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REVIEW

Unwanted disorders and xenogeneic graft-versus-host disease in experimental immunodeficient mice: How to evaluate and how to report

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Abstract

Human-derived tumor models are essential for preclinical development of new anticancer drug entities. Generating animal models bearing tumors of human origin, such as patient-derived or cell line–derived xenograft tumors, is dependent on immunodeficient strains. Tumor-bearing immunodeficient mice are susceptible to developing unwanted disorders primarily irrelevant to the tumor nature; and if get involved with such disorders, reliability of the study results will be undermined, inevitably confounding the research in general. Therefore, a rigorous health surveillance and clinical monitoring system, along with the establishment of a strictly controlled barrier facility to maintain a pathogen-free state, are mandatory. Even if all pathogen control and biosafety measures are followed, there are various noninfectious disorders capable of causing tissue and multiorgan damage in immunodeficient animals. Therefore, the researchers should be aware of sentinel signs to carefully monitor and impartially report them. This review discusses clinical signs of common unwanted disorders in experimental immunodeficient mice, and how to examine and report them.

KEYWORDS
animal models, graft-versus-host disease, health surveillance, preclinical drug evaluation, xenograft model antitumor assays

1 | INTRODUCTION

Human tumor xenograft models are at the core of preclinical development of new anticancer drug entities.[1,2] Despite histologic and molecular similarities between cancers of humans and other mammals, human-derived xenograft-bearing animals, recapitulating intrinsic and phenotypic features of human tumors, are superior to the cancer models of animal origin. In other words, for drug response assays of novel pharmaceuticals designed for human malignancies, preclinical models featuring tumors of human origin are preferred.[3,4] To generate animal models bearing tumors of human origin, such as patient-derived xenograft (PDX) or cell line–derived xenograft (CDX) models, immunodeficient strains are required. Various strains of immunodeficient mice with different levels of immunodeficiency exist.[1,5] The most commonly used conventional strains include nude, which lacks only T cells, followed by severe combined immunodeficient (SCID; knocked out for *Prkdc* gene) and Rag-deficient (knocked out for *Rag1* or *Rag2*

genes) mice, which lack B and T cells. The hybrid strains of non-obese diabetic (NOD), and SCID, i.e., NOD-SCID, or *Rag* knockouts, i.e., NOD-*Rag*^{null}, lack T and B cells and have impaired natural killer (NK) cells. Strains with NOD-SCID background, but additionally knocked out (total or truncated) for *IL2RG* (encoding gene of IL-2 receptor γ chain), identified as NOD-SCID gamma-knockout mice marketed under NSG (The Jackson Laboratory, USA), NOG (Taconic Biosciences, USA), B-NDG (Biocytogen, China), NXG (Janvier Labs, France), NCG (Charles River Laboratories, USA), and NIG (GHBIO Inc., South Korea) brand names, and those with NOD-*Rag*^{null} background, and additionally knocked out for *IL2RG*, also known as "NOD-Rag-gamma," abbreviated and marketed as NRG brand name, are all characterized by the lack of T, B, and NK cells, reduced function of macrophages and dendritic cells, and the absence of complement activity.[1,2,6–9] With a high degree of immunodeficiency, NSG/NOG/NDG/NXG/NCG/NIG/NRG mice are appropriate recipients of human-originated xenografts and lymphoid lineages of hematopoietic cells. Next generation of these strains, which are additionally transgenic for forced expression of human IL-3 and GM-CSF ± M-CSF and thrombopoietin, such as NSG-SGM3, NOG-EXL, B-NDG-MGMT3, and MISTRG, were developed to be used for generating fully humanized models or humanized immune system mice, as they are primed for robust and stable engraftment and differentiation of hematopoietic stem cells, both lymphoid and myeloid lineages.[8,9]

Xenograft-bearing animals are appropriate tools for predicting clinical response to investigational anticancer agents, provided that the impacts of confounders are prevented or controlled in experimental research.[1] For these immunodeficient animal models, a rigorous health surveillance system, in addition to establishment of a strictly controlled barrier facility to maintain a disease-free state, is mandatory.[1] Even if all hygienic precautions and sterilizing practices are followed, the immunodeficient animals are prone to develop unwanted disorders inevitably undermining the validity of models and confounding the research outputs.[10] Some of these disorders are exclusive to immunodeficient strains, and some are not (i.e., some disorders may develop in both immunodeficient and immunocompetent experimental mice). Because preclinical studies using immunocompromised animals require considerable funding and are of paramount importance for their sponsor companies, the responsible laboratory animal technician(s) should be aware of all potential disorders that can involve such animals. That is to say, the research team should be vigilant for alarming signs of unwanted disorders, as failure to notice them may result in xenograft failure causing tumor formation arrest, premature death of animals, and, above all, distortion of the study findings. Consequently, it might become necessary to redo the whole project, and thus, a major part of the grant, funding, or company's investment will be lost.[4,11,12] Therefore, these disorders should be carefully and regularly monitored and impartially reported. The aim of this review is to explore and discuss the clinical signs of common unwanted disorders in experimental immunodeficient mice, and how to examine and report them.

2 | INFECTIOUS DISORDERS

Immunodeficient animals are highly susceptible to various pathogens. Potential infections of laboratory mice caused by various bacterial, viral, mycotic, and parasitic pathogens have been adequately reviewed elsewhere.[10,13–19] Microbial diseases can decrease the lifespan of animals, reduce the transplantability (take rate) of xenografts, obscure the genuine clinical presentations of disease models, and pervert the preclinical studies. In addition, some pathogens can affect the external validity of the model, especially in immune-oncology studies, owing to their immunomodulatory (impeding the investigational adoptively transferred immune cells), immunoreactive (reactive expansion of effector immune cells and increase in pro-inflammatory cytokines, resulting in augmented antitumor effects), myelosuppressive (engraftment failure of adoptively transferred immune cells in the animal model), and oncosuppressive (decreased proliferation of tumor cells causing early rejection of the xenograft or extra shrinkage of tumor xenograft irrespective of the actual effect of a test agent) properties.[20,21]

Therefore, the pathogen status of a facility should be regularly monitored with reliable testing. In advance, the facility should be washed, disinfected, and irradiated prior to establishing a colony. Moreover, traffic patterns and standard of operations (SOPs) should be devised and practiced to prevent the entry of pathogens into the facility and cages. Besides, transplantable tumors and cell lines may be contaminated with infectious agents before xenotransplantation.[22] This necessitates special care and concern over transplantable materials in particular and any biological material in general, which are going to be used in the facility. Common signs that may be suggestive of infectious diseases in mice include ruffled fur (also called scruffy hair coat), diarrhea, dermatitis, conjunctivitis, weight loss, reduced body condition, and poor growth of the pups. Less commonly, hyperkeratosis, retro-orbital abscess, mastitis, inflammation and cysts in accessory sex glands, and alopecia may occur.[10,19,23] Severe and prolonged conjunctivitis may cause keratitis. In advanced stages of infections, ascites, nasal/rectal/vaginal discharge, dyspnea, necrotic amputation of limbs and tails, rectal prolapse (secondary to infection with *Helicobacter* spp. or intestinal parasites), hunched posture, emaciation, and lethargy may be seen. Rare cases of otitis should be suspected when circling behavior and head tilt are observed.[10]

3 | NONINFECTIOUS DISORDERS

3.1 | Spontaneous endogenous tumors

Masses, lumps, and protrusions should be carefully examined to determine the etiology and significance. Common causes include tumor, cyst, and abscess, whereas lymphadenopathy and salivary gland hyperplasia are less frequent.[10] DNA repair deficiency and the lack of a competent immune system to eliminate endogenously arising tumor cells have made immunodeficient mice vulnerable to

developing various spontaneous neoplasms of murine origin, causing unwanted comorbid conditions in xenograft-bearing models, especially in older ages. These include lymphoma, mammary tumors, intestinal adenomas and adenocarcinoma, hemangiosarcoma, primary pulmonary tumors, hepatoma, myoepithelioma, plasmacytomas, leukemia, and osteosarcoma.[24–29] Preneoplastic lesions have also been identified in laboratory mice that can undergo malignant transformation with age, depending on the mouse's genetic background.[30,31] Axillary lumps in immunodeficient mice are often lymphomas. Mammary tumors are most commonly seen in female mice.[10] Swann and Smyth have summarized a strain-specific list of spontaneous tumors, potentially affecting some strains of immunodeficient mice.[26] It is noteworthy that by comparing various immunodeficient strains, the prevalence of lymphomatous neoplastic lesions is remarkably higher in the SCID and NOD-SCID mice, potentially owing to defective DNA repair mechanisms or genetic modifiers affecting lymphocyte homeostasis or an endogenous murine retroviral infection,[28,32,33] thereby causing shorter lifespans of these strains. This and other reasons urged subsequent strain crossing and generating NOD-SCID-gamma strains, in which the prevalence of lymphoma is evidently very low.[28,33]

3.2 | Oncogenic virus–induced neoplasms and proliferative disorders

Spontaneous neoplastic development owing to infection with inherently oncogenic viruses is a potentially unwanted disorder in immunodeficient animal models. Murine leukemia virus, mouse papillomavirus type 1, mouse γ-herpesvirus 68, murine sarcoma viruses, and mouse mammary tumor virus are among the potential causal agents of neoplastic lesions in murine models,[34,35] which can be controlled through strict pathogen control and health surveillance of the colony and biological materials. However, despite a comprehensive health monitoring, transplantation of xenografts containing tumor infiltrating lymphocytes (TILs) preinfected with human lymphotropic viruses, such as Epstein–Bar virus (EBV), Kaposi sarcoma–associated herpesvirus (KSHV), human T-cell leukemia virus type 1 (HTLV-1), and human immunodeficiency virus (HIV), to immunodeficient mice may lead to neoplasms and lymphoproliferation.[34–37] Lymphoproliferative disorders that originate from EBV-infected lymphoid cells, either from co-transplanted TILs within solid tumor xenografts or human hematopoietic xenografts, can transform the nature of the xenograft resulting in benign lymphoid lesions or lymphoma.[38,39] That is to say, the proliferation of EBV-infected lymphoid cells supersedes the proliferation of the tumor cells of the xenograft, resulting in partial or sometimes complete transformation of the phenotype of the PDX into a lymphocytic disorder.[3,39] Coinfection of EBV/KSHV has been linked to increased incidence of primary effusion lymphoma in the humanized mouse models.[40] It is important to note that engraftment of human B cells is trivial to none in nude mice,[9,41] and thus infection with B-cell tropic viruses, such EBV and KHSV, and consequently lymphoproliferative disorders are not expected in this strain. Humanized models of HTLV-1 infection are vulnerable to T-cell leukemia, lymphoma, and thymoma.[36,37] HIV infection has been shown to be associated with increased epithelial cell proliferation in humanized mice.[42] Therefore, it has been recommended that prior to xenotransplantation, any solid tumor or hematopoietic xenograft should undergo reliable testing to ensure that the specimen is not contaminated with the mentioned viruses.[39]

3.3 | Xenogeneic graft-versus-host disease

Xenogeneic graft-versus-host disease (xGvHD) in immunodeficient animals occurs as a result of (1) transplantation of a T-cell–containing product to intentionally induce and develop GvHD models or to evaluate its anticancer effects; or (2) reactivation, extravasation, and expansion of the TILs co-transplanted with a solid tumor PDX. Although the former situation is an expected event in systemic administration of immune cells of human origin to immunodeficient mice, the latter situation is rare and arises spontaneously (compared to the former one that can be considered iatrogenic). The spontaneous xGvHD is more likely to be seen after transplantation of non–lymphocyte-depleted hematopoietic grafts or lymphocyte-predominant solid tumors.[43–45] Common manifestations of xGvHD include ruffled fur, conjunctival erythema, hair loss, erosive/scaling dermatitis, and reduced body condition. In severer xGvHD cases, emaciation, weakness, severe paleness, hunched posture, neuropathies, diffuse alopecia, ulcerative/crusting dermatitis, and necrotic amputation of digits may occur.[44–47] Reduction in the animal's lifespan, shortening of the experimental window, exaggerated therapeutic effect of the investigational treatment (due to the destruction of murine-originated embedding of PDX tumors and systemic effects of xenoreactivity, causing an underconditioned and malnourished animal, which therefor result in extra shrinkage of the xenograft tumor), and altogether impeding immunotherapy research are the consequences of xGvHD.[3,48]

3.4 | Intervention-induced lesions and toxicities

The common process of PDX model generation involves the heterotopic implantation of PDX tumor fragments into a surgically created subcutaneous pouch or pocket, though less commonly the fragments can be implanted orthotopically in visceral organs or heterotopically in subrenal capsules.[1,2] If it is performed by an expert veterinarian, the risk of postsurgical wounds, hemorrhage, and tissue damages in immunodeficient mice is very low, but these mice are susceptible to postoperative infections that require prophylactic antibiotic therapy and careful observation. Hypertrophic scaring and overreactive subcutaneous fibrosis at the incision site (pouch) may mimic xenograft tumor growth, appearing as a false-positive tumor formation. However, in such a case, only a small fibrotic mass forms that does not grow or shrink over time. Scratching wounds and dermatitis caused by pain and/

or irritation in surgical sites, implanted devices, and ear tags may develop in experimental mice.[10] Blepharospasm, mucoid discharge, periorbital edema, puncture wounds, ocular ulcerations, infection, keratitis, and blindness are the complications associated with retro-orbital blood collection.[49] Per oral administration of substance (oral gavage) may result in passive reflux, local irritation or infection, aspiration pneumonia, severe stress, and esophageal or gastric rupture.[50] Complications associated with parenteral substance administration include local irritation, pain, infection, and damage to the surrounding tissue.[50] Although rare, extravasation injuries, injection-site sarcomas, and mesenchymal neoplasms may occur following parenteral administration of investigational chemotherapies.[51,52] Hyperreaction at the injection site, especially to adjuvants, may cause subcutaneous lumps, ulcerating to small dry, open lesion on the skin.[10] Moreover, pulmonary granulomatosis is a complication associated with the administration of complete Freund adjuvant in rodents.[50] Intraperitoneal injection of irritating substances, in particular, and large volumes of any substance, in general, can cause pain, peritonitis, fibrous tissue formation, adhesions, perforation of abdominal organs, hemorrhage, and respiratory distress.[1,50,53] Local pain, irritation, and dermonecrosis may be caused by intradermal and subcutaneous injection of irritants or large volumes (above the maximum volume allowed for each route) of any substance. Vascular occlusion, emboli, and thrombosis of local and distant capillary systems are expected after intravenous injection of cellular products with very high cell density, as well as compounds containing particulate material having low pH that precipitate when mixed with blood.[50]

3.5 | Strain-specific and age-related disorders

Some strain-specific disorders (comorbidities) and anomalies in immunodeficient mice have been described. Nude mice are athymic and characterized by congenital total hair loss and nail dystrophy. Spontaneous alopecia areata may be seen in C3H/HeJ strain.[27] NOD mice are inherently susceptible to autoimmune insulitis causing insulin-dependent diabetes mellitus, which grounds the nomenclature of this strain.[1] Immunodeficient mice on the background of BALB/c strain (i.e., NOD, NSG, NOG, SCID, etc.) are albino, and thus visually impaired and sensitive to light; additionally, they are vulnerable to age-related hearing loss.[54] NOD-SCID-gamma strains are prone to spontaneous early-onset neurodegeneration with age-related progression in the brainstem and spinal cord, though it is associated with unremarkable clinical neurologic findings in approximately the first 5 months of age.[55] Spontaneous dystrophic cardiac calcinosis might be developed in BALB/cByJ mice.[27] Development of acquired immunity or leakiness, which means generating some functional lymphoid cells and increase in serum immunoglobulin with age, is a relatively common phenomenon in nude, SCID, and NOD-SCID mice, especially if the mice are housed in less-controlled environments.[1,56,57] The resultant immune function restoration hinders the PDX-based studies.

Decreased body condition, mucoid nasal discharge, rapid shallow breathing, mild-to-moderate scruffy hair coat, less interaction with peers, and pallor or paleness might be normal in an aging mouse, though they are suggestive of disease in younger mice.[10,58,59] Other dermal pathologies in aging mice include dyskeratosis, hyperkeratosis, hair thinning, patchy hair loss, loss of whiskers, and dermatitis. Dryness of mucosal membranes, most commonly dryness of lacrimal glands, can be seen in aged mice, sometimes causing keratitis. Neuromuscular disabilities may emerge in aging mice, including ataxia, head tilt, spinning, circling, urinary retention, muscle tremors, seizures, reduced reflexes, and activity.[10,58,59] Age-related abnormalities of genitalia and genital system include ectasia of accessory sex glands, cysts in reproductive organs and adnexa, prolapse of vaginal or uterine tissue, uterine masses, polyps, and tumors in older females and retired breeders.[10,60,61] Malocclusion, decreased liver function and ascites, abdominal distention due to intraperitoneal fluid accumulation, hypertriglyceridemia and atherosclerotic lesions, constipation, rectal prolapse, hunched posture, and arthritis are the other disorders and abnormalities of aging.[10,27] It is worth noting that the rectal prolapse might be secondary to prolonged constipation, which can be prevented by modifications in the mouse diet. In addition, anorexia may be seen in terminally ill or aged mice.[10]

It is necessary to emphasize that many of the clinical presentations listed in this section may be due to other etiopathogenesis. In fact, it may be difficult to differentiate aging from other underlying causes in an aged mouse. Therefore, before attributing any sign to aging in a mouse, it should be assessed in the context of mouse cagemates having similar chronological age, considering the infectious etiologies, the collateral effects of experimental interventions, and the impacts of common procedural and environmental stressors.

3.6 | Traumatic injuries

Traumatic injuries of laboratory mice might be caused by fighting, abnormal behavior of cage-mates, self-injury, or in contact with environment. Fight wounds are mostly seen in co-housed males and are typically presented as a cluster of wounds on the rump, hips, and/or genital region, which may extend to the trunk of the body or forelegs. Fighting can also cause conjunctivitis, keratitis, and genitalia wounds. Patchy alopecia might occur due to barbering or overgrooming, especially in a group of co-housed mice.[10] Unintentional friction with the nesting materials, especially when the orifice of the igloos or paper/plastic tunnels is too narrow for the mouse body size, may cause a mild coat ruffling and slight hair fall in the absence of any organic pathology.

4 | CLINICAL EXAMINATION AND REPORTING PRINCIPLES

Directors of preclinical anticancer research are advised to implement a surveillance plan for the experimental animals to ensure

the best practice. In this context, researchers are encouraged to check animals at a regular basis to inspect clinical signs secondary to the tumor and the intervention and to monitor adverse events, general welfare, and health of the animal models.[62] Careful reporting of the clinical condition of an experimental mouse requires both in-cage observation and hands-on examination.[10,63,64] The in-cage observation provides information about the animal's general appearance, posture, locomotion/activity level, and behavioral interaction with cage-mates and environment. Through hands-on examinations, body condition based on the scale developed by Ullman-Cullere and Foltz,[65] any abnormal clinical signs, hydration status, neurologic reflexes, including grasping and righting, and any palpable mass, abnormality, and anomaly, especially on mammary chain, abdomen, flanks, genitalia, and rectal area can be checked.[10] To assess the hydration status, the skin over the shoulder blades can be pinched gently. In a normal well-hydrated mouse, the skin quickly returns to its original shape, whereas it takes longer in dehydrated mice. Sunken or recessed eyes and fuzzy facial fur might be signs of moderate-to-severe dehydration. Generalized weakness, muscle tremors, reduced neurologic reflexes, hypoactivity, and hindlimb paralysis are also suggestive of severe dehydration,[10] whereas they may be due to other underlying causes.

In tumor-bearing mice (solid tumor models), measuring the tumor size should be performed on a regular basis. The volume of a subcutaneous xenograft tumor (heterotopic implantation) can be readily estimated by the sizes gauged using caliper via this formula: $volume\ (\text{in mm}^3) = length \times width^2 \times 0.52$.[66] For orthotopically implanted visceral tumors, a sensitive imaging or reporter systems/radiolabeling or multimodal method is required to estimate the tumor volume.[62,67,68] Body weight may not be a good representative of the clinical condition of a solid tumor-bearing mouse, as the tumor adds to the body weight despite the potential animal's fat and muscle breakdown. Therefore, estimating the carcass weight might be a better alternative. Carcass weight at each time point can be calculated as the total body weight minus the concurrent solid tumor weight, when the tumor weight is approximated based on the volume considering the average tissue density.[48] An updated checklist for complete evaluation of the clinical status of tumor-bearing experimental mice is devised in Table 1, which is based on previous recommendations and guidelines.[62–64] Monitoring the clinical status of experimental mice is crucial in preclinical anticancer research.[62] Regular visual inspection of the mice will help quickly diagnose any potential problem before spreading to the entire colony or any critical condition necessitating immediate veterinary care or humane termination.[62,69–72] The frequencies of evaluations specified in Table 1 should not be looked as must-do routines, as they are only suggested on the ground of best laboratory practice.[62,72,73] Moreover, researchers may determine narrower intervals for evaluating each parameter based on their study objectives, xenograft tumor size, and previous experiences. For instance, many studies assess the size of xenograft tumor and weight of animal models daily. For models reaching advanced stages of disease, more frequent clinical evaluations have been recommended to identify those approaching humane endpoints.[10]

In the context of routine clinical monitoring (Table 1), observing some symptoms, such as mild coat ruffling and conjunctival erythema, is of little importance and requires watchful surveillance, whereas inspecting some behaviors or clinical signs demands immediate action of a laboratory operator. If discharge from any orifice, diarrhea, keratitis, or abscess is noticed, separation of the diseased mouse from its cage-mates and treatment with antibiotics might be helpful, though, humane termination has been the recommended intervention.[62] In case of patchy alopecia (especially when only a mouse within co-housed mice is involved) and barbering, separation from cage-mates and enrichment of cage environment has been advocated.[10] Hypoactivity may be linked to dehydration, poor diet, infection, or advanced disease, which, therefore, requires careful checking of these parameters. If any of the symptoms suggestive of humane endpoint in laboratory animal experimentation, such as cyanosis, jaundice, limb paralysis, persistent recumbency, or lethargy, no response to manual stimulation, dyspnea (labored breathing), head tilt/circling, significant abdominal distention, anuria, urinary retention (due to enlarged xenograft tumor obstructing bladder outlet), tumor burden exceeding 10% of body weight, enlarged tumors interfering with ambulation, emaciated or poorly conditioned state, hunched posture impairing movement, is noticed, euthanasia becomes mandatory.[10,27,62,72]

In addition to the routine examinations listed in Table 1, pain and distress in laboratory mice can be graded based on a scale recommended by Burkholder et al.[10], and weakness or frailty can be scored based on an index recommended by Whitehead et al.[58] Furthermore, similar to the Karnofsky performance status scale that is used for the assessment of functional impairment and the prognosis of an individual in clinical (human) studies,[74,75] performance/activity of an experimental mouse can be graded according to a scale shown in Table 2. Because no specific clinical sign can be attributed to xGvHD, in a strictly controlled barrier facility, emergence of a set of immunoinflammatory signs in young immunodeficient mice, undergoing a kind of immunotherapy or adoptive transfer of immune cells, is likely to be due to xGvHD, unless infectious pathologies or traumatic etiologies can be identified. The clinical severity of xGvHD can be assessed based on a grading scale (Table 3). Although grading the xGvHD might be helpful but in a semiquantitative manner, for studies seeking a quantitative assessment of this disorder, a scoring system is more applicable (Table 4).[46] Table 4 illustrates an updated version of a scoring system for xGvHD proposed by Cooke et al.,[76] which encompasses a more comprehensive evaluation of organ/systems with amended definitions. It should be noted that although evaluations based on Table 1 may be included in the routine surveillance plan of an animal laboratory, the use of Tables 2–4 is optional (generally for research purposes) and based on objectives of a study. Moreover, the items mentioned in Tables 2–4 are already included in Table 1, and

TABLE 1 Checklist of clinical status monitoring in tumor-bearing experimental mice.

Step	Parameter	What to report/examine[a]	Frequency of evaluation[b]
In-cage observations	General appearance	Fur texture, pallor/cyanosis/icterus (color of skin, ear, or conjunctiva), fascial expression of pain (grimace), any abnormality on the body (wound, bulging, deformity, discharge)	Daily
	Posture	Hunching	Daily
	Locomotion and self-directed behaviors	Normal/hypo−/hyperactivity, jumping, bar-mouthing, climbing, gait, limb paresis/paralysis, ataxia, persistent recumbency or lethargy, eating/chewing/drinking, grooming, scratching	Daily
	Interaction with cage-mates and environment	Aggression/biting, chasing, barbering, exploratory behaviors, group sleeping, mating, nest building, digging (burrowing), vocalization	At least weekly
Hands-on examinations	Body condition	Palpating the sacroiliac region of a mouse to estimate the degree of flesh and fat covering the bones	At least semi-weekly
	Hydration status	Pinching the skin over the shoulder blades	At least semi-weekly
	Neurologic reflexes	Grasping and righting	At least semi-weekly
	Total body weight	Measurement of mouse weight using an accurate balance	At least semi-weekly
	Tumor volume and weight	Measurement of the length and width of the tumor xenograft using caliper to estimate the volume, and estimate the tumor weight based on the average tissue density (≃1 g/mL)	At least semi-weekly
	Carcass weight	Subtracting the estimated tumor weight from the total body weight	At least semi-weekly
	Visible organ systems and clinical signs	Respiration (tachypnea, dyspnea), body temperature (hyper−/hypothermia), eye and conjunctiva (squinted/sunken eyes, protruded eyes, conjunctival erythema/conjunctivitis, keratitis), skin (erosion, wound, ulcer, rashes, papules, alopecia, scleroderma, dyskeratosis), mouth, nose, and orifices (malocclusion, rectal/vaginal prolapse, bloodstained or mucopurulent discharge from any orifice; e.g., nasal/vaginal discharge), stool consistency (diarrhea, constipation), urine color, abdominal distention, response to manual stimulation (moves away with agility, moves away slowly/with difficulty, no reaction)	At least semi-weekly
	Palpable swelling/mass	Inspection and palpation of the body, especially the mammary chain, axilla, accessible lymph nodes, abdomen, flanks, genitalia, and rectal area	At least weekly

[a]There are also other less common manifestations not listed in this column (e.g., ringtail, desquamation and flaky skin, ascites, amenorrhea, route tracing, head tilt, circling, stereotypic behaviors). However, any abnormal sign (other than what are listed in this table) that is inspected in any visit (irrespective of the recommended frequency) should be noted down and reported.

[b]This column indicates the recommended frequency of evaluation for each clinical parameter in routine laboratory works. Researchers may determine narrower intervals for their evaluations.

TABLE 2 Performance status scale for experimental rodents (disease models).

Disability progression	Description
Mild	Normal ambulation, normal feeding, normal grooming, healthy interaction with cage-mates (including allo-grooming), still and alert, normal exploratory behaviors (including search, attend, approach, investigate), group sleeping and normal sexual behaviors (if group-caged), mildly reduced reflexes, normal response to manual stimulation, no or mild symptoms of disease
Moderate	Reduced activity, decreased feeding, reduced self-directed behaviors, not well groomed (mild-to-moderate coat ruffling), less interaction with peers, reduced social and exploratory behaviors, remarkably reduced reflexes, muscle tremors, hunching at rest, reduced response to manual stimulation, evident signs of the disease
Severe	Extremely reduced activity, inability to stand and drink from sipper tube of water bottle, poor feeding, lethargy/persistent recumbency, poorly groomed (severe coat ruffling), hunching impairs movement, limb paralysis, ataxia, lack of response to manual stimulation, advanced stages of the disease

Note: In the description column, the Boolean operator between items is "AND/OR."

TABLE 3 Clinical severity grading for xenogeneic graft-versus-host disease (GvHD).

Grade	Description
I	Mild coat ruffling (mild ruffled fur), conjunctival erythema[a], diarrhea, normal or mildly reduced reflexes, normal activity, well-conditioned mouse (BC3)
II	Moderate coat ruffling, local (patchy) alopecia, conjunctival erythema[a], scaling dermatitis, constipation, hunching at rest, reduced activity, remarkably reduced reflexes, mild paleness, mild weight loss, and underconditioned mouse (BC2)
III	Severe coat ruffling, diffuse alopecia, conjunctival erythema[a], necrotic amputation of digits, crusting dermatitis, reduced skin elasticity, hunched posture (that impairs movement), difficulty breathing, cyanosis, nail loss, severe paleness, hindlimb paralysis, polyneuropathic walking pattern (ataxia), urinary retention, severe constipation, lethargy or persistent recumbency, emaciated/poorly conditioned mouse (BC1)

Note: In the description column, the Boolean operator between items is "OR."

[a]Conjunctival erythema is expected to occur in all grades of xGvHD.

TABLE 4 Xenogeneic graft-versus-host disease (xGvHD) scoring system.

Parameter	Description	Score
Activity and general appearance	Normal activity and appearance	0
	Reduced activity, paleness, muscle tremor, conjunctival erythema	1
	Lethargy (no walking unless being touched), dyspnea, dehydrated, cyanosis	2
Posture	Normal	0
	Hunching at rest	1
	Hunching impairs movement	2
Reflexes and neuropathic features	Normal ambulation, normal reflexes	0
	Mildly reduced reflexes, slow gait, constipation	1
	Remarkably reduced reflexes, ataxia, hindlimb paralysis, urinary retention	2
Body condition	BC3	0
	BC2	1
	BC1	2
Fur texture	Normal	0
	Mild-to-moderate coat ruffling	1
	Severe coat ruffling	2
Skin integrity	Normal	0
	Erosive/scaling dermatitis	1
	Ulcerative/crusting dermatitis, reduced skin elasticity	2
Alopecia	None	0
	Local (patchy)	1
	Multiple (diffuse)	2

Note: Because, the maximum score in each category is 2, the highest possible xGvHD score for a mouse is 14. In the description column, the Boolean operator between items is "OR."

so, if clinical status of a mouse is evaluated and reported properly based on Table 1, determining the status of that mouse based on Tables 2–4 is easily feasible and straightforward.

5 | DISCUSSION

The poor translatability of preclinical research is a major problem and a tough challenge in the development pipeline of novel anticancer drugs,[77–79] which is partly due to the confounding effects of unwanted disorders (comorbidities) in the tumor-bearing animals compromising the validity of preclinical research.[80,81] In well-designed, scientifically sound experimental research on cancer models, the health and well-being of the animals should be carefully monitored.[62] Under ordinary conditions, the real effects and safety issues of an investigational treatment or a personalized therapy can be preclinically revealed using valid animal models. However, if an unwanted disorder occurs, it may hamper xenograft tumor engraftment or cause unexpected tumor shrinkage or reduce animal lifespan and, above all, confound the data, which therefore make the analyses erratic and create misleading results. Therefore, with lack of reliability and low reproducibility, such studies are inevitably a waste of medical resources.[82,83] In this review, a catalogue of potentially unwanted disorders in cancer models (with a focus on symptomatology) was discussed. Such disorders are recommended to be considered, and if noticed being recorded in the animal care (animal monitoring) log, as it is imperative to avoid the loss and misuse of valuable CDX/PDX models.[29] Moreover, a checklist of minimal information on clinical status of experimental mice (Table 1) and three grading scales (Tables 2–4) have been recommended for calibrating preclinical cancer research and promoting reproducibility in the use of tumor models. Although regular recording of these signs and unwanted disorders may seem challenging in a large-scale study, ignoring them may lead to study failure making the whole project futile. Nonetheless, when a laboratory operator becomes used to notice and note these signs and perform such evaluations, this may not add a significant time to their laboratory workload (in contrast, negligence that leads to repeating a project will definitely cause a greater workload).[64] If an unwanted disease is diagnosed in a group of experimental immunodeficient mouse models with the potential of confounding the research objectives and outputs, researchers can halt the research to observe ethical principles of 3Rs and to prevent waste of funding resources. For when these confounding factors cannot be prevented, documenting these unwanted disorders is necessary in preclinical

phase, as by removing their effects in statistical analyses, the actual efficacy of an investigational drug be explored.[80]

Although there are other signs and disorders affecting laboratory mice,[64] it should be noted that we compiled a list of common disorders, with a focus on what potentially confounds research on xenograft-bearing immunodeficient mice. Moreover, we concentrated on the disorders that can be observed or identified via routine clinical examinations, whereas there are other disorders with no apparent or minimal symptoms that can be diagnosed after necropsy, presenting with histopathologic changes in internal organs. For instance, cataract may occur in aging mice or after prolonged xGvHD.[59,84] Furthermore, in xenograft models with limited ascites and/or organomegaly, which both can be grossly witnessed after necropsy, assessment of body condition scoring that appraises muscle wasting is superior to body weight and carcass weight, as there is unmeasurable added weight by the ascitic fluid or the enlargement of an organ. It is important to note that the xGvHD grading and scoring systems recommended in this paper consider xGvHD symptomology that can be easily assessed with routine clinical examinations, while there might be clinically invisible internal pathologies. For example, lymphadenopathy, which is not included in the grading and scoring systems, may only be identified after necropsy, especially if it affects the tracheobronchial, lumbo-aortic, and renal lymph nodes in PDX models having xGvHD.[44,45] Mice involved with lymphoproliferative disorders, as well as leukemia and lymphoma xenograft-bearing models, may also develop lymphadenopathy.

Although the pathogen-induced unwanted disorders (discussed in Sections 2 and 3.2) can be prevented by devising laboratory SOPs concerning strict pathogen control, preimplantation pathogen testing and biosafety measures, other noninfectious disorders may or may not have a feasible solution to be averted. To avoid the development of age-related, aging-related, and strain-specific disorders that generally appear at older ages (most commonly mice >9 months old),[85,86] and compromise the internal validity of research; young mice (8–12 weeks old as the optimal age) are recommended to be used for cancer model generation.[86,87] To prevent spontaneous xGvHD, lymphodepleting methods of xenografts and use of β2m knocked-out strains lacking MHC class I expression have been recommended. To prevent iatrogenic xGvHD, studies exploring the minimum dose of immune effector cells concerning xGvHD induction should be performed for each species. That is to say, in preclinical studies, a cell dose below the minimum xGvHD-inducing dose of immune effector cells or the dose inducing mild (tolerable) xGvHD should be administered, if development of moderate to severe xGvHD in the models is undesirable. Besides, in case of unwanted noninfectious disorders in cancer models, if a feasible solution is not available, the confounding effects should be estimated through various statistical methods and are recommended to be removed through multivariable analyses.

In conclusion, preclinical studies using xenograft-bearing immunodeficient animals may be confounded by disorders (comorbidities) that undesirably develop in such models, resulting in undermining the internal and external validity of research. Therefore, researchers and laboratory technicians should primarily consider measures to prevent the development of such unwanted disorders and should carefully monitor health and clinical status of the models to avert study failure or loss of translatability of findings. In this review, we aimed to compile the clinical presentations of common unwanted disorders. Determining true nature and cause of the condition by only inspecting visible signs is often impossible, as discovering the exact underlying etiology requires post-investigation necropsy and subsequent histopathological and molecular testing in the vast majority of cases. Therefore, and more importantly, it has to be reminded that many conditions discussed in this paper only add to the list of differential diagnoses, and thus should be evaluated in an individual mouse in the context of the intervention it underwent, its age and genetic background, and clinical status of its cage-mates and the colony (in overall) to approach the exact cause.

AUTHOR CONTRIBUTIONS

Conceptualization: SMM, SM; Methodology: SMM, SM, NA; Investigation: All authors; Data curation: SMM, SM, HA; Visualization: SMM; Validation: SM, SMM, NA; Writing – original draft: SMM, SM, VM; Writing – review & editing: All authors; Supervision: SM, NA.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Not applicable in this article, as no new dataset was generated in this study.

ETHICS APPROVAL

Animal Welfare and Ethics Committee approved this research and discussed the implications for research.

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