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Using the canine microbiome to bridge translation of cancer immunotherapy from pre-clinical murine models to human clinical trials

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The microbiome has clearly been established as a cutting-edge field in tumor immunology and immunotherapy. Growing evidence supports the role of the microbiome in immune surveillance, self-tolerance, and response to immune checkpoint inhibitors such as anti PD-L1 and CTLA-4 blockade (1–6). Moreover, recent studies including those using fecal microbial transplantation (FMT) have demonstrated that response to checkpoint immunotherapies may be conferred or eliminated through gut microbiome modulation (7, 8). Consequently, studies evaluating microbiota-host immune and metabolic interactions remain an area of high impact research. While observations in murine models have highlighted the importance of the microbiome in response to therapy, we lack sufficient understanding of the exact mechanisms underlying these interactions. Furthermore, mouse and human gut microbiome composition may be too dissimilar for discovery of all relevant gut microbial biomarkers. Multiple cancers in dogs, including lymphoma, high grade gliomas, melanomas and osteosarcoma (OSA) closely resemble their human analogues, particularly in regard to metastasis, disease recurrence and response to treatment. Importantly, dogs with these spontaneous cancers also have intact immune systems, suggesting that microbiome analyses in these subjects may provide high yield information, especially in the setting of novel immunotherapy regimens which are currently expanding rapidly in canine comparative oncology (9, 10). Additionally, as onco-microbiotic therapies are developed to modify gut microbiomes for maximal responsiveness, large animal models with intact immune systems will be useful for trialing interventions and monitoring

adverse events. Together, pre-clinical mechanistic studies and large animal trials can help fully unlock the potential of the microbiome as a diagnostic and therapeutic target in cancer.

KEYWORDS

microbiome, immunotherapy, cancer, comparative oncology, canines

Success and limitations of the murine model

Murine models represent the most used model for studying host-microbiome physiology at both functional and mechanistic levels. Due to genetic homogeneity and laboratory environments, mouse models allow for high levels of control and improved experimental reproducibility (11). In cancers with representative murine models, mice have uncovered cancer promoting microbiota, and microbiota associated with improved response to treatment. Murine colon cancer models, for example, have contributed to understanding the influence of the gut microbiome on colorectal tumorigenesis *via* modulation of inflammation. In these models, transfer of the microbiome from tumor-bearing mice to germ free mice accelerated tumor growth, demonstrating causality (12, 13). Similarly, TLR4 knockout mice have been critical in understanding the oncogenic effect of gut microbiota in liver cancer (14). Sivan et al (15)demonstrated key differences in tumor kinetics and responses to immunotherapy between genetically similar mice bred in two different environments. One cohort showed anti-tumor effects following anti-PD1 checkpoint inhibitor therapy, while the other cohort showed no benefit. However, co-housing the mice together reversed the differences in anti-tumor responses, which were then restored by the administration of oral Bifidobacterium spp (15). Similar observations were made for mice receiving anti-CTLA checkpoint inhibitors with effects modulated by Bacteroides species, (specifically *B. fragilis* and/or *B. thetaiotaomicrom*) (16).

Despite the strength of these mechanistic murine studies, there are limitations to mouse models, particularly when considering the complex interactions between microbiome, immune response, and cancer. Laboratory mice demonstrate different microbial compositions to their wild counterparts. Attempts to convert lab-type to wild type mice have successfully altered, but not completely recapitulated, natural microbiome profiles (17). Moreover, animal facilities themselves significantly impact microbiome composition. For example, key differences in dominant taxa have been observed between identical mouse lines from different animal locations (18). Similarly, the use of germ-free mice is both a strength and a weakness of mouse models (19). These sterile conditions allow mice to be born without microbial colonization prior to the introduction of species of interest. However, this germ-free environment also leads to significant impairment of immune system development and responses. For example, mice raised in sterile conditions develop higher levels of IgE and fail to induce the same levels of immune reactivity seen during the "weaning period" of wild-type mice (20–23). This results in susceptibility to certain bacteria and increased risk of immunopathologies, complications which are not resolved with instillation of an "adult" or "wild-type" microbiome (21, 22, 24). Additionally, mouse studies of the microbiome show a large genetic influence which is distinct from humans where <10% of taxa are thought to be heritable (24–28). The high level of genetic homogeneity present in most murine models raises questions of generalizability to the human situation where genetic backgrounds and environmental exposures are vastly more diverse. This concern is reinforced by studies which have demonstrated the impact of environmental influences especially diet and drugs, as the most prominent factors influencing the microbiome in humans (29–31).

Additionally, as laboratory animals, mice do not receive the multi-faceted cancer care that is provided to humans and companion animals such as dogs. While pre-clinical murine models of checkpoint blockade immunotherapy have demonstrated impressive ability to prevent disease progression in various cancer cell lines, translation to human medicine has shown mixed results (32). Clinically meaningful survival benefits of PD-1 & CTLA-4 checkpoint inhibitor therapy have been demonstrated in some cancers, such as melanoma and non-small cell lung cancer, but only in a subset of patients (31–34) While observations from murine studies have demonstrated microbiome-associated factors for responsiveness to treatment, another limitation of murine models is their tendency to be reductionistic in their capacity to explore all potential intersecting variables which influence the cancer-immunity cycle (5–8, 15, 35, 36).

Similarly, murine models are able to strictly control diet and environment, both of which are key drivers of microbiome plasticity and composition, but as with germ-free studies, this can be both a strength and a weakness. For example, Matson et al. and Gopalakrishnan et al. (5, 6) used FMT to study whether human commensal microbes would potentiate anti-tumor T cell

responses in germ-free mice in two separate studies. Matson et al. demonstrated slower tumor growth in some mice after transferring microbiota from human responders. Gopalakrishnan et al. (6) showed changes in tumor infiltrating cells and upregulation of PD-1 in the tumor microenvironment after FMT. Although these mouse experiments demonstrated significant proof-of-concept results, it is not clear why effects were seen in some, but not all, mice. Given that the mice were evaluated to be highly homogenous and genetically inbred, the mice are expected to respond similarly. It is unclear whether the effects on the treatment would persist when using models with more heterogenous genetic backgrounds and environmental exposures. Additionally, the fact that similar mechanisms were identified in these studies but the precise microorganisms were different highlights potential concerns regarding generalizability and extrapolation as noted above.

Microbiome studies in dogs with spontaneous cancers receiving immunotherapy offer an additional avenue for exploration. As the development of canine immunotherapy advances, including the development of novel caninized anti-PD1, anti-CTLA-4 and anti-PD-L1 monoclonal antibodies, microbiome correlative studies and attempted therapeutic intervention in canines with intact immune systems and a lifetime of commensal gut microbiome symbionts may yield useful observations for human applications (37–39). Human clinical immunotherapy trials are impacted by immune-related adverse effects such as colitis and myocarditis (32, 33, 39, 40). While murine models have been developed to help study these immune-related events, these models are prone to develop auto-immune responses which may limit translational potential (41). Canine models represent an opportunity to bridge mechanistic studies in mice with descriptive studies in humans, and microbiome studies in companion animal dogs can help advance our understanding of how the gut microbiome shapes immunotherapy responses as well as toxicity and adverse events.

Human vs canine microbiome

As in humans, studies of the canine microbiome have evaluated the relationship of the microbiome with inflammatory disease states, development of malignancies, and more recently, response to oncologic therapies (42–47). The "normal" adult microbiota comprises thousands of bacterial species across mucosal and skin surfaces and is determined by environmental and genetic factors. The colonization of both the human and canine gut microbiome, one of the densest bacterial environments on earth, increases in alpha diversity and decreases in beta diversity during the weaning period before stabilizing (0-3 years in humans, 0-9 weeks in canines) (45, 48–50). Exposure to microbes during delivery, early diet, and antibiotic exposure have all been shown to impact gut microbial development (22, 51, 52). Early influences have clinical impact – colonization with *C. difficile* during the first months of life is associated with increased risk of atopic disorders such as asthma and eczema in children (53). Microbiome compositions in pre-weened puppies display instability of prominent taxa. Puppies that displayed these "immature" microbiome profiles are more suspectilble to diarrheal illness compared to those who have developed stable more "adult" compositions (54). Environmental factors such as diet, drugs, and living conditions also exert key influences on subsequent adult microbiota composition (29, 30). Microbiomes of genetically unrelated co-habitants are consistently demonstrated to be more similar than those of relatives who do not cohabitate, and genetic ancestry does not predict compositional similarities (29, 55). While environment is the primary influencer of composition, there appears to be heritability in human and canine gut microbiomes as well. A study of 400 human twin pairs raised in the same household compared monozygotic vs dizygotic siblings. Interestingly, monozygotic twins demonstrated more similar gut microbiota compared to their dizygotic counterparts (56). Canine comparisons of dog breeds show similar findings, although studies of breed variation indicate that breed does not cause major shifts in microbiome composition or diversity but may influence abundance of specific taxa (46). Although not to the extent of humans or mice, the canine gut microbiota has been surveyed in some detail (50, 57–59). Most large studies agree on the five most prominent phyla in dogs: *Firmicutes, Fusobacteria, Bacteroides, Proteobacteria and Actinobacteria*, the composition of which is more similar to humans than other commonly studied mammalian species. Coelho et al. (57) compared 129 stool samples from 64 dogs against previously published gut microbial gene catalogs based on similar sequencing methods. The phylum level distribution of genes in the dog was more like the published human data than that of a mouse or pig. When genes were clustered by each species pool, the dog gut gene pool overlapped most with the human gut microbiome (23%) compared to only 4.9% for the murine gene catalog. As in prior human data, the majority of the composition appeared to be influenced by environmental exposures, diet, and disease states. Importantly, certain bacterial species have been found to exert similar effect across species. Obesity, which is a growing problem in both humans and canines, is correlated with a change in Firmicutes/Bacteroides ratios in both humans and canines (60–62). Canine and human inflammatory bowel disorders are associated with a reduction in microbial community diversity and structure characterized by loss of key species or overgrowth of species with genotoxic potential such as *Bacteroides fragilis* and *E. coli* (45, 63). In humans, these microbiome shifts have been associated with colorectal cancer (12, 64, 65). While colorectal cancer is less common in dogs, increased *E.coli* is associated with canine intestinal lymphoma (42). Importantly, human and dog differences in microbiome interactions are also informative. Fusobacterium, for instance,

plays a role in energy utilization in the gut as a fermenting species. In humans, *F. nucleatum* is associated with colorectal cancer and thought to trigger inhibitory T cell receptors that suppress anti-cancer immune response (66, 67). In dogs, however, fusobacteria are associated with maintaining gut health (67).

While similarities in the development and composition of the gut microbiome present one advantage of the canine model, perhaps the most important is that of a shared environment with humans. As companion animals, dogs inhabit the same environment as humans. Therefore, shifts in composition caused by disease states or therapeutics must be robust enough to compete with the myriad of "real life" influences that patients encounter. Shared environments may also lead to shared taxonomy; canine housemates show similar microbiome profiles, similar to what is observed between human cohabiting with a spouse or partner (68). Interestingly, co-living between species influences microbiome profile as well (69). Companion dogs share more skin flora with their owners than with other dogs and oral microbiomes, which may show relevance in oral melanoma, as there is evidence of oral spread of symbionts between humans and dogs (68, 69). Some have hypothesized that pet ownership may influence the human gut microbiome as well (70). Small differences in abundance for two OTUs have been associated with pet ownership, although additional studies are needed given the risk of type I error (69).

Microbiome in canine therapeutics

Attenuation of immune surveillance and development of immunological tolerance to tumor-derived antigens contribute to cancer development and progression. Novel immunotherapies target one of these two mechanisms to stimulate the subject's immune system to recognize and target cancer as non-self. Overcoming both intrinsic and acquired resistance to these treatments are critical areas of study, and although several species of interest have been identified, no universal "responder signature" has been identified. Canine analysis that identifies similar bacterial enrichment as in human studies would greatly enhance the chances of replicable and generalizable effects.

Microbiome modulation has demonstrated exciting promise in increasing the number of human patients who may respond to PD-1/PD-L1 checkpoint inhibitor immunotherapy (4–6). Demonstrated first by Gopalakrishnan & Matson et al. (5, 6) in a murine model, and recently by Baruch et al. (7) in a human clinical trial, FMT from a drug-responsive subject to a nonresponsive subject was able to convert a subset of prior non-responders, allowing them to benefit from treatment. Importantly, when considering the human trial of FMT, extensive pre-and post-treatment analysis regarding microbiome composition, local metabolism, and expression of immune-related genes failed to show significant differences between those who became responsive to treatment and those who did not (7). Thus, while successful in proving the concept of FMT to be safe and potentially beneficial, the effects remain correlative. These types of immune-microbiome relationships could benefit from additional studies in dogs with spontaneous cancers in the setting of an intact immune system. FMT is accepted in community veterinary practice making high-powered studies possible once commercially available caninized ICI's are readily available (59). Repeating FMT trials with canine immunotherapy provides an opportunity to integrate observations of gut microbial alterations with longitudinal changes in metabolism and immune surveillance. Dogs, unlike mice, allow for multiple collections of adequate blood samples that can detect metabolites of short chain fatty acids that can act as ligands for G-protein coupled receptors. Iosine and hypoxanthine have recently been associated with bacteria of interest, for example (71, 72).

While veterinary medicine is waiting for the widespread commercial availability of canine specific PD-1/PD-L1/CTLA-4 mAbs to usher the area of immunotherapy in dogs, other immunotherapy approaches are in use, and comparison of microbiome profiles between responder status in other immune-based therapies should be considered (73, 74). Longitudinal stool and blood sampling of these canine patients for microbiome and metabolic investigation presents a rich area of investigation. Standard treatments for dog cancers include chemotherapy and radiation, which may allow for evaluation of the impacts that traditional therapies have on the microbiome alone or in combination with immunotherapy. Canine lifecycles are also inherently shorter, so rapidly progressing canine cancers allow for collection of samples linked to critical end points such as disease-free interval and time to progression which are highly relevant to the human situation.

Microbiome in the tumor microenvironment

The gut microbiome represents the most extensively classified community in humans and animal models, yet increasing studies have demonstrated that microbial communities in other compartments have clinical implications (74–76). Tumor-associated microbiomes have been identified in multiple human cancers including breast, lung, pancreas, and melanoma tumors (74–79). While tumor infiltrating lymphocytes (TILs) have emerged as a key feature of the immune tumor microenvironment (TME), a deeper molecular and cellular signals responsible for immune-tumor-microbiome communication are not yet understood. While some studies demonstrate a higher number of tumor infiltrating lymphocytes (TILs) in the TME project more positive patient outcomes, others suggest that the upregulation of regulatory T cells in the TME is associated with a more negative outcome (80–82).

Although mouse models can provide key insights into the immune-tumor interactions in the TME and are important for mechanistic studies, these models have limitations given the nature of transplanted highly retroviral tumors. Known differences in tumor initiation and promotion are likely to impact the fidelity of the model for microbiome studies (83, 84).

Therefore, dogs represent an important translational opportunity for studies of the cancer-immunity cycle, including microbiome studies. Canine immunotherapy, including evaluation of novel therapies in the context of clinical trials, has received significant attention from researchers and funding agencies, including the Cancer Moonshot, and examples of ongoing canine immunotherapy trials can be seen in Table 1 (9, 10). Dogs are diagnosed with naturally occurring cancers which are highly similar to humans, and genetic studies have demonstrated notable overlap in the tumor genetic makeup of human and dog OSA, melanoma, mammary tumors, gliomas and lymphoma (84–90). Canine OSA recapitulates several feature of human OSA including frequent TP53, PI3K, and MAPK pathway mutations with low expression of immuno-associated genes and a trend toward higher mutation burden in metastatic disease (88). Canine mammary tumors and lymphomas share major gene alterations with their human counterparts in addition to having similar tumor mutation burdens (91). Gliomas have been shown to share specific alterations in receptor tyrosine kinases such as TP53 and cell cycle pathways with human pediatric gliomas (89). Overall, the incidence of these canine cancers is believed to be as high or higher than their human counterparts, although precise case numbers are not known since no national database exists for dogs. For example, soft tissue sarcomas make up 10-15% of malignant tumors in dogs with estimates of approximately 7700-31,800 new canine cases per year in the United States compared to soft tissue sarcoma in humans which represents approximately 1% of cancer cases and 10,000 – 12,000 new cases per year in the US (91). Similarly,

TABLE 1 Ongoing Clinical Trials in Dogs.

Institution	Clinical Trial	Cancer Type
University of California Davis	Enhancing Natural Killer Immunotherapy with First in Dog Trials of Inhaled Recombinant IL-15 and Super agonist IL-15 in Naturally Occurring Canine Cancers	Osteosarcoma Melanoma
University of Alabama at Birmingham	Canine Immuno-Neurotherapeutics (Combination immunotherapies for canine brain tumors)	Glioma
Colorado State University	Optimizing Novel Immunotherapy Combination Targeting the Tumor Microenvironment in Canine Spontaneous Osteosarcoma	Osteosarcoma
Tufts University	Enhancing the Efficacy of Immunotherapy in DLBCL Using Rational Combination Approaches	Lymphoma
University of Minnesota	Novel Combined Immunotherapeutic Strategies for Glioma: Using Pet Dogs as a Large Animal Spontaneous Model	Glioma

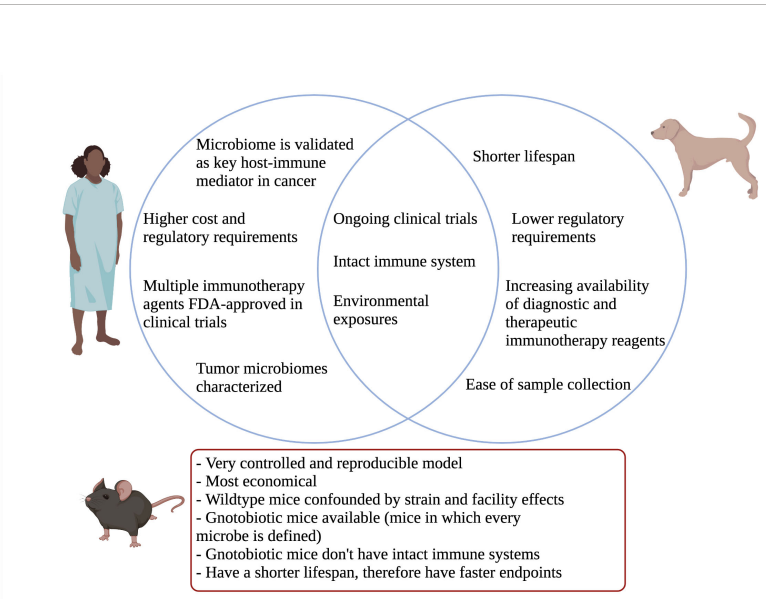

FIGURE 1
Venn diagram comparing relevant aspects of canine model to humans. Important mouse model differences highlighted below.

canine OSA is estimated at approximately 25,000 – 50,000 cases per year, whereas the number of human OSA is approximately 3000 per year in the US (92–94). Data such as these highlight the value of the canine model as an important asset in tumor immunology and immunotherapy studies, especially where an intact immune system is critical for understanding tumor and host biology.

Conclusion

Murine models will always have utility as pre-clinical models, including for microbiome studies. However, due to the complex interactions between the immune system, the TME, and metabolism, utilization of canines for microbiome research is a promising strategy to yield additional data that can bridge our understanding from "proof of concept" to "proof of mechanism". Further classification of the canine microbiome in cancer, changes in the canine microbiome in response to immunotherapy, and characterization of microbiomes outside the gut will all be important in deriving the most from canine models. This is particularly important now as veterinary medicine has made advances in canine specific immunotherapy agents that mirror human ICI. Canine clinical trials should consider collection of stool samples for microbiome biomarkers, as well as tissue and blood samples as well to correlate microbiome changes with immune infiltration and metabolic changes. As we increase our understanding of the interplay between specific commensal bacteria and the targets of immune therapy, the next step is therapeutic application. Through diet, antibiotics, fecal transfer, or other methods, reliably tailoring gut microbiomes to create maximal responsiveness with minimal side effects is the future of immuno-oncology. Dogs present a step-in bench to bedside science where their developing interventions can be tested on immune intact models exposed to complex environmental influences to ensure they demonstrate durability and utility.

Author contributions

KK and RC conceptualized the manuscript. KK, KI, LP, SC, and AR reviewed current literature. KK and KI wrote the manuscript and generated the associate figure. WC, MK, JE, and RR edited the manuscript and provided context for veterinary literature. RC edited the final paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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