Am J Clin Exp Urol 2019;7(5):313-320
www.ajceu.us /ISSN:2330-1910/AJCEU0102806

Review Article

Humanized mice for immune checkpoint blockade in human solid tumors

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Received September 25, 2019; Accepted October 13, 2019; Epub October 15, 2019; Published October 30, 2019

Abstract: Immunotherapy, specifically research involving immune checkpoint blockers (ICBs), has become a popular trend in anticancer research over the last three years. Due to the difficulties and often poor translation of results from *in-vitro* models, *in-vivo* models have become more relevant than ever. With the discovery of NOD, *Prkdc*^{scid}, and *Il2rγ*$^{-/-}$ mutations, patient-derived xenograft (PDX) mouse models were developed, providing an ideal environment for ICBs testing. By implanting a PDX with either CD34^{+} or peripheral blood mononuclear cells, we can create a human immune system capable of mounting a response against tumor burden. These animal models are currently being used to study molecular mechanisms, test drug efficacy, and trial drug combinations. Others have found use for these humanized mouse models as surrogates to represent otherwise uncommon diseases. Limitations remain with regards to what the models are capable of, but in the short amount of time between the development of these models and heightened interest in ICBs, these mice have already shown utility for future developments in the field of immunotherapy.

Keywords: Humanized mice, patient derived xenografts, immunotherapy, immune checkpoint blockers

Introduction

In June 2019, the National Vital Statistics Report of the Centers for Disease Control and Prevention (CDC) reported that in 2017 malignant neoplasms were the second leading cause of death in the United States since 1958 and have the second highest rate of incidence as recorded via the International Classification of Diseases (ICD) [1]. With many types of cancers currently lacking a consistent and reliable treatment plan, the development of newer approaches for treatment such as immunotherapy have received greater focus [2-4]. However, in the testing of novel ideas, difficulties arise when translating *in-vitro* results to *in-vivo* and from animal models to human therapy. In order to better replicate the immune system environment in which neoplasms develop, humanized mouse models have grown in popularity.

What is the humanized mouse model?

Simply defined, a humanized mouse is an immunocompromised mouse engrafted with a human immune system. Thus, it is capable of mounting an immune response to foreign insults such as bacteria or virus and also intrinsic defects such as cancer, which is the focus of this review. Interest in grafting human immune systems to immunocompromised mice started with the discovery of the nude mouse in 1962. However, this early mouse strain was unable to cultivate human bone marrow suspensions and thus failed to establish human immune cell growth [5, 6]. In 1983, another breakthrough came with the discovery of immune deficient mice with the Prkdc^{scid} mutation, which in humans is called Severe Combined Immunodeficiency (SCID) resulting in the C.B-17-*Prkdc*^{scid} mouse [7]. When C.B-17-*Prkdc*^{scid} mice were given a sublethal dose of radiation they were able to support purified CD34^{+} human hematopoietic stem cells (hHSCs) from bone marrow and umbilical cord [8]. However, these mice also had limitations from the innate immune system. Specifically, the natural killer (NK) cell was still present which resulted in rejection of implanted cells. Eventually the C.B-17-*Prkdc*^{scid} mice were back crossed onto the non-obese-diabetic (NOD) strain. This resulted in creation

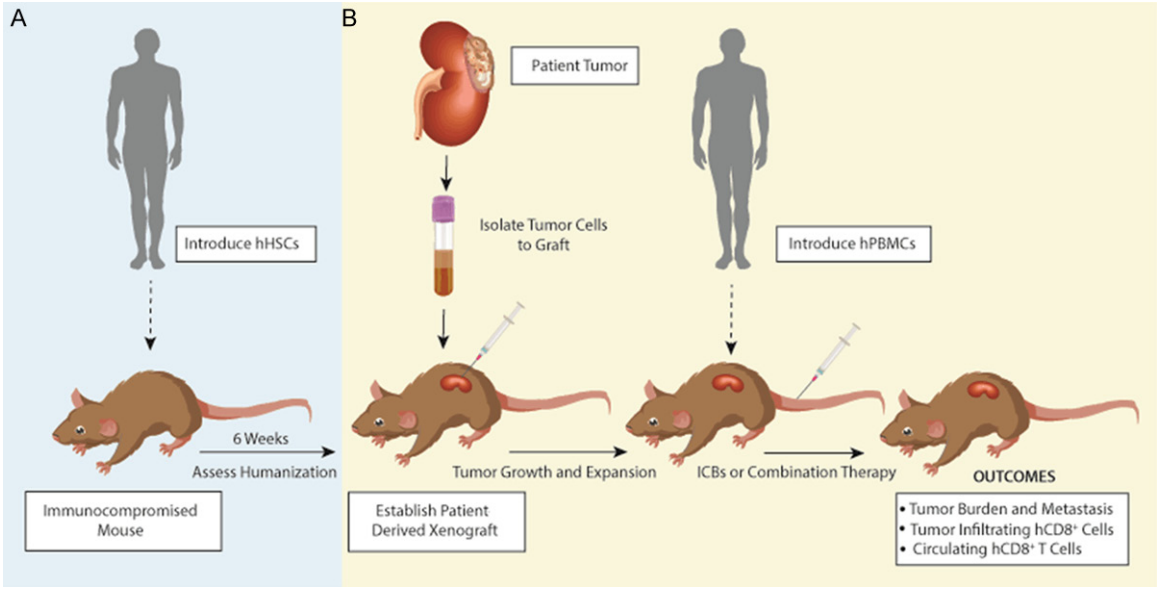

Figure 1. The major steps involved in the production of humanized mice. Process (A) demonstrates the humanization process using $CD34^+$ hHSCs. After implantation and allowing at least 6 weeks to establish, patient tissue sample can then be grafted. After 6 weeks, humanization can be verified by flow cytometric analysis, ultimately aiming for at least 25% $CD45^+$ human cells in the blood. Note that if proceeding with method (A), human PBMCs (hPBMCs) will not be used. Process (B) starts with implantation of patient tumor cells into immunocompromised. Additional experimental therapies may also be used once the mice have been allows to sufficiently humanize and xenograft has been established.

of NOD/SCID mice [8]. Although these mice were much more capable of supporting implanted cells, they were prone to developing thymomas and continued to demonstrate "leakiness" - the eventual production of T and B cells [9]. In 2002, a targeted deletion in the interleukin-2 receptor gamma (*Il2rγ*$^{-/-}$) resulted in a new generation of now commonly used immunocompromised mice: BALB/C, RAG2$^{-/-}$γc$^{-/-}$, and NOD/SCID/γc^{null} [8]. Typically, these mice share the three previous cumulative defects leading to their immunocompromised state. The first is a defect originally found in the NOD mice which leads to a defect in complement and $CD47^+$ cell function. This decreases both the ability to lyse foreign cells and the functional capacity of macrophages. The second is the presence of the SCID mutation which is akin to the one found in humans and leads to a lack of production of T and B cell production. The third is a defect in *Il2rγ* gene which results in a defective IL-2 gamma chain receptor that is involved in at least six interleukins and NK cells [10]. Ultimately, the lack of a mouse immune system is what allows us to graft and grow a human immune system in mice. As humanized mouse models increase in demand, newer strains of mice will be required and bred for specific experimental needs and designs. Due to trans-species limitations, only severely immunocompromised mice are able to tolerate the humanization process.

How are humanized mouse models created?

Although the creation of humanized mouse models is relatively straight forward, as shown in **Figure 1**, there are several variables that are up to the investigator's discretion which are usually based on preference and resources available. Additionally, depending on what the investigator is researching, the degree of mouse humanization can also be altered. There are methods which allow pseudo-humanization or humanization of specific genes that can be used to check the function of ICB which will be touched on briefly, but the ideal goal would be to have a mouse with a long term human immune system and diverse immune response [10].

A pseudo-humanized state can be achieved by implanting a patient tumor sample with stroma and already present immune cells into the mouse. Over time, the tissue will be replaced by

mouse cells but for a brief period of time, the patient derived xenograft (PDX) can be studied. It is a simple model which only allows for the study of immune cells already present within the PDX and there will be limited tissue sample within the one generation of PDX transferred to the mice [11].

Humanizing a gene is useful when probing systems which require specific molecular interactions within the immune system. Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is a protein receptor of the immune system whose main function is to down-regulate the immune response [12]. When CTLA-4 is deficient or the gene encoding the protein has a mutation, the down regulation of the immune system is removed and an autoimmune syndrome occurs [13]. The anti-CTLA 4 drug Ipilimumab (Yervoy) is benefit because it creates a hyperinflammatory response state in the body making tumor cell evasion more difficult.

A step further from this approach involves the injection of peripheral blood mononuclear cells (PBMCs) along with implantation of the tumor into mice (**Figure 1B**). This is a relatively easy way of establishing a fully humanized mouse, but it does have the downside of an eventual graft versus host disease between injected PBMCs and host, typically seen around week 4. This creates a limitation for experimental length [14].

A more complicated procedure is to humanize mice via $CD34^+$ hHSCs (**Figure 1A**). This requires several variables that must be decided upon by the investigator. First, a source of hHSCs must be identified that will be able to support the number of mice required for the experiment. hHSCs are available for purchase which are ready to use and can be ordered to arrive as needed but can become quite costly. Human umbilical cords are generally a good source of hHSCs as they are considered waste and do not require informed consent by most institutional review boards [15]. Bone marrow is an obvious source as this is where the cells are generated, but this requires a stimulus before harvest in the form of granulocyte-colony stimulating factor. In both cases the cells need to be collected and isolated, most commonly with apheresis [16]. In our lab, we have found it easiest to use donor blood collected by the blood bank. This reduces cost and blood is provided at our convenience. The downside of this method makes it impossible to HLA match the hHSCs with the tumor source. Another limitation to growing the PDX in humanized mice is the volume of blood required. In order to account for graft failure, ensure proper power for analysis, and have enough cells to test multiple variables on mice, large amounts of blood are needed. Some blood collection sites are capable of rendering these services for fees which would negate some of these disadvantages [15]. Culturing and freezing the collected hHSCs should be explored and optimized as circumstances may result in the mice and cells not being ready at the same time. In a study by Lang et al., they found that pre-culturing hHSCs between one day to one week in the presence of IL-1, stem cell factor, and *McDonough strain of feline sarcoma virus-like tyrosine kinase 3 ligand* (FLT3L) increased frequency of cell chimerism and lymphoid tissue development [15]. If longer storage of these cells is needed, the same study found that frozen and fresh cells provided similar yields. Once cells are ready and the mice are prepared, the age of the mice must be considered. Both adult and newborn mice can be used. In newborn mice, the facial vein becomes less visible after 48 hours [17, 18]. Currently, the prevailing thought is that younger mice adapt to the transplanted tissue more readily [15]. The mice must be whole-body irradiated with 240cGy for adults and 100cGy for young mice in order to ablate the immune system. After waiting for a minimum of 4 hours to a maximum of 24 hours, 3×10^4 cells to 2×10^6 $CD34^+$ hHSCs can be injected through the lateral tail vein in adult mice, or injected into the spleen, facial vein, or liver in young mice [17-19]. Because of the sensitive location of the injection sites in newborn mice, previously published protocols call for the use of anesthetics as approved by respective animal ethics boards. After injection, the mice are allowed to develop hHSCs for a minimum of 6 weeks [17-19]. Jackson Labs, one of the largest sellers of immunocompromised mice in the United States, sell mice reconstituted with at least 25% human $CD45^+$ cells in the peripheral blood [20]. In order to confirm successful humanization of the mice, cells isolated from peripheral blood can be tested using human specific antibody staining and flow cytometery. At necropsy splenic, bone marrow, and peritoneal lavage cells can be gath-

ered as well to reconfirm successful humanization [15].

Benefits of patient derived xenografts

In 2016 the National Cancer Institute switched from its "NCI-60" panel which can screen more than 100,000 compounds and over 3,000 compounds yearly for anti-cancer activity in favor of PDXs in mice [21, 22]. Despite being in use for over 25 years, as cancer research continues to develop, scientists will need to develop their own screening tools. *In-vitro* models are very useful when testing single components, as the model focuses on the cell lines at hand [23]. The shortcomings of *in-vitro* versus *in-vivo* arise when data is extrapolated into a larger, more complicated system. The difference in drug efficacy has often been blamed on the homogeneity of the cell line as opposed to the heterogeneity of the tumor sample as well as failure to replicate the tumor microenvironment seen *in-vivo* and in patients [10, 23, 24]. Cell lines lack the complex mechanisms of molecular interplay between the cancer and human environment in which it grows. This drives the development of drug resistance [25]. When companies use cell lines to develop drugs which target specific markers or mechanisms, this can lead to a false efficacy as there are no other factors that would typically be present in a patient or *in-vivo* model [25]. PDX models are able to overcome many of the shortcomings of an *in-vitro* model. One of the big advantages of using *in-vivo* models, especially PDXs, is that it is known that what was implanted into the mouse is what is growing [26, 27]. A study found that cell lines from tumor samples that had been cultured and then implanted, as compared to those that had been directly implanted, had developed mutations unrelated to either the source tumor or the original cell line [27]. PDX models are able to be passed down generationally to mice and retain fidelity to their tumor, as others have established by observing morphologic similarities, and measuring both mutations and stable gene expression patterns [28]. Additionally, translation of drug efficacy in patient drug responses has been claimed as one of the most accurate *in-vivo* models currently available [29-31]. Substantiating these claims, others have done trials to test the reproducibility of results with known mechanisms and offer more relevant predictive information [30, 31].

Humanized mouse models in immunotherapy

With Drs. James Allison and Tasuku Honjo winning the Nobel Prize in 2018 in the field of physiology or medicine for their work on immune checkpoint molecules, immune checkpoint blockers (ICBs) have come into vogue as the new field of drug development research. These drugs are designed to block ligand interactions between cancer cells and T cells. Various cancers have been shown to upregulate inhibitory molecules which sabotage T cell function by inducing apoptosis during recognition of aberrant peptide fragments - a process which normally leads to destruction of the offending cell. Inhibition of this interaction via an immune blocking antibody has led to significant therapeutic benefits for a number of malignancies, including small cell lung cancer, breast cancer, leukemia, and melanoma [32].

A PubMed search for the key words "immune checkpoint inhibitor" reveals a continuous increase in the number of articles being published in the field (**Figure 2**). As of September 11, 2019, the search query resulted in a total of 1802 articles versus 2017's total of 1314 articles and has surpassed 2018's total of 1789 articles. Following the trend of immunotherapy research is the United States Food and Drug Administration's (FDA) approval of many immunotherapy drugs.

In 2011, Yervoy became the first ICB to be approved by the FDA. In 2014 pembrolizumab (Keytruda) gained FDA approval and was quickly followed by nivolumab (Opdivo) in 2015 [33-35]. Then, in 2017, three ICB's were approved within months of each other: avelumab (Bavencio), durvalumab (Imfinzil), and atezolizumab (Tencentriq) [36-38]. The most recent ICB to gain FDA approval was cemiplimab (Libtayo) in 2018 [39]. The availability of drugs to the public suffers from an inherent delay after their research due to the need for final clearance. However, with the increase in volume of research, it is likely that more drugs and approvals will be coming out in the near future.

Other studies using humanized mouse models include dose response trials to medications [40], drug combination regimens [41, 42], and targeting novel immunotherapy markers and mechanisms [3, 41-47]. We are using PDXs in humanized mouse models testing various che-

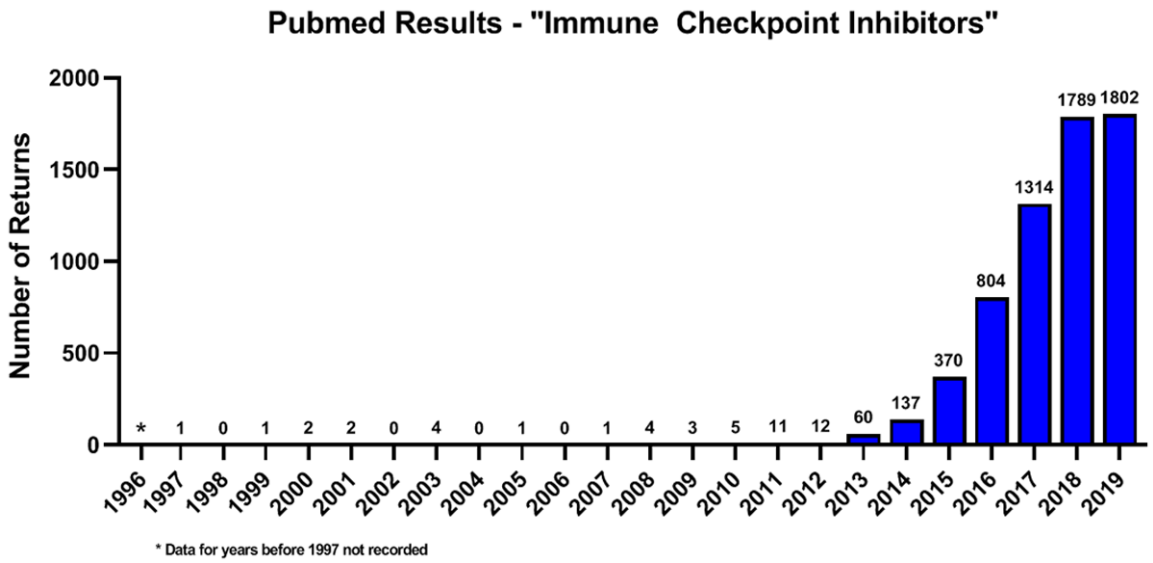

Figure 2. PubMed search results. There has been an increase in the quantity of research being published in the field of ICIs. Data from PubMed was able to be collected as far back as 1997, publications to be released in the year 2020 were excluded from this search result.

motherapy courses to validate the model's use in renal cell carcinoma and colorectal cancer. Similarly, Pyo et al. published a paper validating their usage of a humanized mouse to model the efficacy of ICB drugs (anti-PD-L1) for lung cancer treatment [4]. In 2013 Alcantar-Orozco published a study which aimed to validate the utilization of humanized mice in melanoma. This demonstrated promising results but also revealed that the model was not perfect and that there is room for improvement with regards to how these models are produced, and how the experiments which employ them are conducted [48].

Future directions and limitations

The often-repeated phrase in the science, technology, engineering, and mathematics fields is that "necessity is the mother of invention". This is quite apparent with regard to the humanized murine model. With the advent of immunotherapy for cancer we are rapidly discovering limitations in the development and overall availability of tools which we can use to investigate our ideas. Animal models served as analogues for humans, especially the humanized mouse model as discussed earlier. The humanized mouse model is an evolution of the ordinary mouse model which has enabled us to adapt to more technical and quantitative needs. With so much interest in novel drug therapies and new models to test these therapies, the burning question is how far can we take this? Our lab and many others are working on using mice as avatar models for patients in which we can see the beginnings of medicine being specifically tailored. This idea of personalized medicine is being refined in order to treat patients on an individual level [49]. In an experiment done by Wang et al., immunocompromised mice were implanted with PBMC and tumor from the same patient. $CD8^+$ T cells were taken from the patient, expanded *ex-vivo*, and rendered insensitive to transforming growth factor-beta (TGF-β) type II receptor via retroviral transfection [50]. TGF-β is a potent immunosuppressor associated with the tumors ability to hide from immune surveillance. The desensitized $CD8^+$ T cells were tested against control naïve $CD8^+$ T cells to confirm resistance. After introducing the desensitized T cells, Wang et al. found a decrease in tumor burden, an increase in INF-γ, a potent anti-tumor cytokine, and complete prevention in pulmonary metastasis. The desensitized T cells however did not cause a complete resolution of the RCC, but it does leave room for future investigation and pathways to explore [50]. In an editorial done by Ilmer and Berger, they laud how much benefit these avatar models are able to provide. As a rare disease, hepatoblastoma is generally below the level that draws the attention of drug

developers and designers. This results in an increased disease impact despite the overall rarity. By taking a rare cancer line and effectively increasing the incidence rate, Ilmer and Berger now have a larger sample size to work with. As a result, they have discovered a possible treatment regimen that would have otherwise been impossible to test. These avatar experiments representing experimental personalized human models are an idea that is growing as people strive for more tailored, patient specific treatments which are able to avoid the physical and ethical issues of experimentation [51].

Other studies have also posed similar questions regarding how to better improve the humanized mouse model including using base mice that more closely resemble the patient population in age (converted to mouse age), body habitus, and microbiome [2]. During the humanization process, although many protocols call for HLA matching, it may not always be possible to perform an HLA match depending on when and what hHSCs and graft samples are available [5, 16, 51]. Graft failure is an issue that must be considered when creating the xenografts as whether the graft will survive for the entirety of the experiment is a major concern. From previous experience we have also seen patients become understandably hesitant about any possible delays or extra procedures that they feel may detract from their treatment. This can result in denial of research participation. Although such research has the potential to benefit many, it is also a fledgling field and subject to intense scrutiny. Still, it is reasonable to imagine that these current issues will eventually be addressed so that the field may move forward and show that humanized mice can play a role in the development of immunotherapy for cancer. The FDA continues to grant new approvals to currently approved drugs demonstrating that progress is being made with our current understanding of this mechanism. More papers are being published on the use of humanized mice, ICBs, and testing of ICBs in the murine models, indicating promise for the development of this crucial field.

Acknowledgements

We would like to thank Barbara Siede for assistance with the excellent medical illustration.

Disclosure of conflict of interest

None.

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