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Novel Mouse Models for Cancer Immunology

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Keywords

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Abstract

Mouse models of cancer immunology provide excellent systems in which to study and test biological mechanisms of the immune response against cancer. Historically, these models were designed to have different strengths based on the current major research questions at the time. As such, many mouse models of immunology used today were not originally developed to study current questions in the relatively new field of cancer immunology, but instead have been adapted for such purposes. In this review, we discuss various mouse models of cancer immunology in a historical context in order to provide a fuller perspective of each model's strengths. From this outlook, we discuss the current state of the art and strategies for tackling future modeling challenges.

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INTRODUCTION

Checkpoint inhibitor (CPI): a class of blocking antibodies specific for checkpoint receptors such as PD-1/PD-L1 or CTLA4

Tumor-specific antigens (TSAs): antigens arising from novel peptide sequences representing neoantigens expressed only on cancer cells, unlike tumor-associated antigens, which are also expressed by normal cells

The ground-breaking anticancer responses to checkpoint inhibitor (CPI) immunotherapy in patients across many cancer types have forced us to reconsider long-held assumptions about the antitumor immune response. Likewise, this impressive clinical success has revealed many important unanswered questions in cancer immunology regarding the potential of patients' natural anticancer immune responses. The study of human tumor samples, while extremely valuable, is largely correlative by nature. Thus, exploring the mechanisms of these immunotherapies and investigating ways to improve their efficacies require mouse models that appropriately reflect the natural development of an anticancer immune response. Most available models have been less than ideal for such studies, and this, in large part, is due to the fact that many human cancers were previously thought to be nonimmunogenic; thus, models of these cancers were not designed for the study of cancer immunology. This is par for the course when it comes to mouse model development, as the combination of technological advancements and new research findings is constantly driving a need for more advanced models, and vice versa. The cyclical nature of this process is apparent when viewing the development of mouse models of cancer through a historical lens in order to provide context for the current state of available models. The ideal mouse model of cancer immunology will likely be ever evolving and, as such, remains just out of reach. Therefore, choosing the best model or models for a particular research question should be a decision made based on the current understanding of cancer immunology, as well as the requirements of the research project in question. While animal models besides the mouse have been useful for the study of cancer (Davis & Ostrander 2014, Dewi & Cline 2021, Schachtschneider et al. 2017, White et al. 2015), this review exclusively focuses on mouse models of cancer immunology. We discuss what we have learned so far and reflect on the model evolution that helped us get here. Finally, we address current modeling challenges and efforts to overcome the limitations of available models. An immense amount of research has contributed to what is now the current state of cancer immunology mouse modeling. Here, rather than provide an exhaustive account of all contributions, we focus on highlights as a means to discuss larger concepts. For those interested in diving deeper into topics mentioned throughout this review, we reference various in-depth reviews found elsewhere.

PREEXISTING ANTITUMOR IMMUNITY DRIVES THERAPEUTIC RESPONSIVENESS

The concept of immune-surveillance, in which a natural antitumor adaptive immune response shapes tumor development and cancer progression, was first proposed by Paul Erlich in 1901. Yet, the role of the immune system in cancer development remained underappreciated and highly debated until the late 1980s. That is not to say that the use of immunotherapies in general was discounted, as investigations into such therapies date back to a century ago (reviewed in Zhang & Zhang 2020), but rather that the presence of a natural antitumor immune response in the absence of therapy was uncertain. The discovery that tumor-specific antigens (TSAs) in melanomas could be recognized by T cells helped to establish an active role for the adaptive immune system in antitumor responses (Brichard et al. 1993, Bruggen et al. 1991, Lee et al. 1999, Wölfel et al. 1995). Additionally, the discovery that cytotoxic T lymphocyte–associated protein 4 (CTLA4) functioned as an inhibitory molecule on T cells helped to revive the theory of immunosurveillance (Leach et al. 1996, Tivol et al. 1995). At this time only a handful of human cancer types were considered to have immunogenic potential, such as melanoma and renal cell carcinoma, whereas other types of solid tumors were considered nonimmunogenic.

THE USE OF THE TERM "IMMUNOGENIC"

In the purest sense, "immunogenic" refers to anything that elicits an immune response in the host. In the context of therapy, "immunogenic" has often been used to refer to a tumor with a clear therapeutic response to immunotherapy. This latter use of the term reflects the historical uncertainty of the existence of natural immune responses in some types of cancers, which were often only revealed following successful immunotherapeutic strategies. Thus, immunotherapeutic responses have been the guide for attempting to discover the presence of immunogenic potential.

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Of note, determinations of the immunogenicity of different cancer types are nuanced, and discrepancies exist among the clinical observations of human cancer types and their corresponding mouse models. For example, while melanoma in humans is considered immunogenic, the murine melanoma cell line B16 is not (Zhong et al. 2020). Additionally, certain cancer types are considered immunogenic in some instances and not others. For example, the immunogenicity of individual human colorectal cancers (CRCs) can vary greatly and has been determined using immunoscores based on the density and location of lymphocytes in the tumor (Galon et al. 2006). Concordantly, immunoscores have been used both as a prognostic marker and to predict responses to therapies in human CRC (El Sissy et al. 2020). In spite of this, the commonly used murine colorectal cell lines MC38 and CT26 are known to be very immunogenic (Lau et al. 2017). These discrepancies bring to light important considerations for the use of existing mouse models of cancer (see sidebar titled The Use of the Term "Immunogenic").

The impressive responses in some patients to PD-1/PD-L1-blocking immunotherapies, across human cancer types previously considered both immunogenic and nonimmunogenic (Raez et al. 2005, Schreiber et al. 2010), have forced us to reconsider how we think about the relationship between cancer and the immune system (Brahmer et al. 2012, Topalian et al. 2012). CPI therapy targets inhibitory ligands or their cognate receptors and therefore acts on T cells expressing the specific checkpoint molecules targeted. We now appreciate that tumor-infiltrating lymphocytes are heterogeneous, ranging from bystander naïve cells (tumor nonresponsive), which do not express inhibitory markers, to tumor-specific T cells at various stages of differentiation, which exhibit a range of inhibitory marker expression (van der Leun et al. 2020). Importantly, the unexpected success of these therapies across many cancer types confirmed the long-debated hypothesis that an ongoing immune response to cancer already exists in patients and, if bolstered therapeutically, has the potential to cure disease.

Tumor-infiltrating lymphocytes: lymphocytes, including B and T cells, present within the tumor microenvironment

Hot tumors: tumors with a microenvironment that is highly infiltrated with lymphocytes

Cold tumors: tumors with a microenvironment that is largely absent of lymphocytes

Early indications of natural antitumor immunity came from studies in transplantable and carcinogen-induced mouse models of cancer, as well as from biopsies of patient tumors, which led us to begin classifying tumors either as hot tumors (having lymphocytic infiltrate) or as cold tumors (having little to no infiltrate) (Binnewies et al. 2018, Galon et al. 2006). However, recent technological advances in genome sequencing and variant prediction methodologies have shown that tumors traditionally classified as cold, and thus thought to be nonimmunogenic, possess neoantigens capable of inciting an adaptive antitumor immune response (Vareki 2018). Likewise, in cancer types traditionally considered to be nonimmunogenic, such as certain subtypes of non-small-cell lung cancer, CPIs have now become first-line therapy (Gardiner et al. 2015, Hui et al. 2017). Yet, long-term response rates remain low. Therefore, a new challenge for researchers is to elucidate the factors distinguishing tumors that respond to checkpoint therapy from those that do not. To fill this critical gap in our knowledge, we need new mouse models of cancer immunology that will

allow for the careful study of the complicated and evolving antitumor immune response over the progression of disease from initiation to metastasis.

A HISTORICAL PERSPECTIVE OF ESTABLISHED MOUSE MODELS OF CANCER

Many components of the developing tumor immune landscape over the course of disease can impact responsiveness at the time of therapy. The goal of early cancer studies was to identify effective therapeutics. Thus, researchers relied heavily on the in vitro study of cancer cells and mouse models that lacked a sufficient immune component, such as human cancer cell xenografts. These models were not designed with the immune system in mind. As a consequence, cancer research has focused primarily on tumor cell–intrinsic characteristics of disease. These intrinsic factors, such as the role of various genetic mutations on therapy responsiveness and disease progression, have been reviewed elsewhere (Ashworth et al. 2011). Comparatively less is understood regarding the contributions of tumor cell–extrinsic factors impacting disease outcomes. As mentioned, below we discuss the complicated dynamics of tumor cell–extrinsic components of cancer immunology through the lens of the historical evolution of mouse models, as depicted in **Figure 1**. In so doing, we hope to highlight why mouse models of cancer are critical to understanding these factors and their impacts on developing tumors.

Syngeneic Transplant Models

Syngeneic transplant models of cancer dominated the field of early cancer immunology. These models utilize cancer cell lines, generated from a primary or metastatic tumor, arising spontaneously or by induction, that are grown in a mouse with the same genetic background as the mouse from which it was generated (see the sidebar titled Syngeneic Transplant Models). Importantly, these models straddle the line between having adequate genetic similarity to not cause rejection and harboring enough mutations to generate an antitumor immune response that can be studied. Nevertheless, this largely occurred by happenstance and it took many years of trial and error for these concepts to be fully appreciated.

Thankfully, efforts to transplant tumors within and between species had begun decades before the development of tumor cell lines. These efforts would eventually lead to suitable hosts essential for syngeneic transplant studies. One of the first successful transplantations of a tumor

SYNGENEIC TRANSPLANT MODELS

The concept of syngeneic tumors comes from the field of transplant biology. A syngeneic tissue graft is the transplant of tissue from one area of the body to another in the same individual, or an identical twin in some cases. This term has been adopted by the field of cancer research to refer to the transplantation of tumor cells into the same inbred mouse strain as the strain from which the original tumor was derived. Crucially, syngeneic tumor cell lines can be generated from tumors regardless of the manner by which the original tumors arose (spontaneous, carcinogen-induced, etc.). Such cell lines are established after the original tumor is dissociated, cultured, and expanded in vitro or passaged in vivo. For example, CT26 and MC38 were derived from carcinogen-induced tumors while B16 and derivatives originated from a spontaneous melanoma, but all are examples of syngeneic tumors (B16 and MC38 on the C57BL/6 background and CT26 on the BALB/c background). Whether injected subcutaneously or orthotopically, used to model metastasis, or generated into organoids prior to transplantation, such cell lines are all examples of syngeneic transplant models when grown in the inbred mouse strain of their origin.

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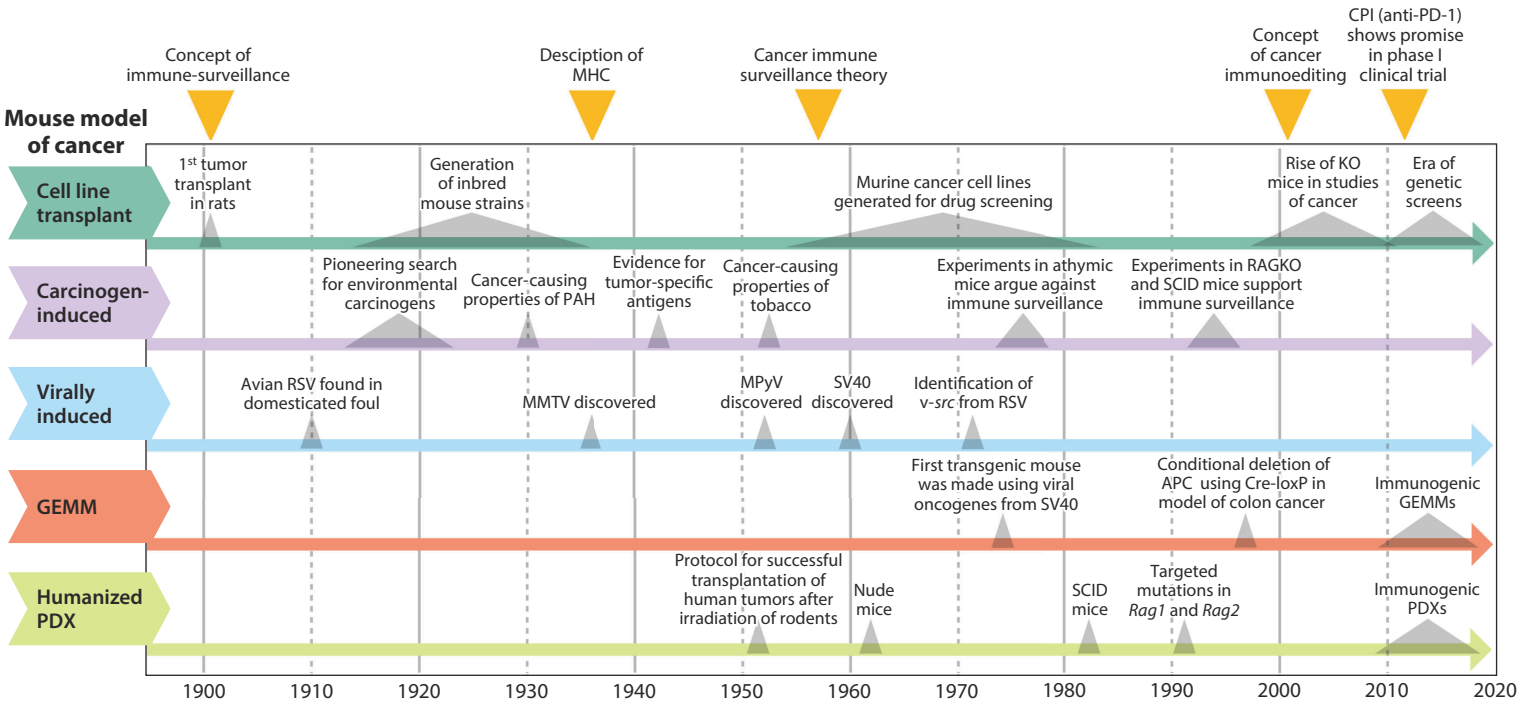

Figure 1

Juxtaposed timelines of notable advances generated in models of cancer: syngeneic cell line transplant models (*dark green*), carcinogen-induced models (*purple*), virally induced models (*blue*), GEMMs (*orange*), and humanized PDX models (*light green*). These timelines are set in the context of major findings and theoretical advances in the study of cancer immunology, marked above with yellow triangles. Figure adapted from an image created with BioRender.com. Abbreviations: APC, adenomatous polyposis coli; CPI, checkpoint inhibitor; GEMM, genetically engineered mouse model; KO, knockout; MHC, major histocompatibility complex; MMTV, mouse mammary tumor virus; MPyV, mouse polyomavirus; PAH, polycyclic aromatic hydrocarbon; PDX, patient-derived xenograft; RAGKO, *Rag*-knockout; RSV, Rous sarcoma virus; SCID, severe combined immunodeficiency; SV40, simian virus 40.

was demonstrated in 1901 between rats (Loeb 1901), but only a portion of recipients accepted the transplants. Around this time, Leo Loeb and Abbie Lathrop observed that mice with different genetic backgrounds spontaneously developed cancers with varying rates of incidence (Lathrop & Loeb 1915a,b, 1918; Steensma et al. 2010). Clarence Cook Little, after carefully mating the progeny of mice obtained from Lathrop, developed some of the first inbred strains of mice: first the DBA (dilute, brown, and nonagouti) inbred mouse strain and, eventually, the C57BL mouse strain widely used today (Little 1911, 1913, 1927; Murray & Little 1935). In the years to follow, Little, Leonell Strong, and John Bittner tested various tumor and strain combinations for tumor incidence (Bittner 1936). Together with Little, Ernest Tyzzer used these inbred strains to provide an explanation for the previous failures of tumor transplantation and showed a genetic and Mendelian basis for rejection of cancer grafts, later described as the major histocompatibility complex by George Snell and Peter Gorer (Little 1914, Little & Tyzzer 1916, Gorer 1936, Snell 1948, Tyzzer 1909).

The generation of inbred mouse strains in the 1900s set the stage for all later mouse models, creating reliable hosts for syngeneic transplant studies, as well as studies of chemical and genetic tumorigenesis. In the mid-1950s, the U.S. National Cancer Institute would begin promoting efforts to produce cancer cell lines in which to perform drug screenings for new cancer treatments. Since then, hundreds of cancer cell lines have been established and propagated. Tumor cell lines isolated from mice in the 1970s and 1980s as a part of these efforts (e.g., MC38, B16) continue to be widely used today. The potential of manipulatable and transplantable systems generated excitement in the field, and a model in which to grow these cell lines with consistency and reproducibility was essential. Due to their ease of availability, presence of an intact host tumor environment, and reproducible growth kinetics, transplanted syngeneic tumors became the most favorable way to screen drugs for therapeutic efficacy. In fact, syngeneic transplant models played an important role in the discovery and early mechanistic studies of CPIs (Dong et al. 2002, Leach et al. 1996, Townsend & Allison 1993).

While many transplanted mouse tumors were immunogenic, their relevance to human disease was uncertain. Notably, this created a rift in the scientific community and many grew skeptical of the role of immunity in cancer. Furthermore, early experiments by Medawar and colleagues supported Thomas Burnett's theory of acquired immunological tolerance put forth in 1949, which stated that self-reactive cells were deleted prior to adulthood (Billingham et al. 1953, Parish 2003). These data supported a model in which the immune system could not recognize malignant cells as nonself, as they would be indistinguishable from healthy tissues. This was in contrast to findings by Edward Foley, who demonstrated in 1953 with C3H mice that tumor grafts were rejected in genetically identical mice after they had been immunized against the cancer (Foley 1953). The role of the immune response in cancer would eventually be clarified after many years of studies using carcinogen-induced cancer models, as discussed in the next section titled Carcinogen-Induced Models.

Many varieties of syngeneic transplant tumor models exist with different strengths and weaknesses. Subcutaneous transplants are used widely, as they provide a relatively quick and straightforward way to gauge tumor growth kinetics using simple caliper measurements over a period of a few weeks. These are an excellent choice for testing the effectiveness of different therapeutic strategies in immunocompetent hosts. Additionally, transplanting tumor cell lines into the tissue type in which they originated, known as orthotopic transplantation, better reflects tissue-specific microenvironments and thus could be more relevant to the study of human disease. Nevertheless, depending on the tissue, tumor measurements are more complicated and often require the addition of a bioluminescent marker or CT (computed tomography) scans. Organoid models offer somewhat of a compromise between orthotopic and subcutaneous models, as they have been

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shown to maintain architectural and molecular characteristics of the original tissue microenvironment. Nevertheless, as cell lines and organoids are generated from established tumors and passaged multiple times, they represent a more advanced stage of disease and therefore are not ideal for studying early events, such as tumor initiation or early priming of the immune response.

Methylcholanthrene (MCA): chemical carcinogen used in cancer research to induce tumors in mouse models

Athymic (nude) mice: mice homozygous for a mutated *Foxn1* gene that do not develop a thymus and lack most mature T cells

RAGKO mice: a strain of mice lacking one or both of recombination-activating genes (*Rag1* and *Rag2*), resulting in the absence of mature T and B lymphocytes

Severe combined immunodeficiency (SCID) mice: a strain of mice homozygous for a mutated *Prkdc* gene, causing deficiency of both B and T lymphocytes

Carcinogen-Induced Models

In the late 1900s, carcinogen-induced tumor models, extensively reviewed elsewhere (McCreery & Balmain 2017), became critical for conversations surrounding the role of the immune system in cancer. The ongoing search for environmental carcinogens, pioneered by Yamagiwa & Ichikawa (1918), began to gain more traction as genetically similar mice were utilized in experiments. The cancer-causing properties of polycyclic aromatic hydrocarbons were demonstrated in 1930, adding validity to this pursuit (Kennaway & Hieger 1930). Around this time, Murray Shear set up a drug-screening program, the first of its kind, to test thousands of compounds for cancer-causing properties in the murine S37 model of sarcoma (DeVita & Chu 2008). Shear's program later served as a model for future cancer drug screening programs. Inbred mice were also used to demonstrate the cancer-causing properties of tobacco (Wynder et al. 1953). One of the most reproducible models to come of these efforts was the application of the chemical methylcholanthrene (MCA) to the skin of inbred mice for the induction of tumors, and this model is still used for cancer immunology studies today. Ludwig Gross, followed by E.J. Foley, R.W. Baldwin, R.T. Prehn, J.M. Main, and others, soon showed that chemically induced tumors were recognized by the immune system, and soon after, the rejection of MCA-induced tumors was shown to be immune cell mediated (Rosenberg et al. 1986, Schreiber & Podack 2009, Scott 1991). We now appreciate that carcinogen-induction results in many genetic mutations, and thus, resulting tumors contain various neoantigens, or new proteins expressed by transformed cells, that can be recognized by the immune system. Work in these models led, serendipitously, to the discovery of TSAs not present in healthy cells (Gross 1943, Old 1981, Prehn & Main 1957). This forever shifted the dogma and further legitimized the field of cancer immunology. As a result, Burnet, together with Lewis Thomas, proposed a model of immune surveillance of cancer (Ribatti 2016). These working models came with defined hypotheses, including the existence of TSAs and immune cell–dependent tumor growth restriction. To test these new hypotheses, researchers in the field once again looked to new mouse models.

This period of time serves as a cautionary tale about the applicability and drawbacks of chosen models. Experiments aimed at testing the idea of immune surveillance were first conducted in athymic (nude) mice as a way to measure the impact on tumor growth in the absence of adaptive immunity. These experiments erroneously disproved the immune surveillance hypothesis, showing no differences in tumor growth regardless of the presence or absence of adaptive immune cells (Outzen 1975; Stutman 1974, 1979). Once again, it appeared that immunology and cancer biology had little overlap. Later, it was discovered that athymic mice possess macrophages and natural killer (NK) cells with enhanced cytotoxic activity as compensation for the lack of thymically derived T cells and are therefore not completely immunodeficient (Hasui et al. 1989). Additionally, while these mice lack most mature T cells, an appreciable T cell population remains as a result of extrathymic maturation, a natural process that was not universally accepted for many years (Torfadottir et al. 2006). The same experiments, repeated in improved immunodeficient mouse models, including RAGKO mice, lacking the gene *Rag* that encodes key enzymes responsible for the generation of all mature T and B cells, as well as severe combined immunodeficiency (SCID) mice, demonstrated that tumors grow more robustly in immune-deficient mice, thus supporting the immune surveillance hypothesis (Engel et al. 1997, Shankaran et al. 2001, Shinkai et al. 1992).

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Auspiciously, the concept that adaptive immune cells could recognize tumor antigens had been demonstrated by this time using in vitro studies of patient tumor cells and autologous lymphocyte cultures (Brichard et al. 1993, Bruggen et al. 1991, Wölfel et al. 1995). Meanwhile, Robert Schreiber and others developed increasingly sophisticated MCA-induced cancer models (Dunn et al. 2005, Koebel et al. 2007) and, in the late 1990s, were able to demonstrate that $CD8^+$ T cells and NK cells produced effector molecules that serve as the primary mediators of the anticancer immune response (Smyth et al. 2001). For more details on immune surveillance in cancer, readers are referred to the review by Swann & Smyth (2007).

The development of athymic, RAGKO, and SCID mice aided the development of a new model of cancer. Severely weakened immune systems allowed for the transplantation of human cancer tissues without rejection by the immune system, providing a means by which to quickly screen new anticancer agents in human cancer tissue (Bogden et al. 1984). These were the first xenograft models, named for the transplantation of tissue from one species into another without rejection (see the section below titled Humanized Patient-Derived Xenograft Models).

Overall, carcinogen-induced tumor models provide a means by which to study cancer immunology that may better reflect the mutational backdrop of environmentally induced human tumors. These models result in nonspecific mutagenesis and thus make it difficult to separate immune cells specifically responding to the tumor from nonspecific bystanders. However, the higher number of point mutations and genetic alterations in the resulting tumors may provide a model that is better able to reflect the genetic complexity of human tumors.

Virally Induced Models

The relationship between infection and cancer has been investigated for well over a century in cancer patients but could not be proven until the early 1900s, and this line of investigation unintentionally led to the eventual design of another class of mouse models of cancer. Peyton Rous is considered the first to demonstrate the cancer-causing potential of viruses after spreading cancer from one domesticated foul to another via an infectious agent in 1910. Rous would eventually earn the Nobel prize for these findings. Later, the infectious agent discovered by Rous was identified as respiratory syncytial virus (RSV). Notably, the search for an RSV-induced tumor antigen led to the discovery, in the late 1970s, of the phosphoprotein Src. The viral *src* gene elicited much excitement in the research community, as it was determined to be a protein kinase necessary for malignant transformation but dispensable for viral replication (Goldé 1970, Toyoshima et al. 1970). Bridging the divide between the fields of virology and cancer genetics, the discovery of *src* created a flurry of excitement for the study of proto-oncogenes—genes that, when mutated, cause normal healthy cells to divide uncontrollably. Soon after the discovery of RSV, other cancer-causing viruses were uncovered in various species including rabbits, mice, cats, nonhuman primates, and humans (Weiss & Vogt 2011). For example, in the 1920s, after crossing a male DBA mouse with a female mouse from Halsey Bagg's albino colony, scientists observed that the resulting inbred strain, the C3H strain, developed spontaneous mammary tumors by 6 months of age (Strong 1935). The cause was soon found to be transmission of mouse mammary tumor virus (MMTV) in breast milk (Bittner 1937, 1939; Visscher et al. 1942). These early findings led to the discovery of human endogenous retroviruses that are homologous to MMTV (Ono et al. 1986). A more comprehensive review of oncogenic viruses and their contributions to our current understanding of cancer immunology has been given by Javier & Butel (2008).

The fields of virology and cancer genetics overlapped further upon the discovery and genome sequencing of polyomaviruses including simian vacuolating virus 40 (SV40) and mouse polyomavirus (Cheng et al. 2009). The discovery of these small DNA viruses led to many years of

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research around cellular transformation and viral oncogenes (Levine & Oren 2009, Weiss 2020). Consequently, viral tools for manipulating genomic DNA were introduced and employed across research disciplines, and in vivo modeling advances proceeded rapidly. For example, the discovery of restriction enzymes in the 1960s quickly led Paul Berg and colleagues to demonstrate that pieces of foreign DNA could be inserted into cells in culture (Goff & Berg 1976, Mulligan & Berg 1980, Mulligan et al. 1979). In the 1970s, Jaenisch & Mintz (1974) microinjected viral oncogenes from SV40 into the blastocoel of mouse embryos, purposefully integrating viral DNA into the mouse genome and consequently generating what are considered by many to be the first transgenic mice. The resulting growth in recombinant DNA technology led to the wide accessibility and use of lentiviral and adenoviral vectors for the induction of cancer. An early example of this was the transfer of the SV40 large T antigen (TAg) oncogene linked to the rat insulin promotor (RIP) into fertilized eggs resulting in RIP-Tag mice (Hanahan 1985). This tissue-specific expression of TAg led to proliferation and spontaneous development of tumors in pancreatic beta cells. Similar techniques continue to be used today to build new mouse models of cancer (see the section below titled Genetically Engineered Mouse Models) and have contributed to our knowledge of cancer immunology, as the induced expression of viral proteins by transformed cells can facilitate the study of the antitumor immune response. A detailed review of viral oncogene models and how they have been used in the study of cancer immunology has been provided elsewhere by Guerin et al. (2020).

Genetically engineered mouse models (GEMMs): strains of mice that have been genomically manipulated using gene-editing techniques

Tumor microenvironment (TME): the cellular and molecular composition and structural architecture of the environment surrounding and supporting a tumor

Somewhat counterintuitively, virally induced genetically engineered mouse models (GEMMs) of cancer have been found, in general, not to elicit robust immune responses in the host, and as such, early GEMMs were less than ideal models for tumor immunology (see the next section). This can be explained, in part, by the fact that, while viruses are technically foreign or nonself to their host organism, once viral DNA integrates into the host genome, in a sense it becomes self and does not elicit a marked immune response. That being said, there are exceptions, including the use of SV40 T antigens such as TAg, which have been shown to induce interferon-stimulated genes that could directly impact immune cell function (Forero et al. 2014).

Genetically Engineered Mouse Models

Efforts to understand the mechanisms by which oncogenes cause cancer initiation and progression converged with a growing frustration in the late 1990s and early 2000s over the poor clinical translatability of drugs shown to cure cancer in mice. This led to an increased appreciation for the role of the native tumor microenvironment (TME) in cancer development and progression. For these purposes, models that recapitulated the microenvironment seen in patient disease were required. Carcinogen-induced models, while generated in situ and therefore present in the tissue environment in which they originated, result in genetically heterogeneous tumors within groups of mice, which increase variability and make it difficult to determine the biological mechanisms or therapeutic efficacy of candidate drugs. Transplant models, while genetically similar and less variable from tumor to tumor, do not adequately recapitulate the natural TME, due in large part to their rapid growth (**Figure 2**). Importantly, they also lack the early phases of progression seen in patient disease (Olson et al. 2018). To overcome this, cancer biologists turned to GEMMs.

There have been many iterations of cancer GEMMs, and advanced GEMMs will continue to be generated in order to meet the needs of novel research questions. They can be broadly categorized as germline or nongermline/conditional (van Dyke & Jacks 2002). Taking advantage of new viral integration systems and gene recombination, researchers genetically engineered early germline GEMMs to express dominant oncogenes (e.g., *Kras*) and to provide evidence that oncogene expression in normal cells could lead to cell transformation and tumors (Hanahan et al. 2007).

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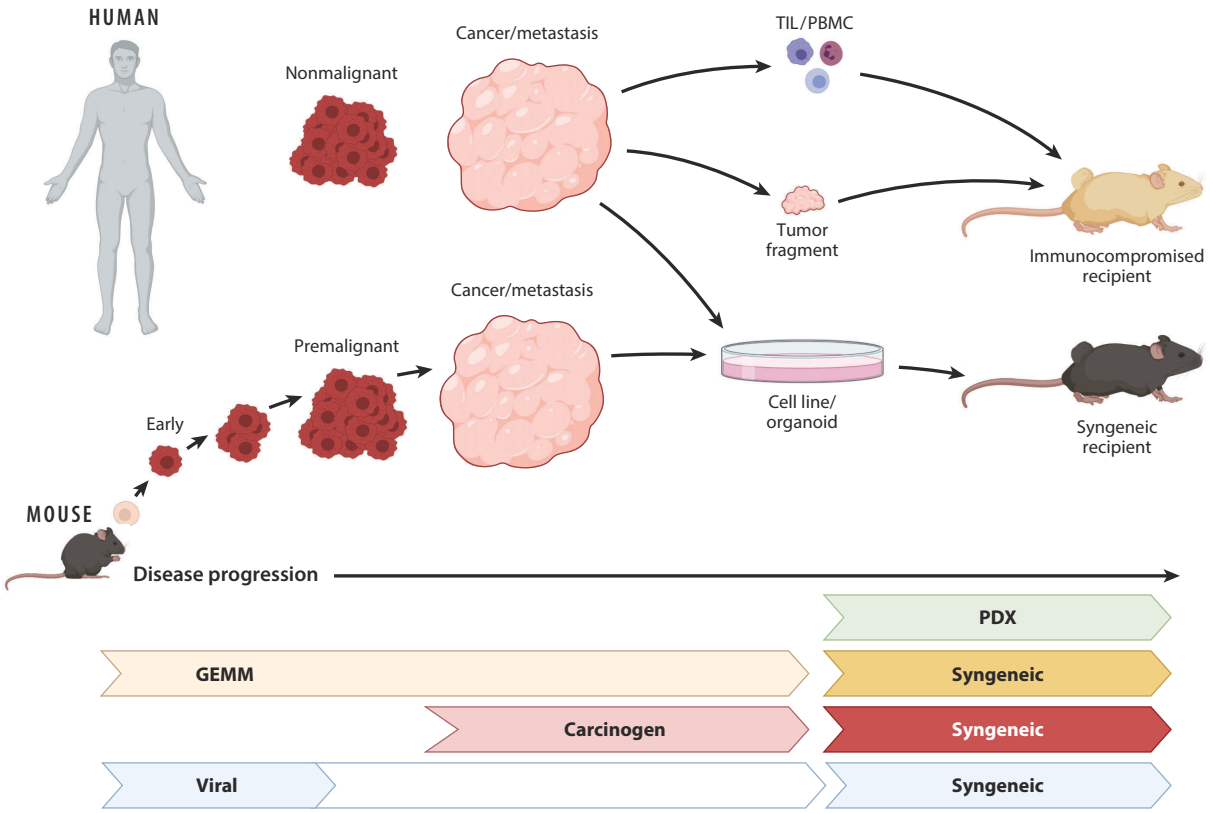

Figure 2

Stages of human and mouse cancer that have been observed. On the right, techniques used to transplant tumors into mice are depicted. Mouse models of cancer best able to recapitulate various stages of disease shown below. Figure adapted from images created with BioRender.com. Abbreviations: GEMM, genetically engineered mouse model; TIL, tumor-infiltrating lymphocyte; PBMC, peripheral blood mononuclear cell; PDX, patient-derived xenograft.

Some systems also use specific recombinases to delete tumor suppressors in desired tissues (e.g., *Trp53*). These models, comprehensively reviewed elsewhere (Politi & Pao 2011, Walrath et al. 2010), illustrated the important role that oncogenes play, not just in the initiation of cancer but also in the maintenance of disease. The ability to turn on or off mutant oncogenes and tumor suppressors, respectively, thus mimicking what is seen in patient tumor progression, allowed the histopathological recapitulation of early and advanced disease in cancer mouse models and laid the groundwork for current advanced GEMMs (Boutin et al. 2017, Frese & Tuveson 2007). The observation of cancer progression through the stages of disease from transformation to metastasis is a notable strength of these model systems.

Advances in genetic techniques paved the way for the creation of conditional GEMMs, in which the expression or deletion of oncogenes could be controlled both spatially and temporally. For example, the Apc^{Min} mouse model of intestinal and mammary cancer was generated by a germline mutation in the *Apc* gene, originally induced by a mutagen, ethylnitrosourea. Due to the expression of *Apc* in many cell types, this mutation results in multiple spontaneous tumors throughout the intestinal tract, as well as in mammary tissue (Moser et al. 1995). Building on this, an inducible model of CRC was created in which the tumor suppressor Apc, flanked by loxP sites,

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was inactivated following adenovirus-mediated delivery of Cre (P1 bacteriophage cyclization) recombinase to the colorectal region, resulting in the rapid, controlled onset of colorectal adenomas (Shibata et al. 1997). Further spatiotemporal regulation was achieved following the induction of somatic mutations with Cre-ERT fusion proteins under the control of tissue-specific promoters. In this setting, a mutated hormone-binding domain of the estrogen receptor is fused to Cre recombinase. Administration of the estrogen analog tamoxifen leads to posttranslational activation of Cre recombinase activity and excision of the targeted gene in a tissue-specific manner (Vooijis et al. 2001). Conditional GEMMs primarily rely on induced transformation by utilizing tetracycline-enabled promoter accessibility or virally delivered Cre or FLP (flippase) recombinases to target genomically encoded *loxP* or *FRT* sites, respectively. These genetic tools allow for control over when and in which tissues/cell types tumors are induced (Jonkers & Berns 2002). One such GEMM is the Tyr::CreER^{T2} mouse model of melanoma, in which the simultaneous activation of the *Braf*^{V600E} oncogene and deletion of the *Pten* tumor suppressor is driven by a melanocyte-specific promoter (Tyr) following treatment by tamoxifen (Dankort et al. 2009).

GEMMs can also take advantage of more diverse methods of tumor induction like RNA interference against tumor-suppressor genes and CRISPR/Cas9-mediated somatic mutations (Livshits & Lowe 2013, Yang et al. 2013). Studies in such models demonstrated that the impact of any single oncogene is heterogeneous, creating genomic diversity similar to what is observed in human disease (Chung et al. 2017). Inducing genetic comutations by mimicking multistep carcinogenesis can further increase the relevance of a GEMM to a particular type of human cancer. For this, the combination of the Flp-*FRT* and Cre-*loxP* systems allows for the sequential induction of multiple gene changes with unique recombinase actors and, thus, temporal control over each gene change separately [i.e., the inducible dual-recombinase system (Schonhuber et al. 2014)]. Overall, cancer GEMMs have supported a rapid expansion of our understanding of the development and, in some cases, therapeutic vulnerabilities of diverse cancers [readers are referred to Kersten et al. (2017) for a comprehensive review of GEMMs].

Cre (P1 bacteriophage cyclization) recombinase: enzyme that recognizes *loxP* sites and recombines DNA between these sites to initiate deletions, inversions, or translocations

FLP (flippase) recombinase: enzyme derived from yeast that recognizes FLP recombinase target sequences and recombines DNA at these sites to initiate deletions, inversions, or translocations

Genetically Engineered Mouse Models of Cancer Immunology

In the flood of excitement surrounding CPI therapies in the early 2000s, it was inevitable that the fields of cancer biology and tumor immunology would become permanently intertwined. Together, findings that demonstrated the importance of CD8$^+$ T cells for tumor control (first noted in the MCA model and tumor transplants) and the interpretation of CPI therapy as a reversal of T cell exhaustion created a frenzy of investigation of T cell responses in the traditional cancer GEMMs described above. Some of these experiments were fruitful and deepened our understanding of the interplay between tumor cells and CD8$^+$ T cells (Goel et al. 2017, Li et al. 2018). Nevertheless, GEMMs were found to incite very low levels of immune responses to tumors and consequently poor responses to CPIs, in contrast to human patients treated in the clinic. Furthermore, it has been shown that many tumor-infiltrating immune cells are bystanders that are not actually involved in antitumor responses, making it necessary to distinguish tumor-specific T cells from the rest (DuPage et al. 2011). The weak immunogenicity of GEMMs is generally thought to be due to the low tumor mutational burden resulting from the precision of oncogene/tumor suppressor mutation (Lee et al. 2016, McFadden et al. 2016). As an example, studies using a GEMM of autochthonous liver cancer, in which SV40 TAg is expressed after tamoxifen-inducible, albumin-specific production of Cre recombinase (AST mice), have resulted in a better understanding of T cell dysfunction in cancer (Runge et al. 2014, Schietinger et al. 2016). Nevertheless, for most studies this model requires that transgenic T cells specific for TAg be transferred into mice prior to tumor induction, as endogenous T cell responses are minimal.

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Several strategies have been used to increase the immunogenicity of cancer GEMMs. Increasing bulk tumor mutational burden by destabilizing the genomes of transformed cells and targeting DNA mismatch repair machinery is effective at mirroring the mutational burdens of human cancer and eliciting immune responses (Germano et al. 2017, Sen et al. 2019). Likewise, a similar approach to increasing mutations has led to improved models to study the immune response against melanoma, in which transplantable cell lines generated from GEMMs were irradiated with ultraviolet B radiation (Wang et al. 2017). A drawback to these approaches is that the variability of antigens in each tumor makes it difficult to determine which immune cells are relevant and specifically responding to tumor antigens. The various T cell clones with different receptor specificities that could be at play between mice and experiments provide further difficulties. Antigen has also been added into advanced tumors via electroporation. This approach is mainly relevant for studying responses to established tumors and generates a great deal of delivery-related inflammation, but it provides a means for studying tumor-specific T cell responses (Radkevich-Brown et al. 2010). Tissue-specific promoters were also bred into GEMMs for constitutive expression of antigens in the tissue/cellular location of choice. Unfortunately, the expression of antigens prior to tumor induction leads to immunological tolerance, precluding the study of the endogenous antitumor immune response (Huijbers et al. 2012). To avoid tolerance against germline antigens, researchers have introduced conditional antigen expression systems, most of which operate similarly to the recombinase-inducible oncogene models described in the previous section (Cheung et al. 2008). However, the slight infidelity of the Cre recombinase on/off switch is a drawback to this approach, as it results in low levels of so-called leaky antigen expressed by thymocytes or in peripheral tissues. This low-level, leaky expression can be recognized by T cells and consequently results in the induction of tolerance prior to tumor establishment.

One successful method of generating tumors that trigger a robust immune response against a known antigen are the lenti-neoantigen-Cre systems. These utilize established Cre-*loxP* cancer GEMMs and add neoantigen into the lentiviral vectors for codelivery with Cre-recombinase at the time of tumor induction (DuPage & Jacks 2013, DuPage et al. 2011). These approaches allow for studies of tumor-specific T cells at many stages of tumor development. However, as lentiviruses and adenoviruses can infect many types of cells, cell-type-specific targeting is an important consideration when programming tumors to express neoantigens in this manner. Neoantigens introduced by nontargeted viruses can be expressed in infected epithelial cells, immune cells, endothelial cells, and more. This creates aberrant sources of neoantigen expression outside of the transformed cells, thus impacting the subsequent neoantigen-specific T cell response. However, the addition of a tissue-specific promoter or micro-RNA to the Cre recombinase and neoantigen-expressing viruses can substantially limit off-target expression (Alonso et al. 2018). Furthermore, lentiviral silencing can in some cases cause tumors to become neoantigen negative by nonimmunological means and thus extinguish any neoantigen-specific antitumor immunity (Cherry et al. 2000). Of note, the likelihood of lentiviral silencing seems to vary based on the promoters used and is therefore relatively simple to avoid (Xia et al. 2007). Another caveat to consider in these models is that lentiviral delivery of antigen means that antigen is expressed during infection-induced inflammation, which could adversely impact the development of the antigen-specific immune response (Fitzgerald et al. 2021). Nevertheless, the controlled introduction of model neoantigens still results in tumors containing relatively low mutational burdens. As such, models mentioned above that target DNA mismatch repair machinery may be preferred, as they better mirror the mutational burden in human patients. While the lenti-neoantigen-Cre vectors have caveats, they successfully generate immunogenic tumors and have proven useful in the investigation of antitumor T cell responses, helping to expand the field of cancer immunology (DuPage et al. 2012, Joshi et al. 2015).

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Like the models that preceded them, neoantigen-expressing GEMMs serve the field well for questions within their scope, but they can always be improved upon to meet the next scientific challenge. Advanced generations of GEMMs with inducible neoantigen tumor models are being developed with multiple lock-and-key induction mechanisms acting in combination to avoid the immunological tolerance and leaky recombinase systems described above (Damo et al. 2020, Hegde et al. 2020). One such model was designed in our lab to overcome the challenges mentioned above and is called the inversion inducible joined neoantigen (NINJA) mouse (Damo et al. 2020). The model neoantigen in this case, NINJA, contains GP33 and GP66 derived from the lymphocytic choriomeningitis virus. Crossing NINJA mice with the widely used KP (*Kras*$^{LSL\text{-}G12D/+}$;*p53*$^{fl/fl}$) GEMM of lung adenocarcinoma to produce KP-NINJA mice allows for the study of endogenous tumor-specific T cells in autochthonous lung cancer throughout cancer progression (Connolly et al. 2021, Fitzgerald et al. 2021). A similar model, designed by the lab of David DeNardo, allows for the inducible expression of the neoantigen chicken ovalbumin, driven by Cre recombinase expression and tetracycline repression. The novel mouse, called OG, was crossed to the KPC (*Kras*$^{LSL\text{-}G12D/+}$;*p53*$^{LSL\text{-}R172H/+}$;*Pdx-Cre*) mouse model of autochthonous pancreatic ductal adenocarcinoma to generate KPC-OG mice (Hegde et al. 2020). The antigens expressed in these models qualify as neoantigens because they are tightly controlled and expressed only by tumor cells following transformation, but they are not strong enough to cause rejection, thus mirroring what is seen in human disease. These genetically programmed, neoantigen-inducible models improve upon existing GEMMs while still histopathologically recapitulating human disease, ultimately resulting in antitumor immune responses that more closely mirror human cancer physiology.

Lymphocytic choriomeningitis virus: a virus that results in a well characterized infection in its primary host, the mouse, and is commonly used to study the T cell response to infection

Humanized Patient-Derived Xenograft Models

In sharp contrast to the other mouse models of cancer immunology discussed above, efforts to design a mouse with little to no immune system have been underway since the 1950s. The original objective for these immunodeficient mouse models was to design a suitable host for the implantation and growth of human tumors; these would become known as the first patient-derived xenografts (PDXs). These models allow human tumor tissue to be serially transplanted or made into cell lines or organoids that have been shown to retain the genetic complexity of human disease. Of note, Todd Golub and colleagues have revealed that human tumors grown in mice long-term may undergo genetic changes that they would not normally undergo in the human body (Ben-David et al. 2017). Now, after many advances in technology and in our knowledge of immunology, researchers are reconstituting these mouse models with human immune components. This is an emerging area of research with the potential for better translatability than has been seen historically for studies of murine cancer.

Helene Toolan, an important pioneer of these efforts, established a successful protocol in 1951 for the irradiation of mice and rats prior to transplantation of human tumors and demonstrated superior results (Toolan 1951, 1958). Around the same time, a growing emphasis on screening possible anticancer agents shifted the intended application for these models, and concerns grew that preconditioning mice with irradiation might lead to erroneous results (Gallily & Woolley 1958, Palm et al. 1958). Thus, this field benefitted immensely from inbred mouse strains designed to be immunodeficient and generated over the next several decades, including the nude, SCID, and RAGKO mice. Aspects of the immune system that had not been appreciated previously were a recurring obstacle for successful transplantation of human tumors. Advanced models of immunodeficiency were generated that contained fewer and fewer immunological components and resulted in increasingly successful engraftment [for a comprehensive review of these models, readers are referred to Shultz et al. (2012)]. With the use of these advanced models of immunodeficiency,

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researchers have begun working backward to rebuild a human immune system within mice in an effort to study human cancer immunology. Various techniques have been used for this purpose, including the engraftment of human hematopoietic stem cells or peripheral bone marrow cells into humanized mice prior to implantation of human PDX and cell line–derived xenograft tumors (Meraz et al. 2019, Yao et al. 2019, Verma & Wesa 2020). These efforts are ongoing (reviewed in Mian et al. 2020). Such humanized PDX models are useful for testing cancer therapies (reviewed in Guil-Luna et al. 2021) and provide benefits over other available models. They allow researchers to better model the genetic heterogeneity of human patients in the presence of a functional, humanized, immune response (Cassidy et al. 2015). Furthermore, in comparison to GEMMs, tumor development is quick and therefore facilitates the testing of many drug combinations and strategies. Nevertheless, as noted throughout this review, no single model is superior for all purposes. For example, without a known tumor antigen, the study of endogenous T cell responses is challenging, and similar to other transplant models, injected tumor cells are extensively differentiated, resulting in tumors that resemble late-stage disease.

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THE CURRENT STATE OF THE ART

Natural Biology of the Tumor Microenvironment

Investigations of the TME at the earliest stages of transformation and disease could have profound impacts on future diagnoses and therapies by elucidating the early events determining the functionality of the antitumor immune response. The TME is highly complex, with cells from both innate and adaptive immune arms coexisting and communicating as tumors progress through various stages of disease. The "Cancer Immunity Cycle" was proposed by Chen & Mellman (2013) and describes a series of steps necessary for generating a productive antitumor immune response. Still, many important questions regarding the mechanistic details of this cycle remain unanswered. For example, tumor-specific $CD8^+$ T cells are critical to antitumor responses, but much remains unknown surrounding the process of $CD8^+$ T cell cross-priming and activation in situ. The signals received by naïve $CD8^+$ T cells during cross-priming can vary greatly depending on the type of antigen-presenting cell and local inflammatory milieu (Fu & Jiang 2018). Consequently, the strength and effectiveness of the resulting antitumor T cell response are likely shaped by that initial cross-priming event.

Understanding the factors controlling dendritic cell migration and cross-priming dynamics could provide important insights for novel immunotherapies and improve the success of current treatments such as tumor vaccination and CAR (chimeric antigen receptor) T cell therapy (Ma et al. 2019). Modeling challenges to consider for these studies include (*a*) the ability to observe the various stages of tumor development, including the earliest stages when priming likely occurs, and (*b*) the physiological immunogenicity of tumor cells. GEMMs provide an attractive option to address the former challenge, as they would recapitulate early stages of disease immediately following transformation of normal cells. In contrast, available transplantable mouse models of cancer, whether syngeneic or PDX, may be better suited for studies of advanced cancer (**Figure 2**).

Beyond this, other aspects of human TMEs have not yet been adequately recapitulated in mouse models. The role of certain immune cells in the development and maintenance of antitumor immunity, such as B cells and follicular helper T cells, to name a few, remains poorly understood despite data from human patients suggesting their therapeutic importance (Baumjohann & Brossart 2021). Relatedly, a mechanistic understanding of tertiary lymphoid structures, which have been observed across many types of human cancer and correlated with improved therapeutic responsiveness, will require mouse cancer models that faithfully recapitulate these phenomena (Munoz-Erazo et al. 2020). As has been demonstrated throughout history (**Figure 1**), knowledge

gained from clinical samples and current mouse models has laid the groundwork for the design of just such emerging models (Cui et al. 2021, Engelhard et al. 2018, Joshi et al. 2015).

Immune-related adverse events (irAEs): autoimmune toxicities that can arise in various organ systems as a side effect of treatment with immune checkpoint inhibitors

Large-Scale Genetic Screens

Emerging cancer mouse models reflect our technological capabilities and our current understanding of relevant biology. The rapidly increasing number of mutations identified in human cancer sequencing studies calls for novel mouse modeling strategies that enable accelerated in vivo evaluation of candidate cancer genes. Unfortunately, it is expensive and time-consuming to engineer a mouse, and thus, until recently, transplant models have been preferred for these purposes, as they can be easily mutated (**Figure 2**). Nevertheless, genetic alteration of embryonic stem cells (ESCs) can be used to produce cohorts of nongermline GEMMs (Heyer et al. 2010). This strategy employs ESCs that are derived from existing multiallelic GEMMs, allowing for the rapid introduction of additional genetic modifications and the generation of chimeric mice with both characteristics of the established GEMM and additional genetic modifications (Huijbers et al. 2011, 2015). Likewise, CRISPR-Cas9 mice, in combination with lentiviral guide-RNA gene libraries and oncogene-inducible strains, are being increasingly employed to screen for novel gene contributors to cancer, as well as contributors to both immunologic and therapeutic responses (Chen et al. 2015, Dong et al. 2019). Critically, the immunogenicity of a tumor model determines the extent to which an antitumor immune response can be generated against it, and this can be greatly influenced by the way in which a model was designed. For example, even CRISPR/Cas9, which has become a tool widely used in tumor immunology studies, can potentially affect the immunogenicity of tumors and adversely impact research findings. This was demonstrated recently when increased immunogenicity caused by components of the CRISPR/Cas9 system induced tolerance/anergy to tumor antigens (Dubrot et al. 2021). This study details a method by which to remove the offending CRISPR/Cas9 components and therefore avoid the formation of tolerance. Still, techniques used in the design of new models must be carefully considered with the potential for erroneous immunogenicity in mind. Large-scale genetic screens are understandably easier in the setting of syngeneic transplant models and have been designed to manipulate genes in both tumors and immune cells to assess their impact on tumor development (Cortez et al. 2020, Manguso et al. 2017, Pan et al. 2018, Roth et al. 2020, Shu et al. 2020).

Immune-Related Adverse Events

Another challenge currently includes modeling immune-related adverse events (irAEs) in a manner that is physiological and clinically relevant. Notably, the emergence of CPI-induced irAEs was not predicted by available models (Curran et al. 2010). Studying irAEs in human patient tissues is difficult, as certain adverse events can be rare, tissues can be difficult to obtain, and manifestations can be heterogeneous. Currently, preclinical models of irAEs for the systematic testing of therapies do not exist, and treatment strategies have been largely developed through trial and error in patients, rather than through large-scale, controlled clinical trials (Brahmer et al. 2017). irAEs manifest as tissue-specific autoimmunity rather than systemic autoimmunity (Postow et al. 2018), and therefore the pathogenic mechanisms and best treatments may vary depending on the type of manifestation. Early studies of irAEs have relied on autoimmune-prone mouse models, and recent work has highlighted the idea that CPI treatments can accelerate the onset of autoimmunity in autoimmune-prone models [mouse models of irAEs have been reviewed by Liu et al. (2014)]. So far, these models have been used to test therapeutics for irAEs. In line with this, mice treated with DSS (dextran sulfate sodium) and anti-CTLA4 have more severe colitis compared with mice treated with just DSS (Perez-Ruiz et al. 2019), and prophylactic administration

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of TNFα-blocking antibodies ameliorates this phenotype without impacting CPI-induced antitumor responses. While promising, the broad utility of autoimmune models in this context is yet to be determined, as a relationship between autoimmune susceptibility and irAEs in human patients remains uncertain. The ideal models for T cell–driven irAEs require three components: (*a*) a naïve T cell repertoire with T cell receptors that recognize self-antigens, (*b*) inducible expression of target self-antigens in a relevant tissue, and (*c*) disease that emerges only in the context of treatment with CPIs. To these points, our lab has employed the NINJA mouse described above, which allows for the induction of a defined set of self-antigens in an organ of choice (Damo et al. 2020, 2021) and thus may be useful for the development of several translationally relevant irAEs. Of note, modeling the relatively rare antibody-mediated irAEs requires engineering target epitopes into proteins expressed in specific locations such as desmosomes, extracellular matrix, or in neuronal synapses, which is an additional obstacle.

CONCLUDING REMARKS

Presently, as a result of more than a century of cumulative research, there are many mouse models with which to study cancer immunology. Critically, each model has unique strengths and weaknesses that must be considered in order to choose the most appropriate model for testing any one hypothesis. The best mouse model of cancer immunology will continue to be a moving target, as mouse models are constantly designed that improve upon old models to address new findings.

FUTURE ISSUES

1. The disease heterogeneity seen in human patients with a particular type of cancer is not reflected in most mouse models of cancer. Recapitulating the genetic diversity of human disease in new mouse models of cancer could more accurately reflect and inform upon variable responses to therapies seen in the clinic.
2. The tumor microenvironment is extremely complex. Adequately modeling many aspects of the tumor microenvironment will likely require novel findings from, and improved characterization of, the tumor microenvironments of patient cancers. New technologies, such as in situ hybridization techniques that allow for spatially resolved transcriptomes and multiplexed imaging, have the potential to accelerate these modeling efforts.
3. The relationship among the microbiomes of various tissues, cancer cells, and immune cells in the tumor microenvironment undoubtedly plays a role in patients' responses to cancer therapies. Technological advances allowing for improved modeling of these diverse microbiota will be necessary for future studies elucidating the impact of the microbiome on disease susceptibility and therapeutic responsiveness.
4. Primary and secondary resistance to therapy is common across many types of cancer following various treatment strategies. Due to a lack of mouse models, very little is known about the mechanisms controlling these phenomena. Improved mouse models of resistance will undoubtedly lead to the discovery of strategies by which to improve the prognoses of these patient groups.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might affect the objectivity of this review.

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