tolerated and shedding of infectious virus was not detected. While tumor response was not the primary objective, a reduction in tumor size was observed in 5 dogs, supporting further study of potential clinical efficacy as a single agent and in combination protocols [31•].

The oncolytic poxvirus, myxoma virus, is particularly attractive as a therapeutic due to a restricted pathogenicity for European rabbits only [9]. In vitro, myxoma virus lacking the antiapoptotic gene serp2 (MXYVΔserp2), induced cytopathic effects in a variety of different primary and immortalized canine cell lines derived from hemangiosarcoma, soft tissue sarcoma, osteosarcoma, and mammary tumors, while normal fibroblasts appeared to resist infection [85]. MXYVΔserp2 was administered intralesionally to ten dogs with soft tissue sarcoma, including five dogs with gross disease that received a

single dose and five dogs undergoing subsequent surgical resection that received two doses separated by at least 14 days [55]. No clinically relevant adverse events were observed. Dissemination of MXYVΔserp2 was not observed, and neutralizing antibodies were detected in only two of the five dogs that received 2 doses [55]. Inflammatory infiltrates were infrequent in posttreatment tumor biopsies, and no significant reductions in target lesion size were noted. Intratumoral viral DNA could not be detected 4 days after a single treatment, suggesting rapid clearance of MXYVΔserp2 and a need to further characterize MXYVΔserp2 distribution and clearance in the host [55]. Finally, in one of the few studies evaluating oncolytic viral therapy for feline cancers, myxoma virus was found to infect, replicate within, and induce apoptosis of feline squamous cell carcinoma and mammary carcinoma cell lines [54]. The restricted pathogenicity of myxoma virus makes it a particularly attractive candidate for future oncolytic antitumor strategies. Together, these studies highlight the feasibility and therapeutic potential of oncolytic viruses in cancer therapy. Furthermore, they underscore the importance of evaluating such treatments in clinically relevant, immunocompetent large animal models to assess safety and efficacy and possibly uncover predictive biomarkers that can be readily translated amongst species.

Veterinary Perspective on Immune System Responses to Microbial Anticancer Therapy

The predominant mechanism of action for most microbial anticancer therapeutics is thought to be induction of a robust antitumor response. This response may be achieved through multiple mechanisms including potent innate immune system activation, enhanced antigen-presentation, TAA delivery, and/or induction of immunogenic cell death. These immunomodulatory strategies are not specific to veterinary studies and have been reviewed in detail elsewhere [10, 63].

Species-specific variations in immune responses exist however, and their appreciation is necessary for designing and interpreting comparative immunotherapy studies. For example, intravenous bacteria are predominantly cleared from the blood by resident macrophages in the liver (Kupffer cells) in dogs, rodents, and humans, whereas in ruminants, pigs, horses, and cats, this task is predominantly performed by pulmonary intravascular macrophages [83]. These differences have obvious implications on the pharmacokinetics of microbial therapeutics and potentially on their toxicity profiles determined in different species. Furthermore, while many genes within MHC class II and III regions share obvious similarities between species, class I genes vary greatly between species [84]. As such, evaluation of antigen-specific T lymphocyte responses is presently limited to measuring lymphocyte activation and cytokine production after exposure to peptide pools; in humans and mice, frequency and function of antigen-specific T cells may be readily identified with tetramers.

There is presently much to learn about the antiviral immune response in both normal and malignant cells in veterinary species. It will be critical to show that canine and feline antiviral cellular responses are comparable with human cells and to therefore confirm that companion animal patients will accurately reflect safety and efficacy of these approaches in the human clinic. For example in humans, dysregulation of cytoplasmic RNA detection via downregulation of RIG-1, MDA5, OAS2, and RNase L, amongst others, is known to enhance RNA virus replication and cytotoxicity in cancer cells [58]. Meanwhile, the gatekeeper protein responsible for linking cytoplasmic DNA sensing with type I IFN expression, stimulator of IFN genes (STING), is expressed at varying levels in human colon and ovarian carcinoma and melanoma, and downregulation is associated with enhanced susceptibility to oncolytic DNA viruses such as HSV1 and vaccinia virus [12, 92, 93]. Defects in IFN signaling pathways, as well as RNA and DNA sensing, also commonly predispose cancer cells to differential cell killing compared with normal host cells [58]. To the authors' knowledge, RNA and DNA sensing and the associated type I IFN response has not been examined in veterinary cancers to date.

Limitations and Special Considerations

Compared with experimental lab animals, client-owned pets are typically housed with their human owners throughout the duration of clinical trials requiring a reasonable expectation of safety and absence of transmission potential of the microbial-based therapy under evaluation. In addition, consideration of the species most at risk for pathogenic effects specific to that microbe is warranted. For example, the most recent VSV clinical trial stated exclusion of dogs in contact with livestock, while MXYV clinical trials excluded dogs that were exposed to pet rabbits [55]. Furthermore, while client-owned pets offer the ability to study novel therapeutics in an immunocompetent host, there is a diminished opportunity to obtain biologic samples due to ethical considerations and owner tolerance of repeat office visits and sample collections. Added to that is the limited availability of standardized reagents, particularly for evaluating protein expression in domestic veterinary species. These limitations ensure experimental murine studies will remain a mainstay for mechanistic studies that require genetic manipulations or invasive sample collections.

Future Directions

At the time of writing, there are two ongoing fully funded clinical trials exploring the efficacy of microbial-based

immunotherapeutics against canine gliomas. The University of Alabama at Birmingham is leading a multi-institutional clinical trial exploring the utility of M032, an attenuated herpes simplex virus-1 genetically engineered to express human IL-12 [78], while the University of Minnesota is investigating the addition of adenoviral-mediated Flt3/TK gene therapy to a combination immunotherapy protocol consisting of a novel checkpoint inhibitor and tumor lysate vaccine. Both trials are funded by the NIH/NCI underscoring the value of comparative oncology in informing human immunotherapy clinical trials. The Comparative Oncology Trials Consortium, a network of academic comparative oncology centers across North America under the National Cancer Institute's Center for Cancer Research, is also recruiting dogs with OSA to determine efficacy of the HER2/neu-expressing attenuated *L. monocytogenes* in the adjuvant setting in an extended patient cohort that builds on the findings of the related pilot study [64].

A detailed list of all veterinary clinical trials currently underway or recently completed is available at https://ebusiness.avma.org/aahsd/study_search.aspx. Several other microbial-based therapeutics are being evaluated in veterinary species across North America. Researchers at the University of Pennsylvania are evaluating a *Listeria*-based vaccine targeting the V600E B-Raf mutation commonly found in canine bladder cancer in an ongoing clinical trial. At Iowa State University, the benefit of adding Immunocidin, an emulsion of mycobacterial cell wall fractions to standard of care treatment with surgical resection and doxorubicin chemotherapy for canine hemangiosarcoma is being investigated. Meanwhile, Veterinary Oncology Services are evaluating a HER2/neu-targeting adenovirus for its efficacy in feline mammary carcinoma, canine transitional cell carcinoma, and OSA. Furthermore, a vaccine series including the Ad6-dTERT is undergoing further evaluation for various cancers in dogs and cats, and ALVAC-fIL2 is continuing to be assessed for its efficacy in delaying or preventing feline sarcoma regrowth. Clinical trials have also recently concluded to further evaluate treatment with VSV-hIFNβ-NIS in dogs with OSA.

Conclusion

In conclusion, similarities in tumor biology and immunology between naturally occurring veterinary and human cancers underscore the value of comparative studies of microbial anticancer therapeutics for the benefit of all species. Importantly, understanding the necessary considerations for undertaking comparative studies will ensure the best chance for successful collaborations and research outcomes between veterinary and human researchers. The potential of such collaborative efforts is readily apparent by several federally funded clinical trials currently underway to evaluate microbial-based therapeutics in veterinary species with naturally occurring cancers. Ultimately, immunocompetent client-owned pets with cancers can offer a way to bridge the gap between experimental murine studies and human clinical trials.

Compliance with Ethical Standards

Conflict of Interest Drs. Withers, Sparger, and Boudreaux certify that they have no affiliations with or involvement in any organization or entity with any financial or nonfinancial interest in the subject matter or materials discussed in this manuscript. Dr. Mason is a named inventor on the recombinant HER2/neu expressing *Listeria*-based vaccine for use in dogs with osteosarcoma.

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.

All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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