Of Mice, Dogs, Pigs, and Men: Choosing the Appropriate Model for Immuno-Oncology Research

Nana H. Overgaard¹, Timothy M. Fan², Kyle M. Schachtschneider³, Daniel R. Principe⁴, Lawrence B. Schook³,⁵, and Gregers Jungersen⁶

¹Department of Micro- and Nanotechnology, Technical University of Denmark, Kgs Lyngby, Denmark, ²Department of Veterinary Clinical Medicine, University of Illinois, Urbana-Champaign, Illinois, ³Department of Radiology, University of Illinois, Chicago, Illinois, ⁴Medical Scientist Training Program, University of Illinois College of Medicine, Chicago, Illinois, ⁵Department of Animal Sciences, University of Illinois, Urbana-Champaign, Illinois, and ⁶Department of Biotechnology and Biomedicine, Technical University of Denmark, Kgs. Lyngby, Denmark

Abstract

The immune system plays dual roles in response to cancer. The host immune system protects against tumor formation via immunosurveillance; however, recognition of the tumor by immune cells also induces sculpting mechanisms leading to a Darwinian selection of tumor cell variants with reduced immunogenicity. Cancer immunoediting is the concept used to describe the complex interplay between tumor cells and the immune system. This concept, commonly referred to as the three E's, is encompassed by 3 distinct phases of elimination, equilibrium, and escape. Despite impressive results in the clinic, cancer immunotherapy still has room for improvement as many patients remain unresponsive to therapy. Moreover, many of the preclinical results obtained in the widely used mouse models of cancer are lost in translation to human patients.

To improve the success rate of immuno-oncology research and preclinical testing of immune-based anticancer therapies, using alternative animal models more closely related to humans is a promising approach. Here, we describe 2 of the major alternative model systems: canine (spontaneous) and porcine (experimental) cancer models. Although dogs display a high rate of spontaneous tumor formation, an increased number of genetically modified porcine models exist. We suggest that the optimal immuno-oncology model may depend on the stage of cancer immunoediting in question. In particular, the spontaneous canine tumor models provide a unique platform for evaluating therapies aimed at the escape phase of cancer, while genetically engineered swine allow for elucidation of tumor-immune cell interactions especially during the phases of elimination and equilibrium.

Key words: cancer immunoediting; canine cancer models; comparative oncology; immunotherapy; porcine cancer models; translational immunology

Introduction

Cancer has recently surpassed cardiovascular diseases as the leading cause of death worldwide.¹ The increasing cancer incidence combined with the emergence of improved therapeutic strategies has driven research into fields such as how the immune system influences cancer development and progression. The term immunosurveillance has traditionally been used to describe how the immune system can protect the host from tumor development.¹ However, because immunocompetent individuals still develop tumors, the hypothesis of immunosurveillance being a
fully protective mechanism is challenged.\(^1\) It has become well-recognized that the interplay between tumor cells and the immune system is extremely complex, and the ability of tumor cells to avoid immune destruction has been included as an official hallmark of cancer.\(^2\) Cancer immunoediting describes a complex interplay in which the immune system not only protects against cancer but also induces tumor-sculpting mechanisms leading to reduced immunogenicity of tumor cell variants.\(^3,4\) The concept of cancer immunoediting is composed of 3 phases: elimination, equilibrium, and escape\(^5,6\) (Table 1). The kinetics by which each of the 3 cancer immunoediting steps occurs is speculated to differ between tumors, with aggressive tumors accelerating faster through these phases.\(^5,7\)

The elimination phase encompasses the original concept of immunosurveillance, where the innate and adaptive immune systems collaborate to destroy the developing tumor.\(^5,8\) Although more work is needed to fully elucidate the mechanisms behind this antitumor immunity, it is known to be partly mediated by release of cytotoxic granules from CD8\(^+\) T cells and Natural Killer (NK) cells in addition to cytokine release from CD4\(^+\) T cells and Natural Killer T (NKT) cells\(^9\) (Table 1). A more detailed mechanism behind the elimination phase has been proposed by Dunn et al (2002).\(^8\) In brief, the tumor becomes invasive when reaching a size that requires a distinct blood supply controlled in part by the production of angiogenic proteins.\(^9\) Such invasive growth results in small disruptions in the adjacent tissue, thereby inducing inflammation, which leads to intratumoral infiltration of innate immune cells like dendritic cells (DCs), NK cells, NKT cells, \(\gamma\delta T\) cells, and macrophages. Upon recognition of tumor cells, these innate immune subsets produce interferon (IFN)-\(\gamma\), which can induce tumor cell death by antiproliferative and apoptotic mechanisms. Moreover, these innate immune cells produce chemokines with the capacity to limit blood vessel formation. Tumor cell debris is then taken up by DCs, which migrate to the draining lymph node and induce tumor-specific CD4\(^+\) T helper cells and tumor-specific CD8\(^+\) T cells. Finally, these activated T cells home to the tumor, where the CD8\(^+\) T cells in particular mediate antitumor activities.\(^5\)

If the immune system succeeds in completing this phase, the host is cleared of cancer with no clinical symptoms or progression to the additional editing stages\(^5,6\) (Table 1).

However, as well as protecting the host, antitumor immunity can also induce tumor-sculpting mechanisms resulting in tumor editing.\(^5,6,8,10-13\) Consequently, tumor cell variants with increased capacity to avoid immune recognition can develop, thereby entering the equilibrium phase (Table 1). This is a dynamic equilibrium that can last for several years and is believed to be the longest of the 3 phases.\(^8,13\) Several underlying molecular mechanisms at the genetic and epigenetic level have been suggested to contribute to reduced immunogenicity of cancer cells during the equilibrium phase. In particular, increased genetic instability, reduced Major Histocompatibility Complex (MHC) class I expression, and defective antigen processing have been implicated in reducing tumor immunogenicity and facilitating tumor escape.\(^8,10,16-23\) Enhanced secretion of immunosuppressive cytokines by tumor cells, increased induction of regulatory T cells, and tumor insensitivity towards IFN-\(\gamma\) have also been reported as important factors\(^24-27\) (Table 1). After a prolonged suboptimal immune response, selected tumor cell variants with reduced immunogenicity can become insensitive to immune recognition resulting in uncontrollable tumor growth. This is referred to as the escape phase,\(^5,8,28\) and the tumor is now capable of proliferating in a fully immunocompetent host environment (Table 1), although the degree of immune cell infiltration still affects the prognosis of the patient.\(^29-31\) Additional work is required to fully understand the complex interplay between cancer and the immune system, highlighting the need for animal models appropriately mimicking the human situation.

Different animal models can provide unique insights into the distinct immunoediting stages (elimination, equilibrium, and escape) of cancer progression and empower cancer researchers to rationally combine various modeling systems necessary to generate high-value and translationally relevant immunobiologic data from future research investigations.

### Table 1 Common Immunological, Tumoral, and Clinical Characteristics of Cancer Immunoediting

<table>
<thead>
<tr>
<th>Phase</th>
<th>Immunological Characteristics</th>
<th>Tumor Characteristics</th>
<th>Clinical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elimination</td>
<td>Active immunosurveillance. Initial infiltration of tumors with DCs, NK cells, NKT cells, (\gamma\delta T) cells, and macrophages. Production of IFN-(\gamma) and chemokines. Recruitment of adaptive immune cells followed by antitumor reactivity mediated by CD8(^+) T cells, NK cells, CD4(^+) T cells, and NKT cells.</td>
<td>High expression level of MHC class I, efficient antigen processing, and presentation of tumor antigens to T cells. Production of angiogenic proteins, tissue disruption, and induction of inflammation.</td>
<td>No clinical symptoms. Potential full regression of the developing tumor.</td>
</tr>
<tr>
<td>Equilibrium</td>
<td>Dynamic equilibrium between the tumor and the immune system. Anti-tumor immunity remains present.</td>
<td>Expansion of tumor cell variants with reduced immunogenicity. Lowered MHC class I expression and increased genetic instability and avoidance of immune recognition. Enhanced secretion of immunosuppressive cytokines. Increased induction of Tregs and insensitivity towards IFN-(\gamma).</td>
<td>The longest of the three phases, which may last for several years.</td>
</tr>
<tr>
<td>Escape</td>
<td>Suppression of antitumor immunity and/or lack of recognition. T cells impaired by inhibitory cytokines and checkpoint molecules, limitations in nutrient availability, metabolic competition, reduction of oxygen levels, and increase in lactate production by the tumor cells.</td>
<td>Defective antigen processing and reduced antigen presentation to T cells. Insensitivity to immune recognition. Immunosuppressive tumor microenvironment.</td>
<td>Uncontrolled tumor growth in an immunocompetent host.</td>
</tr>
</tbody>
</table>

**References.**\(^5,11,13-20,22-28\)

**Abbreviations:** DC, dendritic cell; NK cell, natural killer cell; NKT cell, natural killer T cell; MHC, Major Histocompatibility Complex; Treg, regulatory T cell.
Mouse Models of Immuno-Oncology

Syngeneic Mouse Models

For many years, mice have been the most commonly used animal model for immunological research and have provided a crucial elucidation of complex immunological pathways.32–35 This in part reflects mice displaying reduced genetic variability, short generation intervals, easy maintenance, and the large number of commercially available reagents.32,36 In cancer immunology, the most widely used mouse models involve inoculation of histocompatible (syngeneic) tumor cell lines into recipient mice, often of C57BL/6 or BALB/c background.34,37,38 These syngeneic tumor models offer several advantages including reproducible tumor growth and simplicity in measuring tumor development over time, especially if the tumor cells are inoculated subcutaneously.33,34,39 However, the off-site (heterotopic) injection of tumor cells in the subcutaneous tissues largely fails to recapitulate the normal microenvironment in which most tumor cells develop, and hence the operative mechanisms of immunosurveillance are likewise artificial. Additionally, the tumor cell lines tend to grow aggressively post injection, which causes studies to be terminated within a relatively short time due to ethical considerations and temporarily constrains the time allowed for trafficking of immune cells and the natural development of antitumor immunity. Furthermore, the tumor cell lines differ in their intrinsic immunogenicity, therefore, the resulting tumor microenvironment often does not represent what is seen in human patients.40,41

Orthotopic implantation is administration of a given tumor cell line into the relevant tissue for that specific tumor. In contrast to subcutaneous injection, orthotopic implantation has been shown to better recapitulate the tumor biology, tumor environment, and disease progression.42 In particular, the early steps of metastasis and angiogenesis have been modelled more appropriately using orthotopically implanted tumors.42–45 Moreover, orthotopically implanted tumors have provided a valuable system for evaluation and understanding of checkpoint inhibition in various preclinical cancer models.46–48 To date, several types of orthotopically implanted tumor models have been established amongst others, including transplantation in the brain (GL261 cells), the mammary fat pad (4T1 and EMT6 cells),50,53 intrasplenic (Panc02 cells),52,55 and in the bladder (MBT-2 cells).56 Overall, these models may serve as more clinically relevant systems, although the technicality of transplanting the tumor cells is more complex and labor-intensive compared to subcutaneous administration.42

Genetically Engineered Mouse Models

Although syngeneic mouse models are immunocompetent, they do not offer the opportunity for directly testing human targets. For this reason, syngeneic models are increasingly replaced by genetically engineered mouse (GEM) models, human xenograft, and patient-derived xenograft models.49–51 An almost unlimited number of GEM models exist, with those for cancer research purposes typically produced through deletion, mutation, or overexpression of genes known to be crucial for cellular transformation and malignancy.52 GEM models are very useful for studying the effect of specific mutations on tumor progression in an immunocompetent host.53–55 By changing the genetic profile of these mice, it is possible to introduce mutations resulting in conditional expression overexpression or loss/gain of function of genes known to be involved in transformation and tumorigenesis.55,56 Moreover, tissue-/organ-specific targeting of the mutation or targeting to specific developmental stages during disease progression are valuable research tools for understanding the complex mechanisms underlying transformation and malignancy.53,59

Despite this, GEM models often fail in mimicking the complexity of human tumors that are often driven by stochastic genomic instability.55 Some mouse models of cancer appear to be driven by homozygous mutations, whereas human cancers are most likely heterogeneous with a functional wild-type allele. As such, the knockout of specific genes or pathways in GEM models may fail to recapitulate the chaotic manner in which malignant transformation occurs during spontaneous tumor development in human cancer patients. Although no ideal animal model can fully recapitulate the stochastic nature of human tumorigenesis, certain strategies have been developed to generate GEM models with more heterogeneous tumors of clinical relevance. Such approaches include, for instance, single-cell knockouts to achieve sporadic loss of gene expression and subsequently in vivo mosaics59,60 as well as chemical- or UV-induced models, which can result in heterogeneous tumors arising from a multistep process.51,62

Xenograft Models and Humanized Mice

Xenograft models, which involve the transplantation of human cancer cell lines, or patient-derived tumor cells in the case of patient-derived xenograft models, into immunodeficient mice represent another commonly used mouse model for cancer research.53–65 These models offer a unique tool for testing anticancer drugs targeting human proteins in mutated cancer as well as individualized and patient-specific treatments.66 Moreover, engraftment of surgically resected tumor biopsies into these immunodeficient mice allows for an in vivo system, where interactions between, for instance, tumor cells and stromal cells can be evaluated.55 Xenograft models undeniably add valuable knowledge to the research field; however, they are fairly expensive and labor intensive.66,67 Also, the arising tumor is not exposed to any immune-mediated pressure due to the lack of an endogenous immune system.

To address the limitations associated with using an immunodeficient host, humanized mice have been developed. These mice are either genetically engineered to carry human genes57 or developed through engraftment of human immune cells into an immunodeficient host.66–71 Notably, humanized mice have provided an important tool for obtaining knowledge within the field of checkpoint inhibitors targeting, for instance, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4), programmed cell death-1 (PD-1), and programmed death ligand-1 (PD-L1).72 Moreover, therapies combining chimeric antigen receptor (CAR) T-cell therapies with checkpoint inhibition have been tested in humanized mice.73,74 Despite this, humanized mice are often on the Il2rg−/− background; they lack both lymph nodes and Peyer’s patches,75 which are major secondary lymphoid organs necessary for mature DCs to interact and potentially activate naïve T- and B-lymphocytes. As such, humanized mice are devoid of key organized immune microenvironments critical to initiating robust immune responses. Furthermore, humanized mice are challenged in their capacity to restore MHC class I and II-selecting elements, which are crucial for shaping the T-cell repertoire.76

It is becoming increasingly recognized that mice often poorly mimic human diseases, even when sophisticatedally manipulated with genetic techniques.78,79 An ideal animal model for cancer research should preferably be fully immunocompetent to properly mimic human immune responses.79,81 Although some mouse
models are immunocompetent, they often still display a very narrow MHC class I representation due to inbreeding. Consequently, this might result in unrepresentative results when compared to outbred animals and humans. Overall, no perfect animal model capable of fully recapitulating the complexity of human disease exists. Mouse models have indeed provided the field of immuno-oncology with invaluable insight, but there remains a need for large animal models encompassing a fully competent immune system, which may function as a link between murine studies and the clinic. Given their comparable body size and metabolic physiology to human beings, as well as their well-annotated genomes, canine and porcine models of human cancer are uniquely situated to serve as excellent comparative tumor models.

Canine Models Of Immuno-Oncology

Cancer in pet dogs is common and has been reported as a leading cause of death in aging dogs, accounting for greater than 1 in 4 deaths. As cancer in dogs occurs spontaneously and displays similar characteristics to many specific human tumor histologies, canine models are becoming more widely used in preclinical cancer research. Representative of this research opportunity, in 2003 the National Cancer Institute’s (NCI) Center for Cancer Research established the Comparative Oncology Program to facilitate and support the design, sponsorship, and execution of translational trials in pet dogs to test novel anti-cancer drugs prior to human clinical trials. There are several advantages unique to canine models that were recognized and leveraged to expedite novel drug development ultimately slated for human usage. Because dogs are companion animals, they often live together with humans; therefore, they are exposed to the same environmental risk factors and might to a certain extent have a diet similar to humans. As with humans, a correlation between spontaneous tumor incidence and age is found in dogs. Additionally, from an evolutionary point of view, dogs are more closely related to humans than mice and share more similar physiologic and immunobiologic traits. Lastly, the high degree of homology in the human and canine genome makes analysis of DNA damage as well as epigenetic changes during tumor development and progression more facilely traceable and possible in outbred dogs.

Recently, several canine tumor histologies have been intensely studied using molecular cytogenetic techniques such as comparative genomic hybridization, oligonucleotide arrays, fluorescence in situ hybridization, and gene expression profiling. Based upon these genomic investigations, several conserved genetic similarities have been identified between canine and human tumors, including DNA copy number variations, structural chromosome aberrations, and differential gene expression patterns. These findings of shared genetic perturbations associated with distinct tumor histologies in both dogs and humans further support the potential value of pet dogs with certain types of naturally occurring tumors as a unique model system for human-relevant cancer research. Importantly, canine tumors believed to be immunogenic including osteosarcoma, lymphoma, urothelial carcinoma, mammary gland carcinoma, melanoma, and brain cancers have been the primary focus of most genomic-based investigations.

The Canine Immune System

The canine immune system demonstrates a close homology to the human counterpart and many of the same immune markers have been validated in the canine species. Because tumors in pet dogs arise in an immunocompetent host, canine models enable the design of experiments that elucidate the complex interplay between cancer cells and the immune system as well as the natural progression of malignant transformation under the evolutionary pressures exerted by host immunosurveillance. Using human antibodies toward T-cell markers, it is now possible to distinguish canine activated T cells and central memory T cells by flow cytometry, thus providing an important tool for vaccine research purposes. Adding to the strength of dogs to cancer vaccine research is their recognized breed-specific restriction in MHC expressions thereby allowing cancer researchers to focus efforts on “high-value” neoantigen discovery most likely to elicit potent cytotoxic T-cell responses. Despite being limited in scope to date, some studies have evaluated tumor immune cell infiltrates in canine cancer models. Flow cytometric analysis has shown the presence of both CD4+ and CD8+ tumor infiltrating lymphocytes within canine mammary tumors. Another study using dogs with metastatic lesions showed an increased CD4/CD8 T-cell ratio, which also correlated with decreased survival rate. In studies of canine B cell lymphoma, a worse prognosis was found in dogs with increased representation of tumor-associated macrophages, myeloid-derived suppressor cells, and regulatory T cells and cytotoxic T-cell-mediated killing of autologous lymphoma cells has been demonstrated in vitro. Collectively, these preclinical and clinical findings provide strong support for including the canine species as an immune competent model system for immuno-oncology research.

Immunotherapy Research Using Canine Models

Leveraging the immune system to fight cancer can take many different, yet synergistic, strategies that engage the cellular players comprising the innate and/or adaptive immune systems. Classically, innate immune cells including neutrophils, macrophages, and NK cells can be activated through engagement of diverse cellular receptors with cognate ligands of endogenous (alarmins) nature, while cells of the adaptive immune system including B and T lymphocytes can be activated by primed antigen presenting cells. In addition, eliciting adaptive antitumor immunity can be mediated by both active and passive immunotherapeutic interventions such as vaccines and monoclonal antibodies, respectively. As immunobiologic reagents and therapeutics have become more readily available, many of these different approaches for stimulating both innate and adaptive systems, either passively or actively, have been investigated in pet dogs with cancer and a nonexhaustive list of example strategies are summarized in Table 2, with some of the most recent strategies further described below.

For immunotherapy purposes, canine tumor models offer a very powerful research tool. As monoclonal antibodies blocking CTLA-4, PD-1, and PD-L1 have provided impressive results in the clinic, it is desirable to have a preclinical animal model expressing these molecules. CTLA-4, PD-1, and PD-L1 expression have all been shown in a variety of canine solid and hematopoietic tumors. In fact, the PD-1/PD-L1 pathway in dogs is associated with T-cell exhaustion, as often reported for humans. Due to limitations in commercially available canine reagents, detailed studies with checkpoint inhibitors in dogs remain preliminary in scope and nature; however, early evidence demonstrates that blockade of PD-1/PD-L1 can lead to enhanced T-cell proliferation and cytokine release. Whether these
observed immunobiologic activities will be adequate to produce robust clinical benefit in a substantial fraction of treated pet dogs remains to be determined, yet early results indicate some measurable immunobiologic activity against specific solid tumors including oral melanoma and soft tissue sarcoma.\(^\text{126}\)

Most recently, genetically engineering of CAR T cells has been heralded as an immunologic breakthrough for the management of pediatric acute lymphoblastic leukemia in human beings.\(^\text{124,125}\) Although this genetic manipulation technology remains in its infancy for veterinary medicine, CAR T cells have shown promising results in dogs as a proof-of-concept for the management of both hematopoietic (B-cell lymphoma) and solid (osteosarcoma) tumors.\(^\text{126,127}\) Therefore, pet dogs might in the future serve as an important model in elucidating the design of treatment regimens that maximize therapeutic benefit yet minimize adverse events often observed upon CAR T-cell therapy.\(^\text{128}\)

The establishment of active adaptive immunotherapy through tumor vaccination strategies remains a priority in human cancer patients. Although preventative vaccines against hepatitis B virus and human papillomavirus have dramatically decreased the incidence of hepatocellular and cervical cancers, respectively,\(^\text{129,130}\) the utility of therapeutic cancer vaccines remains limited. In 2010, the FDA approved sipuleucel-T (Provenge), a vaccine that utilizes tumor lysate-loaded dendritic cells to activate the immune system against castration-resistant prostate cancer,\(^\text{131,132}\) and to date this remains the only approved therapeutic cancer vaccine in people. In terms of cancer vaccine trials in dogs, while tumor cell lysate vaccines have been tested either as combination therapy or stand-alone treatment,\(^\text{133–135}\) Most notably, in 2007, a xenogeneic DNA vaccine (Oncept) targeting the human tyrosinase protein was the first therapeutic vaccine to be approved for treatment of canine oral melanoma.\(^\text{136,137}\) Although considered the first of its kind, the definitive immunostimulatory potential and clinically benefit derived from this xenogeneic DNA vaccine strategy would be substantially bolstered through the conductance of a large, prospective randomized phase III clinical trial in pet dogs. In addition, canine vaccine trials targeting telomerase reverse transcriptase, heat-shock proteins, and the human vascular endothelial growth factor protein have been performed.\(^\text{129,136,138}\) Notably, these trials all share the aim of treating cancer in dogs rather than using the canine tumor models as a link between rodent studies and human clinical trials. However, at least 2 examples exist that seek to leverage the pet dog as a comparative model for the development of immunotherapeutic strategies to be employed in human cancer patients. First, a Listeria monocytogenes vaccine strategy has been evaluated in pet dogs with osteosarcoma, and initial results support the generation of a potent adaptive immune response translating into substantive improvements in overall survival time.\(^\text{139}\) Second, a DC-based vaccine in combination with IFN-γ administration has been demonstrated to improve the clinical outcome in tumor-bearing dogs.

<table>
<thead>
<tr>
<th>Immune Arm</th>
<th>Immunotherapeutic Strategy</th>
<th>Specific Methodology</th>
<th>Tumor Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innate</td>
<td>Innate immune cell activation</td>
<td>Localized radiation and autologous NK cell intratumoral transfer</td>
<td>Osteosarcoma</td>
<td>229</td>
</tr>
<tr>
<td></td>
<td>Innate immune cell activation</td>
<td>Localized radiation, TLR activation, and indolamine-2,3-Dioxygenase inhibition</td>
<td>Melanoma, STS</td>
<td>230</td>
</tr>
<tr>
<td></td>
<td>Innate immune cell activation</td>
<td>Liposome MTP-PE infusion</td>
<td>Osteosarcoma</td>
<td>200</td>
</tr>
<tr>
<td>Adaptive (passive)</td>
<td>Exogenous cytokine therapy</td>
<td>Intravenous liposome-DNA complexes with interleukin-2 gene Inhalation therapy with liposome interleukin-2 Intralesional interleukin-2 Intratumoral interleukin-2</td>
<td>Osteosarcoma</td>
<td>231</td>
</tr>
<tr>
<td></td>
<td>Monoclonal antibody therapy</td>
<td>Ex vivo PD-L1 blockade to mitigate T cell exhaustion</td>
<td>Various solid tumors</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>Adaptive transfer of T cells</td>
<td>In vitro PD-L1 blockade to induce TIL activation In vivo PD-L1 blockade in cancer-bearing dogs</td>
<td>STS, adenocarcinoma, Melanoma, STS</td>
<td>122, 120</td>
</tr>
<tr>
<td>Adaptive (active)</td>
<td>Generation of CAR-expressing T cells (CAR-T)</td>
<td>Autologous T cell transfer following cytokine activation</td>
<td>B cell lymphoma</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td>Generation of CAR-expressing T cells (CAR-T)</td>
<td>Autologous lymphokine-activated T cell transfer</td>
<td>Melanoma, others, Osteosarcoma</td>
<td>236, 237</td>
</tr>
<tr>
<td></td>
<td>Generation of CAR-expressing T cells (CAR-T)</td>
<td>Generation of CAR-expressing T cells specific to HER2 epitope-in-vitro</td>
<td>Osteosarcoma</td>
<td>236, 237</td>
</tr>
<tr>
<td></td>
<td>Generation of CAR-expressing T cells (CAR-T)</td>
<td>Generation of CAR-expressing T cells specific to CD20 HER2-targeting Listeria monocytogenes vaccination Adenovirus DNA-electro-gene-transfer targeting dog telomerase reverse transcriptase Lipoplexes with HSV-TK and canine INFγ; tumor extract vaccine + cytokines Xenogeneic human tyrosinase DNA vaccine</td>
<td>B-cell lymphoma, Osteosarcoma, B-cell lymphoma Melanoma</td>
<td>127, 139, 238, 239, 240, 241</td>
</tr>
</tbody>
</table>

Abbreviations: CAR, chimeric antigen receptor; NK, natural killer; PD-1, Programmed cell death-1; PD-L1, Programmed death-ligand 1; STS, soft tissue sarcoma; TIL, tumor-infiltrating lymphocyte; TLR, Toll-like Receptor.
thereby supporting the use of canine models for preclinical testing of human anti-cancer therapies.\textsuperscript{140}

Despite the many benefits of canine cancer models, their use for therapeutic cancer vaccine development has a number of important drawbacks. The low number of known canine tumor antigens,\textsuperscript{138} the increasing ethical regulation of experiments on companion animals,\textsuperscript{184} and the limited number of commercially available reagents undeniably make canine translational research more difficult.\textsuperscript{90} Although dogs are more outbred than mice, modern dog breeds are the results of line inbreeding, thus questioning whether canine models can properly mimic human heterogeneity.\textsuperscript{56} Therefore, although canine models provide some important advantages over murine models, there is still a need for alternative large animal cancer models, and the most robust investigations will likely be derived from the utilization of a panel of animal models.

**Porcine Models of Immuno-Oncology**

Pigs are valuable models for studying immune responses toward infections.\textsuperscript{145-147} Moreover, porcine models are becoming increasingly used for human biomedical research and as unique research tools for surgical procedural training.\textsuperscript{144-146} The advancement in using porcine models is due to the high degree of homology in anatomy, physiology, size, cell biology, key metabolizing enzymes, genetics, and epigenetics between pigs and humans.\textsuperscript{147-157} In addition, the life-span of the pig also offers an opportunity to monitor and characterize disease development and progression over a human-relevant amount of time.\textsuperscript{56,149,158} Importantly for cancer research, porcine somatic cells, consistent with human cells, suppress telomerase activity in most tissues, which is then reactivated during tumorigenesis.\textsuperscript{159,160}

Although mice are closer to humans phylogenetically, pigs and humans share a higher similarity in protein structure.\textsuperscript{161} A detailed comparison of immune-related genes across several species revealed that pigs are more closely related to humans at the immunome level than mice.\textsuperscript{141} In addition, the number of species-unique immune-related genes is considerably lower in pigs than in mice.\textsuperscript{141} Using orthology preservation analysis of the immunome, the authors found 188 genes shared across humans, mice, and pigs. When evaluating species-unique immune-related genes, humans and pigs showed 37 and 16 genes, respectively. In contrast, 174 genes relating to various immunological pathways were found to be present only in the mouse,\textsuperscript{141} clearly indicating crucial differences in the immune system between rodents and larger animals, including pigs and humans. Recently, the same authors compared the inflammasome across humans, pigs, and mice. Here, they clearly showed a murine expansion in the number of 7 different pattern recognition receptors compared to the 2 other species analyzed.\textsuperscript{162} For instance, mice displayed 57 different receptors belonging to the NK cell receptor subfamily of the C-type lectin superfamilies, whereas only 24 and 23 were found in the human and porcine system, respectively.\textsuperscript{165} As NK cells are crucial players of mediating antitumor immunity and limiting tumor metastasis,\textsuperscript{165,166} such differences need to be taken into account when interpreting immuno-oncology research. Combined, these data support the notion that preclinical results obtained in porcine models have several advantages compared to rodent models.

**The Porcine Immune System**

Overall, the porcine immune system comprises the same immune cell populations as demonstrated in humans.\textsuperscript{143,154} For instance, the porcine Treg population expresses markers similar to the human population, namely CD4, CD25, and FoxP3.\textsuperscript{145,156} However, some important differences do exist between the porcine and the human immune system. Porcine peripheral blood comprises a large number of γδ T cells, representing up to 50% of the total blood lymphocyte population in young animals.\textsuperscript{162} In contrast, the representation of γδ T cells in human peripheral blood sampled across the world is less than 10%\textsuperscript{158} Although the functional properties of γδ T cells are not fully understood, it is suggested that these cells display both cytolytic activity and capacity to perform antigen presentation.\textsuperscript{160}

Another notable difference is that the porcine T-cell pool comprises a large proportion of CD4$^+$ T cells coexpressing the CD8α homodimer in peripheral tissues.\textsuperscript{169,170} In pigs, these CD4$^+$CD8α$^+$ T cells are defined as an activated/memory CD4$^+$ T-cell population recognizing antigens in the context of MHC class II.\textsuperscript{165,171} As this CD4$^+$ T-cell population expresses the CD8α homodimer, expression of the CD8α molecule is commonly used to define porcine cytotoxic T cells.\textsuperscript{174,175} In addition, the lymphocyte migration pattern differs slightly between pigs and humans due to the porcine lymph nodes being structurally inverted.\textsuperscript{172,173} Consequently, porcine lymphocytes, similar to humans, enter the lymph node via L-selectin$^+$ high endothelial venules. However, porcine T and B cells leave the lymph node by directly entering the blood stream via high endothelial venules rather than migrating out via the efferent lymph as in humans.\textsuperscript{172,175} Despite the increased representation of CD4$^+$CD8α$^+$ T cells in porcine peripheral blood and the inverted lymph node morphology, there are currently no indications of these differences resulting in any significant functional differences between the human and porcine immune system.\textsuperscript{173}

The porcine MHC molecule is commonly referred to as the swine leukocyte antigen (SLA). As pigs are largely outbred compared to rodents, fully immunocompetent porcine models display a high MHC class I allelic diversity with the number of known alleles continuously expanding with improved typing methods and growing interest in swine for biomedical research.\textsuperscript{174,175} In particular, the development of a Next Generation Sequencing-based SLA-typing approach has allowed a fast identification of expressed SLA class I molecules,\textsuperscript{174} thereby allowing selection of MHC-matched animals to be used for instance in a vaccine protocol or other immunological assays.

**Immunotherapy Research Using Porcine Models**

Although pigs have provided valuable findings for infectious diseases, porcine models have had limited use thus far in experimental oncology. The 2 most common cancer types found in pigs are lymphosarcoma and melanoma.\textsuperscript{176} Porcine skin is very similar to human skin both in terms of morphology and functional characteristics,\textsuperscript{177} providing a unique model for studying skin cancers like melanoma. For many years, the Sinclair minipig and the melanoblastoma-bearing Libechnov minipig (MelIM) model have been the 2 most commonly used porcine spontaneous melanoma models, although the underlying genetic changes resulting in the melanoma development are not well understood.\textsuperscript{178,179} Despite this, a study in the MelIM model has contributed to a better understanding of melanoma progression and identified RAG1 as a potential marker of malignancy in human melanoma.\textsuperscript{179} In recent years, porcine severe combined immunodeficiency models have also been developed.\textsuperscript{180-185} As in the rodent equivalents, porcine porcine severe combined immunodeficiency animals lack T and B cells, allowing them to be used for xenotransplantation studies including engraftment of human tumor and immune cells.
Genetically Engineered Porcine Models
To expand the use of pigs in experimental oncology, several genetically modified porcine models of human cancer have been developed. By overexpressing the human GLI2 gene, it was possible to develop a model with basal cell carcinoma-like lesions.\(^\text{168}\) In addition, colorectal cancer\(^\text{167,188}\) and breast cancer\(^\text{189,190}\) models have been developed, although these animals either lacked in vivo tumor development or displayed lethality issues. Modification of either the tumor suppressor gene TP53 or the oncogene KRAS has enabled the development of porcine models giving rise to various cancer types. Mutational silencing of the TP53 tumor suppressive pathway is observed in approximately 33% of human cancers.\(^\text{189}\) Such mutations in the TP53 gene are often associated with increased cell proliferation, survival, invasiveness, and metastasis.\(^\text{190}\) The porcine models express the TP53\(^{R167H}\) dominant negative mutation, which is equivalent to the frequently observed TP53\(^{R175H}\) mutation in humans.\(^\text{191,193}\) Upon expression of TP53\(^{R167H}\), the pigs develop both lymphoma and osteogenic tumors.\(^\text{194}\)

Furthermore, the RAS gene is mutated in approximately 25% of all human cancers, with KRAS being the most commonly mutated isoform.\(^\text{195}\) The RAS protein is a GTPase driving cellular proliferation, and oncogenic RAS especially promotes pro-growth, proangiogenic, and antiapoptotic signals.\(^\text{196}\) Specifically for KRAS\(^{G12D}\), this oncogenic activating mutation promotes metastasis in human pancreatic cancer in part by downregulating E-cadherin.\(^\text{197}\) Although histopathology is yet to be determined, a porcine model with inducible KRAS\(^{G12D}\) has been developed.\(^\text{198}\) Upon xenotransplantation, in vitro- transformed porcine mesenchymal stem cells expressing both the TP53\(^{R167H}\) and the KRAS\(^{G12D}\) mutation have successfully established tumors in immuno-deficient mice.\(^\text{199}\) However, the only transgenic pig combining both the TP53\(^{R167H}\) dominant negative mutation and the KRAS\(^{G12D}\) oncogenic activating mutation is a model known as the Oncopig.\(^\text{191}\) The expression of the 2 mutations is under control of a CAG promoter. Due to the internal ribosome entry site element, bicistronic expression of the mutated transgenes, KRAS\(^{G12D}\) and TP53\(^{R167H}\), is possible. Because every cell in the Oncopig has this expression construct, the model enables induction of a broad range of cancer types upon exposure to Cre recombinase.\(^\text{191}\)

In vivo induction of sarcomas with regional leiomyosarcomas has been shown upon intramuscular, testicular, and subcutaneous injection of adenoviral vectors encoding Cre recombinase into Oncopigs.\(^\text{193}\) Successful in vitro transformation of 11 different Oncopig cell lines has been established, as described in detail elsewhere.\(^\text{196}\) Although limited in scope, some immunological characterization of the Oncopig intratumoral landscape has been performed. Using immunohistochemistry, infiltration of CD3+ cells was shown in Oncopig hepatocellular carcinoma.\(^\text{198}\) A more detailed and T-cell-focused evaluation of the immunological landscape in Oncopig sarcomas was recently performed, where pronounced T-cell infiltration to the tumor site was demonstrated (Overgaard et al, 2018, submitted). The tumor microenvironment was especially enriched with cytotoxic and activated immune cells. This, in conjunction with RNA-seq analysis revealing elevated gene expression of the immunosuppressive molecules CTLA4, PD1, and indoleamine 2,3-dioxygenase 1 in tumor tissue, supports the use of this transgenic porcine model for evaluation of the complex interplay between the tumor and the immune system of the host.

Ongoing and Future Translational Opportunities
Efforts are made to promote a One Health approach to evaluate new treatment options for cancer in canine animal models through the Comparative Oncology Trials Consortium at NCI as a major clinical trial hub across Northern America (United States and Canada). Further, a group of Academic Veterinary Teaching Hospitals in the United States/Canada recently established the Comparative Brain Tumor Consortium to improve the knowledge, development of, and access to naturally occurring canine brain cancers, specifically glioma, as a model for human disease.\(^\text{199}\) Supporting the merits for the NCI’s (Comparative Oncology Trials Consortium and Comparative Brain Tumor Consortium) translational efforts, existing evidence for the value of pet dogs with cancer in expediting anticaner drug development are multiple. Perhaps the best example for pet dogs to be included in the new drug or biological agent development path is mifamurtide, which is liposome encapsulated MTP-PE.\(^\text{200}\) Although the data packet for mifamurtide was deemed insufficient for FDA approval, the European Medicines Agency was convinced of mifamurtide’s activity and in 2004 approved its use for the treatment of high-grade, nonmetastatic, resectable osteosarcoma in human beings. In addition to mifamurtide, other investigational agents that included pet dogs with cancer in the pathway towards investigational new drug designation and human Phase I clinical trials include GS-9219, KPT-335, and PAC-1.\(^\text{107,201-205}\)

Given the immune competency of pet dogs with cancer, and underscoring the unique and valuable potential of large animal models in cancer research, the NCI recently launched a request for proposals to support canine clinical studies evaluating the feasibility and activity of immunotherapeutic agents and novel drug combinations such as immune modulators, molecular targeted agents, chemotherapy, and/or radiation.\(^\text{206}\) Clinical studies will be accompanied by laboratory correlative studies that seek to describe, characterize, and understand the cellular and molecular mechanisms that determine the antitumor response (or lack of response) in dogs with spontaneous tumors. Specifically, the spontaneous tumor types that have been deliberately targeted as comparative for immunotherapeutic development include lymphoma,\(^\text{106,107,207,208}\) osteosarcoma,\(^\text{106,107,209-212}\) mammary gland cancer,\(^\text{106,107,213,214}\) brain cancer,\(^\text{199,215-217}\) melanoma,\(^\text{218-220}\) and transitional cell carcinoma.\(^\text{221,222}\) (Table 3)

Complementing spontaneous tumor models in pet dogs, the development of genetically modified pigs has allowed for several tumor types to be studied in these large experimental animal models. In particular, basal cell carcinoma,\(^\text{106}\) colorectal cancer,\(^\text{107}\) breast cancer,\(^\text{189,190}\) soft-tissue sarcoma,\(^\text{191,223}\) hepatocellular carcinoma,\(^\text{198}\) pancreatic ductal adenocarcinoma (Principt et al, 2018, submitted), lymphoma,\(^\text{106}\) and osteosarcoma\(^\text{191,197}\) (Table 3) are among the tumor types that are currently in focus. However and as previously mentioned, both the colorectal cancer\(^\text{187,188}\) and breast cancer\(^\text{189,190}\) models currently either lack in vivo tumor development or display issues with lethality. Although there are obvious ethical problems in development of genetically modified pet animals for cancer studies, several genetically modified swine have already been developed to study cancer development as outlined above. With the emergence of precision gene editing tools, such as CRISPR/Cas9 or TALEN technologies, the potential for development of point-mutation models as well as single and multiplexed recombinants using homology-directed repair is a real and accessible option for development of new complex cancer models as well as complex comorbidity models.\(^\text{199}\)

### Acknowledgments

**Financial support.** The authors thank the Danish Council for Independent Research, Technology and Production for the grant (ID: DFF-4005-00428) provided to GJ. Moreover, the authors acknowledge support from the Rural Development Administration, Republic of Korea (538/NU), the US NIH (1-R21-CA-19543-01A1) and the USAMRMC (CA150590) to LBS.

**Potential conflicts of interest.** All authors: No reported conflicts.

### References


The text appears to be a collection of references or citations, likely from a scientific or medical journal. It includes various authors, publication years, and titles, with a focus on mouse models and cancer research. The text is not a continuous narrative but rather a compilation of bibliographic entries.


142. Groenen MAM, Archibald AL, Useni H, et al. Analyses of pig genomes provide insight into porcine demography and
153. Paoloni MC, Khanna C. Comparative oncology today.

154. Breen M. Update on genomics in veterinary oncology.

156. Gray MA, Pollock CB, Schook LB, Squires EJ. Characterization of porcine pregnane X receptor, farnesoid X receptor and their splice variants.


156. Gray MA, Pollock CB, Schook LB, Squires EJ. Characterization of porcine pregnane X receptor, farnesoid X receptor and their splice variants.


