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Stocking the Toolbox – Using Preclinical Models to Understand the Development and Treatment of Immune Checkpoint Inhibitor-induced Immune-Related Adverse Events

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Summary

Cancer patients treated with immune checkpoint inhibitors (ICIs) are susceptible to a broad and variable array of immune-related adverse events (irAEs). With increasing clinical use of ICIs, defining the mechanism for irAE development is more critical than ever. However, it currently is challenging to predict when these irAEs occur and which organ may be affected, and for many of the more severe irAEs inaccessibility to the tissue site hampers mechanistic insight. This lack of understanding of irAE development in the clinical setting emphasizes the need for greater use of preclinical models that allow for improved prediction of biomarkers for ICI-initiated irAEs or that validate treatment options that inhibit irAEs without hampering the anti-tumor immune response. Here, we discuss the utility of preclinical models, ranging from exploring databases to *in vivo* animal models, focusing on where they are most useful and where they could be improved.

Keywords

immune checkpoint inhibitor; immune-related adverse events; autoimmunity; preclinical model; mouse

Structural Damage – the Clinical Impact of Immune Checkpoint Inhibitor (ICI)-induced Toxicity.

Immune checkpoint inhibitors (ICIs), such as clinically approved monoclonal antibodies directed towards programmed death-1 (PD-1)/PD-ligand 1 (PD-L1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and lymphocyte-activation gene 3 (LAG-3), continue to expand their clinical use in the cancer setting (Figure 1)[1,2]. In addition to being employed as standard of care, first-line treatment interventions for many advanced malignancies, ICIs are now also being utilized in early-stage disease, as well as in the neoadjuvant treatment setting, and in a younger patient population[3–6].

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One of the major impediments for the clinical success of ICI treatment, is the risk for developing immune-related adverse events (irAEs). These irAEs can impact all organ systems, with variable timing, frequency, and severity, which causes difficulty in both clinical surveillance and treatment[7]. In addition, a proportion of irAEs can cause hospitalization, and discontinuation of potentially cancer-curing ICI treatment, leading to worse survival outcomes for cancer patients[8,9].

Tissue-specific irAE profiles have emerged, relating to both the ICI target and type of cancer for which a patient is receiving treatment (Figure 2). For example, anti-CTLA-4 preferentially induces hypophysitis and gastrointestinal irAEs such as colitis and diarrhea, while autoimmune diabetes predominantly occurs following exposure to anti-PD-1/PD-L1 treatment[10–13]. Different mechanisms have been suggested for the development of these and other irAEs, including the disruption of unique immunomodulatory receptor-ligand interactions that lead to a breakdown in tolerance, or the potential for complement-initiated cell killing both of which may be caused by exposure to specific ICIs[14]. In contrast, vitiligo, which is an autoimmune attack towards melanocytes leading to depigmentation, occurs predominantly in melanoma patients due to epitope-spreading between the cancer and site of irAE[15]. For many irAEs, both the frequency and severity are amplified, and disease onset accelerated by combining multiple non-redundant immune-based therapeutic targets[1,2]. While some irAEs may be effectively treated by using broad-acting immunosuppressive agents such as corticosteroids, for others, additional lines of treatment, such as cytokine inhibitors, or long-term maintenance of hormone replacement may be necessary.

Understanding who is at risk for irAEs as well as developing targeted treatment strategies to inhibit irAEs without impacting anti-tumor immunity will be essential for improving the care of ICI-treated cancer patients. To do this, mechanistic insight both in the periphery and at the irAE-affected tissue site is needed to identify alterations in immune composition and immunomodulatory pathways that can form robust biomarkers for irAE susceptibility or novel targets to limit immune-mediated damage. As ICI treatment can initiate a broad spectrum of irAEs, the need for a diverse range of preclinical models to expose and analyze multiple aspects of the immune response that replicate clinical observations is necessary, and the utility of current modeling modalities will be explored here.

Selecting the Right Tool for the Job – the Spectrum of Preclinical Models for irAEs.

The breadth of preclinical models may be intimidating when considering which system best reflects the clinical phenomena and accurately captures the etiology for the development of tissue-specific irAEs. Growing use of *in silico* modeling with published datasets, *in vitro* organoid or antigen-reactive systems, *in vivo* mouse and non-human primate models as well as canine patients all play a role in identifying features of and mechanisms for the development of ICI-induced irAEs (Figure 3). Yet, each system has unique advantages and challenges that relate to the implementation of their use for the study of irAEs.

In silico data analysis

With increasing high-throughput data of the immune composition for cancer patients treated with ICIs, this also represents an opportunity to build more robust cohorts to ascertain predictive information relating to ICI-initiated irAEs. Concerted efforts to improve data-sharing and annotation of cancer patients from individual datasets have been growing, although there remains a number of technical, ethical, and intellectual concerns to the implementation of this process[16,17]. To date, in the setting of examining ICI treatment efficacy, there has been an inability to distill mechanistic insight from clinical trials or real-world cohorts to redefine biomarkers for therapeutic response that provide additive value to the presence of PD-L1 in the tumor or the tumor mutational burden (TMB). However, there is increasing emphasis on developing platforms that synthesize readily available datasets to test novel immune signatures that correlate with therapeutic response across multiple distinct ICI-treated cancer cohorts. For instance, the Immuno-Oncology SIGnatures explorer (http://iosig.tanlab.org/) combines response information from over 2,500 patients in a form that can be interrogated freely by other users to compare their signals of interest in additional cancer types and patient cohorts, both preceding or on ICI treatment.

The development of similar databases for irAEs will be critically important, however, will require increased annotation relating to the timing, grade, and treatment interventions that were employed to boyh monitor and attenuate the toxicity. This type of resource will be particularly important in cases where the irAE is rare, as combining datasets will assist in increasing the number of events and help to improve the resolution of mechanisms related to tissue-specific irAEs. In initial studies that have describe the immune response related to irAEs, patients have often been categorized based solely on irAE severity[18,19]. While this may yield differences relating to the overall inflammatory status and may be reflective of a practical constraint relating to the division of patient groups while maintaining meaningful numbers to study, it is likely that more tailored studies that interrogate the association between immune cell composition and function with tissue-specific irAEs will yield a more robust mechanistic analysis. In a comparison of immune cell composition across different irAE-affected organs, it was shown that at baseline ICI-treated cancer patients who developed pneumonitis displayed an enrichment of T helper (Th)2 cells, whereas thyroiditis was associated with increased interleukin (IL)-17-producing Th17 cells in the periphery[20]. This highlights that distinct mechanisms are likely to occur for irAE development in separate tissues, which may not be identified in a non-tissue targeted classification of toxicity. It will also be critical to differentiate tissue-specific immune responses that are initiated by individual or combination ICI treatments, to define potential treatment options for each toxicity and to accurately shape the clinical care of patients.

Combining multiple immune parameters from the periphery, tumor, and when available irAE affected-tissue will assist in providing a holistic overview of toxicity-associated immune responses. Sampling from peripheral blood remains most accessible, particularly for longitudinal assessments, but it is unclear how reflective these changes are to the irAE site. Since patients with irAEs are also often afforded better anti-tumor immunity, it is likely that information from the tumor microenvironment (TME) itself may also reveal differences in systemic immune activation that could underscore the potential for

irAE development. In a multiomic study of various immune indicators that have been previously related to the development of irAEs as well as responsiveness to anti-PD-1/PD-L1, independent of cancer type, $CD8^+$ T cell abundance alongside either the presence of naïve B cells or an individual's T cell receptor (TCR) diversity provided the best combination of predictive parameters for irAEs[21]. This illustrates the additive value that interpreting multiple immune components in unison can provide, with greater sensitivity and specificity for immunotoxicity. In addition to identifying T cell clonality, understanding the antigen recognized by tissue-destructive T cells will help to define new methods to detect irAE risk for ICI-treated cancer patients. For ICI-induced myocarditis, reactivity towards alpha-myosin was shown in both preclinical mouse models and ICI-treated cancer patients experiencing this irAE, highlighting the tissue specificity and autoreactive nature of the T cell response[22]. As datasets relating to the TCR repertoire of irAE patients grow, using analytical tools such as grouping of lymphocyte interactions by paratope hotspots (GLIPH)[23] and Mc-PAS-TCR[24] will assist in defining overlap between pathogenic T cells, including distinguishing TCR repertoire homology between ICI-induced and spontaneous autoimmune responses with clinical overlap. Developing an understanding of the specificity for immune interactions that cause irAEs may lead to new screening methodologies and treatment options to tolerize against autoreactive responses.

In vitro systems

Cell culture systems are becoming increasingly advanced in both their ability to utilize patient samples, and to bring together multiple cell types to reconstruct and examine diverse but coordinated cellular responses. Three-dimensional (3D) cultures that maintain structural integrity consistent with an individual's tumor, such as in patient-derived tumor organoids, have enabled a more sophisticated understanding of personalized treatment approaches that relate to the tumor landscape for an individual cancer patient. These 3D *in vitro* systems have been especially useful for considering responses to targeted therapies that aim to directly inhibit cancer cell survival and to determine mechanisms of tumor recurrence in a heterogeneous tumor setting. However, examining interactions between the tumor and immune response in unison remains difficult as replicating the complexity of the environmental cues as well as sustaining all the diverse, but in some cases rare, cell types is extremely difficult. Two approaches that have been utilized to mimic the tumor microenvironment (TME) involve either dissociating an intact tumor alongside its immune infiltrate prior to reassembling it or using a tumor suspension to form organoids and substituting an abundant source of autologous immune cells, such as from peripheral blood, to reestablish the immune infiltrate[25]. While the former can cause a loss of cellular heterogeneity through the digestion process, the latter can also lead to differences in reconstitution due to differential immune cell survival, polarization, and entry into the tumor organoid, all of which may require additional vasculature, and supportive structures to allow for adequate formation. However, persistence in establishing these intricate culture systems is needed as it provides a unique opportunity to look at patient-derived responses and cellular interactions using limited tissue requirements from the tumor source.

Organoid development is not restricted to examining cellular interactions within the tumor, with increasing interest in the formation of a broad range of patient-derived 3D tissue

structures that recapitulate both healthy and diseased tissue. Many irAE affected tissue sites have been replicated as organoids derived from both adult and induced pluripotent stem cells, including the colon, liver, thyroid, lung, pancreas, and heart[26]. The ability to develop additional organ structures derived from a patient, which importantly retains the genetic identity from an individual alongside matched immune cells, will allow for examination of organ-specific antigen presentation and immune regulation in the presence of a immune cell pool containing a potentially autoreactive T cell response. Treating immune-infiltrated tumor organoids with ICIs has previously revealed dose-dependent therapeutic sensitivity to nivolumab (anti-PD-1) through increased presence of $CD8^+$ T cells and decreased organoid size due to tumor cell death[27]. Similar studies in tissue-specific organoids will potentially assist in understanding changes to immune cell composition in response to ICI treatment that leads to irAEs. It may also provide an opportunity to further understand cross-reactivity of the immune response between the tumor and irAE affected tissue sites, by examining whether there is a similar expansion of T cell clonotypes that establish immune reactivity in both the tissue and tumor organoids. Importantly, access to many irAE susceptible organs is rare, and by recreating immune-infiltrated tissue structures from an irAE-affected individual, it may provide an optimal condition to understand the phenotype and expansion of T cells in these organs. As these *in vitro* systems develop, it may also provide a screening platform to define at-risk tissues for irAEs in response to individual ICIs and to understand regulatory mechanisms that these treatments interfere with, which will help to define the tolerability of single-agent and combination treatment options for cancer patients.

In vivo systems

Mouse models—Mouse models are a stalwart system for complex biological assessments, with their use particularly valuable when undertaking studies that involve the immune response. While syngeneic mouse tumor models have been routinely used to identify the efficacy of ICI treatment either alone or in combination with additional novel therapeutic agents, many of these tumor-bearing systems are performed in mice insensitive to the development of autoimmunity and that consequently lack the ability to accurately model the complexity of irAEs[28]. Therefore, to assess the interface between ICI treatment and irAE development a transition towards using mice primed towards heightened immune activation either by genetic susceptibility to autoimmunity or chemically induced organ-damage has allowed for greater understanding of the genetic, cellular, and molecular mechanisms that contribute to irAEs. Notably, a variety of *in vivo* approaches may be necessary to dissect the mechanism by which individual tissue-specific irAEs occur, with diverse model selection critically important to provide understanding of therapeutic outcomes (Table 1).

Autoimmune-prone mice—Given the rationale that a proportion of irAEs are provoked by disruption of immune tolerance in individuals with subclinical autoimmunity, the use of autoimmune-prone mice to uncover mechanisms of ICI-induced toxicity is of great interest. One of the main mouse strains that has been used to assess ICI-induced irAEs is the non-obese diabetic (NOD) mice. These mice are prone to developing multiple forms of spontaneous autoimmunity, with an emphasis on type 1 diabetes (T1D), many of which can be accelerated in response to ICI treatment[29,30]. Of note, targeting the PD-1/PD-L1 axis in NOD mice rapidly induces ICI-initiated diabetes, whereas anti-CTLA-4 only causes

loss of immune tolerance towards the insulin-expressing β cells when administered to young mice prior to the appearance of immune infiltrate in the islets[30,31]. For the small proportion of cancer patients that develop ICI-induced autoimmune diabetes, this requires at a minimum exposure to anti-PD-1/PD-L1 treatment, highlighting the consistency between the mouse models and clinical observations[10,13]. Mechanistically, it is thought that this may be due to PD-L1 expression in islets engaging PD-1 in T cells to act as a critical regulator of inflammation to limit pancreatic damage, with PD-L1 expression shown to positively correlate with the frequency of immune infiltrate in the islet[32,33]. Additionally other clinically relevant irAEs can be observed in response to ICI treatment within the NOD model. Thyroid dysfunction occurring in the NOD mice following exposure to combination anti-PD-1 and anti-CTLA-4 display increased IL-17-producing cells, and antibody-directed neutralization of IL-17 was able to abrogate thyroid dysfunction in these mice[34]. As highlighted previously, Th17 cells have been identified to be enriched in the periphery of cancer patients that develop ICI-induced thyroid disorders, suggesting the need to evaluate the use of IL-17 inhibitors in cancer patients exhibiting abnormal thyroid function following ICI treatment[20]. Therapies targeting the IL-17 pathway (secukinumab, ixekizumab, and brodalumab) have been FDA approved to treat psoriatic conditions, with secukinumab also having been used in the treatment of skin irAEs, with reports of varying impact to the anti-tumor immune response[35]. This highlights the connectivity between clinical observations and preclinical autoimmune-prone mouse models as a tool to interrogate ICI-driven immune mechanisms of tissue destruction and to identify potential treatment options that may abrogate irAEs.

Additional autoimmune-prone mouse strains have also been utilized to interrogate irAEs. In particular, the Murphy Roths Large (MRL) strain with the lymphoproliferation spontaneous mutation (*Fas*^{lpr}) has been shown to precipitate irAEs across multiple organs[36]. While both the MRL^{lpr} and NOD mice rely on underlying genetic predisposition for spontaneous autoimmunity, genetic manipulation of immunomodulatory pathways in C57BL/6 and BALB/c mouse strains that are traditionally resistant to autoimmunity has also provided understanding for the role these molecules play in regulating immune tolerance. For instance, mice with CTLA-4-deficiency rapidly succumb to severe lymphoproliferative disease, whereas milder autoimmune phenotypes were observed in mice with a PD-1-deficiency[37–40]. This is consistent with observations relating to irAEs in ICI-treated cancer patients, in which ipilimumab (anti-CTLA-4) is considered to have a worse safety profile with higher frequency of severe adverse events that are grade 3 or higher compared to PD-1-targeting therapies[41]. Modulating the expression of these molecules through different genetic combinations also provides unique observations relating to toxicity. For instance, CTLA-4 hemizygosity combined with complete PD-1-deficiency was shown to initiate myocarditis in C57BL/6 mice[42]. Haploinsufficiency of CTLA-4 in humans has previously been related to certain disease outcomes, but this is the first instance in mouse models in which partial loss of CTLA-4 has shown a deleterious outcome. Differentiating disease pathologies that develop from altering immunoregulatory pathways with unique combinations of genetic deficiencies, or that incorporate cell- or timing-specific conditional mutations, represent important tools that provide insight into potential clinical problems due to the expanding range of therapeutic combinations that include ICI treatment.

In addition to studying irAEs in the setting of genetic predisposition, skewing the balance of systemic immunity through cellular depletion can also help to sensitize autoimmune-resistant mouse strains to the development of ICI-induced toxicities. Using transient depletion of immunosuppressive T regulatory cells (Tregs) in C57BL/6 and BALB/c mice causes a fluctuation in pro-inflammatory cytokine responses and broad changes to the immune cell composition in both the periphery, organs, and tumor[43]. Many of these immune changes lead to a heightened state for autoimmune responses exacerbated by ICI treatment, leading to the potential for systemic organ damage like those observed in ICI-treated cancer patients. Using this system, it was identified that antibody agonism of the costimulatory receptor 4-1BB (CD137) was able to initiate a broader range of irAEs with greater severity than anti-PD-1 blockade, even though they displayed equivalent anti-tumor immunity[43]. These findings have also been reflected clinically, as early-stage clinical trials of a 4-1BB agonist (urelumab) were halted due to severe hepatotoxicity[44]. While modulation of the balance of the systemic immunity in autoimmune-resistant mouse strains allows for interrogation of both the development and treatment of irAEs alongside the use of syngeneic tumors, the depletion of Tregs in humans has not been observed in response to ICI treatment[45]. This provides an important consideration when developing models to evaluate clinical concerns, as often there is incomplete overlap with the disease course observed in patients, which highlights the need for multiple preclinical model systems to be employed to define consistent patterns that define tissue-specific toxicity. While an individual's loss of Treg numbers or function reflects an event that may heighten vulnerability to the precipitation of irAEs, it would also be interesting to examine the contribution of other immune cell types by either their depletion or expansion to understand their role in maintaining the balance against ICI-induced immunotoxicity. This may be particularly important as different therapeutic regimens to modulate the numbers and function of cellular components are being designed to optimize the immune response towards the tumor but could also lead to a detrimental pro-inflammatory immune interaction in tissues. This showcases the gamut of modeling systems that involve modifications in genetic control and cellular responses that are necessary to interrogate the interplay between different immune responses that contribute to the mechanism for immunotoxicity.

Chemically induced toxicity in mice—Chemical induction of organ-specific tissue damage in preclinical mouse models may assist in understanding whether changes in the immune microenvironment due to inflammatory conditions can be exacerbated by ICI treatment. Many cancer patients have underlying chronic diseases of varying severity and it remains unclear whether the degree of tissue damage correlates with susceptibility to irAEs. Sensitizing specific tissue sites to toxicity via exposure to chemicals offers some flexibility in relation to the experimental structure that is not necessarily available in autoimmune-prone mice. For instance, it allows for the use of multiple mouse strains, which may display different levels of sensitivity to chemically-induced tissue damage, which may help to emulate differences in susceptibility to irAEs due to underlying genetic differences that contribute to differences in the immune response of cancer patients. Mouse strains, such as C57BL/6 or Balb/c mice, are also able to be used in combination with certain forms of chemical toxicity, and are adept at modeling changes in immune function in multiple immune-mediated diseases due to the tools available. Unlike in many autoimmune-prone

mouse strains that rely on genetic predisposition, chemical induction of toxicity in mice has the advantage that it often can be initiated at different stages of development, including after the immune response has matured and immune homeostasis has been established. This may be consistent with a proportion of organ damage that is observed in adult cancer patients that has been caused due to lifestyle or environmental exposure. Additionally, the timing of initiation and the duration of exposure to chemical toxicity can be modified relative to ICI exposure to reflect both acute and chronic damage.

One of the limitations of using chemical toxicities to heighten risk for irAEs, is that it may lead to restricted organ-specific damage to be exacerbated by ICI treatment. In contrast, autoimmune-prone mice often have the potential to develop a variety of irAEs dependent on the immunomodulatory drug that they are exposed to and whether this disrupts in immune tolerance across different organs, consistent with the clinically diverse combinations of irAEs that are reported. For instance, C57BL/6 mice exposed to dextran sulfate sodium (DSS) to initiate colon inflammation, display increased weight loss and histologic colonic damage when exposed to combination anti-PD-1 and anti-CTLA-4 treatment[46]. Similarly, anti-PD-1 treatment in mice with dietary obesity display greater skin inflammation in areas exposed to topical imiquimod, a chemical used to induce a disease that resembles psoriasiform dermatitis, compared to control-treated mice[47]. For these examples in which ICI treatment enhanced toxicity in the setting of chemically mediated organ damage, additional toxicities were not reported. This demonstrates that chemical-induced toxicity can be a powerful tool to identify tissue-specific immune responses that are exploited to promote irAEs in response to ICI treatment.

Humanized mouse systems—Development of humanized mouse models has been a priority to enable improved understanding of immune responses that recapitulate clinical conditions. Many of these models involve either engraftment of differentiated immune cells, such as from peripheral blood mononuclear cells (PBMCs), or pluripotent stem cells into an immunodeficient mouse to reconstitute the hematopoietic compartment. One of the major limitations of mouse models bearing human cells is the risk for graft versus host disease (GvHD). In fact, in immunodeficient mice humanized by PBMC engraftment there was accelerated multi-organ GvHD due to exposure of nivolumab[48] or combination ipilimumab and nivolumab[46]. In an alternate method of humanization, NOD-severe combined immunodeficiency (*scid*) mice with a mutation in the IL-2 receptor common gamma chain (NOG) mice receiving human bone marrow, liver, and thymus to assist in tolerizing the immune response to mouse organs displayed severe autoimmune responses following treatment with nivolumab, many of which resembled patterns of irAE pathologies[49]. The benefits of this process include the use of the identical drug that is administered within the clinical setting, and ability to assess immune infiltrate from humans that have different cellular compositions. However, the lack of lymph nodes in immunodeficient mice, which are an important reservoir for activation of ICI-driven tumor immune infiltrate, must also be considered when using humanized mice. The immune response for generating humanized animals can either be initiated using a pre-screened donor that has enrichment of certain characteristics or unbiased engraftment, optimally the systems can evolve to use patient donors to assess whether there is consistency between the

humanized mouse setting and clinical outcomes. Additionally with greater understanding of the genetic elements that predispose individuals to irAEs it will be of interest to investigate whether modifying these target genes can amplify irAE risk through altered antigen presentation or immune regulation in the humanized preclinical mouse setting. This highlights a challenge and opportunity in these humanized models, to coordinate broadly diverse characteristics of the human immune response to better understand differences that dictate therapeutic outcomes to ICI treatment.

Rather than humanizing mice by immune reconstitution, selecting certain gene targets to be humanized can also provide an avenue to further understand the signaling that occurs with the equivalent therapies used in humans. For instance, C57BL/6 mice that have a humanized version of the *CTLA4* gene display development of multiple irAEs in response to ipilimumab, with both severity and frequency of irAEs increased with combination treatment with a mouse anti-PD-1 agent[50]. This system was used to identify that an alternate CTLA-4-targeting antibody was able to prevent the severity of irAEs observed with ipilimumab, while still generating an equivalent anti-tumor immune response. Interestingly, to generate a broad range of irAEs, it was necessary to treat young mice with a humanized CTLA-4 with ipilimumab as older mice were less susceptible to developing irAEs[50]. This has similarly been observed in NOD mice, where anti-CTLA-4 can promote T1D only when treated in young mice that have not yet developed islet-specific immune infiltrate[31]. This suggests that CTLA-4 is likely to be more critical in controlling immune homeostasis, with potentially additional immunoregulatory molecules able to control immune infiltrate after it enters the tissue. It will be of interest to see whether this phenomenon is maintained by assessing other humanized immunoregulatory receptors, and whether differences in the irAE profiles that can be observed in younger mice are also apparent in younger patient populations as they begin to be exposed to ICI treatment.

Non-human Primates—While mice are a powerful tool to examine mechanistic interactions between therapeutic modalities and immune-mediated diseases, differences in the cellular repertoire and regulation of the immune system can make it challenging to transition from preclinical studies to clinical utility via first-in-human trials. As described above, humanized mice aim to create a bridge to examine some of these interactions, but also display several challenges relating to the assessment of functionality and toxicity initiated by immunomodulatory agents that have yet to be well addressed. To overcome this obstacle, the activity of many promising therapeutics are often investigated in non-human primates, with a particular emphasis on the use of cynomolgus monkeys (*Macaca fascicularis*). The high degree of genetic similarity between non-human primates and humans leads to homology of many immunomodulatory receptors, which often enables the therapeutic agent destined for clinical use to be tested in pharmacokinetic and toxicology studies, whereas in non-humanized mice a therapeutic surrogate is employed. Dose-escalation studies performed in cynomolgus monkeys revealed excellent tolerability of both nivolumab and pembrolizumab (anti-PD-1) alone[51,52]. However, when nivolumab was given alongside ipilimumab a marked increase in multi-organ lymphoproliferation, including gastrointestinal toxicity and myocarditis was observed across multiple studies[53,54], consistent with the heightened toxicity demonstrated when combining these non-redundant

immunotherapies in cancer patients[1]. Interestingly, nivolumab was able to be tolerated without obvious adverse events in the monkey model at a dose 20 times higher than that employed in the treatment of cancer patients[51]. This demonstrates the need to understand the effects of additional immune stimuli, including interactions with the tumor microenvironment (TME) and immune-shaping events that predispose cancer patients to increased susceptibility to irAEs.

Canine patients—The emergence of immune-targeting drug options for canine patients provides multiple relevant opportunities to inform on the clinical expectations for human patients. Unlike the mouse and non-human primate cohorts, but similarly to humans, dogs receiving immunotherapies are genetically diverse and have been exposed to a broad range of environmental stimuli that shape differential immune responses. In many cases, as dogs are close companions to humans these environmental stimuli, particularly surrounding exercise, microbiome, and pollution, may be strongly aligned. Additionally, canine patients are also susceptible to the development of multiple spontaneous cancers, which unlike most mouse models used to assess the efficacy of ICI treatment, have had an extended period of development under pressure from the immune system through the process of cancer immunoediting[55]. Dogs also have a different profile of frequently occurring cancers to humans, which could create an alternate source to examine difficult to treat and rare cancers, such as sarcomas and mucosal melanoma for which immune-targeting treatment options with curative potential have remained elusive in human patients {[56]}. As increasing canine-reactive drugs become available, as well as tools to interrogate the immune response, this may allow for improved evaluation of differences in the TME that correlate with treatment efficacy, while also examining the risk for irAE development. This may also provide an opportunity to revisit clinical options that have failed in humans to identify TME's that are most receptive to a particular treatment approach, or the coordination of a hierarchy of treatment combinations based on the TME versus irAE risk.

In a clinical study of canine anti-PD-1 treatment across a variety of dog breeds, over half of the animals developed mild treatment-related adverse events, with a much lower frequency of severe grade 3 or higher irAEs observed in dogs compared to human cancer patients[57]. This may be due to underreporting and insufficient screening of clinical markers for toxicity that are performed routinely as part of clinical care for cancer patients. Notably, many of the tumor-bearing dogs did not respond to treatment, with most tumors displaying progressive disease[57]. A similar profile of therapeutic response alongside a well-tolerated safety profile was similarly observed using a chimeric anti-PD-L1 agent in dogs[58]. Nonetheless, a small proportion of dogs did respond, including reduction in the size of metastatic lesions, which highlights that an immune response can be successfully mounted in dogs to fight cancer. This is particularly impressive with many of these dogs receiving multiple lines of conventional treatments (including surgery, chemotherapy, and irradiation) before initiation of ICI treatment. It is possible that the level of toxicity will increase with therapeutic combinations designed to promote greater anti-tumor immunity or by initiating ICI treatments earlier in the therapeutic regimen before conventional therapies have been exhausted.

Of interest, one of the dogs treated with anti-PD-1 developed severe pneumonitis, which was corticosteroid-refractory leading to death[57]. Understanding the mechanism for irAE resistance to corticosteroids will be essential for identifying which alternate immunosuppressive agents could provide greatest treatment efficacy. This type of study is often difficult to perform in the clinical setting as patients receiving critical care for severe toxicities are often unable to provide consent for research. As the irAE profile in ICI-treated dogs continues to evolve, it will be of interest, when possible, to assess irAE-affected tissue for immune composition, which may be performed by biopsy during ongoing treatment or, if the canine companion unfortunately succumbs to either the cancer or irAE, at autopsy. Rapid autopsy programs for cancer patients have been largely difficult to establish, due to the cost involved, particularly for transportation if a patient is away from an academic medical center at the time of death, and sensitivity surrounding the discussion of the subject with both patients and their families. However, for dogs, discussions relating to euthanization due to impacted quality of life are common with this process being performed in a veterinarian clinic or academic comparative medicine facility. This could lead to an opportunity to provide tissue and tumor samples to research efforts to improve the management of both therapeutic response and toxicity.

Only as Good as your Tools – Assessing Anti-tumor Immunity in Models of irAEs.

While various preclinical systems can be employed to investigate mechanistic features relating to irAEs, it is important to generate appropriate tumor models that allow for the interrogation of anti-tumor immunity together with immunotoxicity in response to ICI treatment. Evidence that tumor-bearing mice are more susceptible to ICI-initiated toxicity also emphasizes the need for paralleled assessment of the immune response[59,60]. Therefore, significant effort in designing robust tumor models that can be used to examine both host immune responses and tumor-directed immunity, are necessary to mimic clinical conditions.

Clinical connectivity between anti-tumor immunity and irAEs

Growing clinical reports suggest that the development of irAEs correlates with improved survival benefit for cancer patients receiving ICI treatment[8,61]. At a more granular level, it appears that the organ involved in the irAE may play an important role in dictating the survival advantage a patient may receive. Endocrine irAEs, such as hypophysitis and thyroid dysfunction, have been shown to improve survival for ICI-treated cancer patients, however the mechanism for this connectivity between toxicity and response remains unclear and should be explored in preclinical models[11,61]. In contrast, a proportion of clinically-observed irAEs occur due to antigen-sharing with the tumor. For example, cross-reactivity between malignant and healthy melanocytes leads to vitiligo in ICI-treated melanoma patients, with these patients often displaying an excellent therapeutic response[15]. While it is important to consider the survival bias associated with the development of irAEs, in that patients who respond to ICI treatment have longer exposure to these therapies providing a greater risk for irAE occurrence, the consistency in these patterns of improved survival across multiple treatment and cancer types emphasizes this striking clinical phenomenon. This highlights the need to assess the relationship between ICI-initiated irAEs and anti-tumor immunity

to determine whether they are provoked by similar or distinct immune changes that either pre-exist or evolve in response to ICI treatment.

Tumors in autoimmune-prone mice

As previously highlighted, developing robust transplantable, syngeneic tumors for use in many of the autoimmune-prone model systems used to exacerbate the immune response for studying irAEs remains a significant challenge. However, multiple groups, including ours, are in the process of investing resources into their development[34,62]. Additionally, developing tumors in autoimmune-prone mice provides an opportunity to understand whether underlying autoimmunity modulates tumor immunogenicity through cancer immunoediting, which could influence ICI treatment outcomes. In some cases, tumors engineered to be deficient in machinery relating to antigen presentation have been transplanted into mismatched autoimmune-prone mouse strains. Tumors derived from C57BL/6 mice, including MC38.β2m$^{-/-}$ colon adenocarcinoma and B16.β2m$^{-/-}$ melanoma, were transplanted into autoimmune-prone NOD mice receiving ICI treatment to assess the impact of anti-IL-17A treatment towards both irAEs and anti-tumor immunity[63]. Notably, anti-IL-17A was able to protect against the development of thyroid irAEs, while maintaining the robust tumor control provided by combination anti-PD-1 and anti-CTLA-4 treatment[34]. While this allows for the coordinated assessment of anti-tumor immunity and autoimmunity in response to ICI treatment alongside an anti-irAE therapy, it remains unclear whether using a tumor generated in an alternate mouse strain with defective antigen presentation is an appropriate system for monitoring immune-mediated responses reflective of clinical conditions. Some cancer patients do appear to develop tumors that are β2m-deficient and this has been associated with ICI therapeutic resistance[64]. Nonetheless, growth of β2m-deficient tumor cells in autoimmune-prone NOD mice revealed that the irAE treatment did not appear to be deleterious to tumor control in this setting.

For mouse strains with tumor systems broadly available, changes to systemic immune activation that allow for exploration of irAEs may make it difficult for tumors to either establish or display progressive growth, which could hamper the interrogation of ICI responses. Systems which allow for delayed initiation of irAEs in mouse strains that are otherwise autoimmune-resistant can help to interrogate the impact of immunotoxicity in an established tumor setting. Transient Treg depletion in tumor-bearing C57BL/6 and BALB/c autoimmune-resistant mice heightens the risk for irAEs from ICI and other immunomodulatory treatments and can be used to monitor whether therapies that inhibit irAEs disrupt anti-tumor immunity. An agonistic antibody directed towards CD40, which is a stimulatory receptor that drives immune activation in the TME at least in part through the production of IL-12 by myeloid cells, was able to promote worse toxicity in combination with Treg depletion, which could be impeded by anti-tumor necrosis factor (TNF) but not anti-IL-6R[60,65]. However, if anti-TNF was administered at the same time as anti-CD40 it led to an attenuation of anti-tumor immunity[60]. This loss of tumor control could be overcome if anti-TNF was delayed and administered only when a flare in the autoimmune response was already detected, where it could protect against irAEs without amplifying tumor growth[66]. This highlights the intricate studies that can be performed to discern outcomes for anti-tumor immunity, autoimmunity, and treatments directed towards both immune-mediated responses.

Tumors in humanized mice

Humanized mice are complex systems that are susceptible to developing multiorgan tissue damage in the presence of ICI treatment[49]. By successfully transplanting patient-derived tumor xenografts or human cancer cell lines into humanized mice it is possible to examine the diversity in the immune repertoire that interacts with both the TME and pathological tissue sites, simultaneously. One example of this was the use of the HT29 colon cancer cell line in immunodeficient mice reconstituted with PBMCs that were susceptible to multiple ICI-initiated irAEs[46]. The tumor progressed sufficiently to identify that combination nivolumab and ipilimumab was able to reduce tumor growth and that an irAE-protective treatment, etanercept (TNFR2-IgG), decreased the severity of liver and colon toxicity without disrupting the anti-tumor immune response from ICI treatment[46]. However, PBMCs were engrafted seven days after tumors were established, which coincided with the initiation of both the ICI and anti-irAE treatment. While this experimental design may be necessary to facilitate the short timeframe before the development of GvHD from PBMC engraftment, the acuity of the immune response, in which severe toxicity would rapidly develop with or without ICI treatment, may create a disconnect with the clinical observations relating to irAEs.

Mice humanized with $CD34^+$ hematopoietic stem cells can be used for extended periods without evidence of overt toxicities, including in the presence of tumors. An analysis of tumor-bearing $CD34^+$ humanized mice revealed that diversity in immune infiltrate present within the TME was driven by the tumor cell line rather than $CD34^+$ donor, consistent with differences in immune reactivity towards tumors that can be observed in cancer patients[67]. Using the same humanized mouse system, human tumor cell lines displayed differential therapeutic sensitivity to atezolizumab (anti-PD-L1), which appeared to be related to the presence of T cell infiltrate and the inflammatory status of the tumor[67]. Preceding treatment, the presence of different tumor types also dictated the composition of the immune milieu in the spleen, which highlights the influence of the TME in eliciting immune changes in the periphery. While this study did not mention whether immunotoxicities were observed in response to ICI treatment, this could create an opportunity to interrogate the connectivity between immune recognition of the tumor modulating organ-related immune infiltrate and the ability to exacerbate systemic irAEs.

Extending tumor growth to examine irAE risk

Multiple infusions of ICI treatment are often administered to cancer patients, and while some irAEs have predictable timing, others have a broad spectrum for when they may occur across the treatment course. For instance, autoimmune diabetes can occur years after initiation of ICI treatment without any additional exposure to stimuli that are likely to precipitate its development[13]. This indicates that investing in tumor models in mice predisposed to irAEs, which allow for an extended period of interaction with the immune system may be critical in examining therapeutic outcomes relating to both irAEs and tumor control. In a previous study, C57BL/6 mice that underwent Treg depletion to reduce B16F10 melanoma tumor burden displayed multiple tumor growth phenotypes, including robust tumor growth, tumor rejection, and delayed tumor escape after a period of stable disease. Notably, mice that exhibited tumor suppression without cure, displayed the greatest risk for

developing autoimmune vitiligo[68]. Treating mice that displayed strong tumor suppression with ICIs as their tumors escape would be of interest to examine whether there was not only a regression in tumor growth, but also the development of additional irAEs given the appearance of vitiligo in a largely autoimmune-resistant strain. The persistent immune response towards tumor antigens during a robust equilibrium phase, in which tumor growth is stalled due to the pressure maintained by the immune system through cancer immunoediting[55], may be more representative of clinical responses to tumor and could highlight the type of interaction that modulates the frequency of irAEs. The ability to also coordinate genetically engineered mouse (GEM) models, in which cancer is initiated by a distinct mutational driver, or carcinogen-induced tumors in autoimmune-prone mice would also assist in identifying the impact of heightened immune activation from autoimmunity in changing tumor immunogenicity and the consequences to ICI-induced immunotoxicity and treatment response. However, this type of intricate modeling requires significant support in terms of both time and monetary costs, but may reveal distinct treatment opportunities that should be considered for a growing group of ICI-treated cancer patients, those with underlying autoimmune disease.

Stopping the Rot – Inhibiting ICI-induced Immunotoxicity.

The largest area of investigation in preclinical models that represent clinically relevant irAEs, is to use these tools to identify new therapeutic strategies to inhibit toxicity without impacting anti-tumor immunity[28]. However, a major translational limitation for treatments that have the potential to ameliorate irAEs has been the inability to simultaneously define the impact towards the anti-tumor immune response, which as described above is becoming increasingly accessible. Developing immunocompetent models, in which both the host and tumor can be modified, will assist in interrogating either genetic or pharmacological approaches to inhibit irAEs[28]. Growing numbers of therapeutic strategies to inhibit conventional forms of autoimmunity are entering clinical utility and understanding whether they can safely transition towards treating irAEs that impact similar organs is of interest. In addition, the timing of when these interventions are employed relative to the initiation of ICI treatment for anti-tumor immunity may be relevant, and modeling approaches are necessary to investigate these interactions.

Corticosteroids

For the majority of irAEs that develop in the clinic, corticosteroids are administered as the first-line immunosuppressive agent to broadly dampen the deleterious immune response initiated by ICI treatment. Although initial reports relating to the impact of corticosteroids towards anti-tumor immunity in terms of therapeutic response and survival outcomes suggested limited attenuation of tumor growth, increasing evidence suggests that this may have been confounded by the superior therapeutic efficacy that is afforded to cancer patients that experience irAEs. Established ICI-sensitive MC38 colon adenocarcinoma tumors grown in C57Bl/6^{lpr} mice were treated with anti-PD-1 and anti-CTLA-4 followed by prednisone. The introduction of corticosteroids reduced tumor control while also decreasing multiorgan inflammation[36]. Consistent with this, melanoma patients that developed ICI-induced hypophysitis receiving high-dose corticosteroid treatment displayed worse survival

outcomes compared with individuals receiving low-dose corticosteroids at concentrations representative of physiological replacement[11]. In addition, there may be a timing element as to when corticosteroids interfere with anti-tumor immunity most profoundly, as patients treated with these immunosuppressive agents within two months of initiating ICI treatment have decreased survival benefit compared to patients where corticosteroids were initiated two months after ICI treatment[69]. Nonetheless, understanding the mechanism for tissue-site specific irAE development will assist in uncovering new therapeutic strategies with greater specificity and selectivity to limit irAEs while preserving anti-tumor immunity.

Cytokine inhibitors

Cytokine inhibitors have been increasingly relied upon in the treatment of spontaneous autoimmune and autoinflammatory diseases, which has led to them being repurposed for use in irAEs. Most commonly cytokine inhibitors have been deployed as a secondary treatment option in severe or corticosteroid-refractory cases of immunotoxicity, however, there are also clinical trials that assess their concurrent use alongside ICI treatment as a potential combinatorial partner to improve survival outcomes[35]. The main pathways to be targeted in irAEs include classic markers of inflammation such as TNF-α (infliximab), IL-6 (tocilizumab), and IL-17A (secukinumab)[35]. Interestingly, for each of these cytokine inhibitors, there is also preclinical evidence relating to their ability to inhibit tissue-specific irAEs, including colitis, neuroinflammatory diseases, thyroiditis, either in the context of chemically induced toxicity in autoimmune-resistant mice or autoimmune-prone mice without impacting anti-tumor immunity[34,46,70].

The pleiotropic nature by which cytokines elicit their effects requires an intricate understanding of the direct and indirect signaling of cellular processes in a tissue-specific manner. In particular, understanding the immune response to cytokines in the tumor versus the site of irAEs may identify strategies that skew the immune balance favorably to promote anti-tumor immunity and protection against irAEs. Patients experiencing thyroid irAEs were revealed to have a robust $CD4^+$ T cell infiltrate consisting of IL-21-expressing T follicular helper (T_{FH}) or T peripheral helper (T_{PH}) cells in fine needle aspirates (FNA) taken from the thyroid. Increased polarization of these IL-21-producing $CD4^+$ T cells was able to drive clonal expansion of cytotoxic $CD8^+$ T cells that could elicit thyroid damage in ICI-treated mice, an effect that was lost in mice deficient for IL-21 signaling[63]. The effect towards anti-tumor immunity was not examined in the presence of defective IL-21 signaling, however, it has previously been shown that IL-21 may decrease ICI treatment efficacy and enhance tumor growth, suggesting that neutralizing IL-21 may both promote anti-tumor immunity and decrease irAEs under select therapeutic conditions[71].

Recombinant proteins and protein inhibitors

Assessing the most amenable timing for administering irAE-preventative therapies also requires careful consideration and might identify strategies that can be given transiently to promote tissue-protective responses without interfering with long-term anti-tumor immunity. In a recent study of an IL-2 cytokine-antibody fusion protein with the capacity to specifically enhance Treg expansion, it was shown that a short, prophylactic treatment preceding initiation of anti-PD-1 was sufficient to significantly reduce the development

of anti-PD-1-induced diabetes[72]. This is likely due to bolstering antigen-specific Tregs to limit islet damage within the pancreas prior to initiation of ICI treatment, with extended treatment this therapy may also start to promote pro-inflammatory responses and skew the immune balance in the organ towards being less protective. As we learn more about patient cohorts and can create risk matrices for toxicity relative to the benefit gained towards tumor immunity from ICI treatment, based on appropriate genetic, cellular, and molecular biomarkers, there may be an opportunity to implement prophylactic approaches to mitigate irAEs to ensure that patients minimize their potential for toxicity.

Novel fusion proteins that target molecules aside from cytokines also have the ability to inhibit irAEs. Abatacept (CTLA-4-Ig), designed to engage CD80 and CD86 and divert the ability to initiate co-stimulation of T cells via CD28, was first clinically approved for the treatment of rheumatoid arthritis in 2005 and more recently as a preventative treatment for GvHD in 2021. In mice that are prone to the development of myocarditis, due to CTLA-4 hemizygosity and PD-1 deficiency, giving abatacept significantly prolonged survival and reduced immune infiltrate into the heart over time[42]. Impressively, and in accordance with these preclinical findings, in a large case series of ICI-treated patients that developed myocarditis those treated with abatacept showed significantly improved survival from this irAE, which has a high-rate of morbidity and mortality[73]. Expanding the use of abatacept to other irAEs, particularly those that are induced more frequently by anti-CTLA-4 therapies to limit the potent immune activation that has been bolstered from CTLA-4 blockade, or arthritic irAEs that show consistency with the spontaneous autoimmune disorders for which abatacept is approved could also be of interest.

The immunosuppressive effects of abatacept appeared to be further enhanced with use of a Janus Kinase (JAK)1/2 inhibitor, ruxolitinib. Like abatacept, ruxolitinib has received FDA approval for treating indications not associated with irAEs, including multiple forms of myelofibrosis and both acute and chronic GvHD. Bulk RNA-sequencing of cardiac tissue from preclinical mouse models of ICI-induced myocarditis demonstrated that JAK2 itself and its related downstream pathways were significantly upregulated, which highlights the rationale for combining abatacept with ruxolitinib to minimize inflammatory responses in the heart[73]. JAK1/2 inhibitors have also been shown to have protective capacity against other irAEs, including both protection and reversal against the development of anti-PD-L1 induced autoimmune diabetes in NOD mice[74]. Given the short timeframe to irAE development following anti-PD-L1 treatment, the ability to rescue islet function with JAK1/2 inhibition in mice that display heightened blood glucose is impressive. However, in mesothelioma tumor-bearing Balb/c mice, which were not reported to display irAEs, there appeared to be diminished ICI-mediated anti-tumor immunity with concurrent, but not delayed use of JAK1/2 inhibitors[74]. Clinically, there appears to be a prodrome, in which slight elevation of blood glucose can be observed before precipitation of fulminant autoimmune diabetes in ICI-treated cancer patients[10]. Given that ICI-induced diabetes is also common late in the ICI treatment course after robust tumor control has been established, it is possible that JAK1/2 inhibitors could be deployed in cancer patients with elevated blood glucose to salvage their islet function, without impeding tumor control.

While repurposing clinically approved drugs for the treatment of irAEs creates a somewhat streamlined transition to clinical utility. There are several inflammatory processes that may be specific to some, but not all, irAEs that should be considered to resolve these complex diseases. Targeting induction of the CD24-danger-associated molecular patterns (DAMP) pathway with a CD24-Fc, has been shown to provide protection against multiple irAEs in humanized mouse models[75]. While not significant, there was also a trend that CD24-Fc combined effectively with ICI treatment to reduce tumor growth and extend survival. Adding to the safety profile relating to this therapeutic compound, patients hospitalized due to COVID-19 that were treated with a CD24-Fc showed clinical improvement[76]. This highlights that CD24-Fc may be an approach that is more suitable to dampen broad cytokine-mediated autoinflammatory responses rather than tissue-specific autoimmunity. Given the ability of CD24-Fc to improve clinical outcomes for hospitalized COVID-19 patients that are often receiving corticosteroids, the use of CD24-Fc should also be considered as an additional immunosuppressive agent for at a minimum cancer patients with corticosteroid-refractory irAEs.

Cellular therapies

Developing targeted cellular therapies to modulate the immune response is increasing in multiple immune-mediated diseases, with a particular interest in cancer and autoimmunity. The clinical success of CD19-targeting chimeric antigen receptor (CAR) T cells in the treatment of hematologic malignancies has spearheaded the acceleration of these approaches. Alternatively, engineering patient-derived T cells to express a tumor-specific TCR, such as Melanoma Antigen Recognized by T cells (MART)-1 in melanoma or New York esophageal squamous cell carcinoma 1 (NY-ESO-1) in antigen-expressing tumors, is also being assessed clinically for its therapeutic utility[77,78]. In autoimmunity, developing immunosuppressive Tregs that recognize a tissue-specific cognate antigen or an environmental factor associated to the inflammatory response are being considered as viable options to restore tolerance. Early-stage clinical trials using human leukocyte antigen (HLA)-A2-directed CAR Tregs are being performed in transplantation through recognition of a non-self HLA-A2 protein from a mismatched transplanted organ in an HLA-A2-deficient recipient (NCT04817774, NCT05234190). This tolerogenic treatment option will ideally prevent transplant rejection and eliminate the need for immunosuppressive agents, for which long-term usage is known to increase the risk of cancer and infection. Alternatively, the presence of post-translational modifications that differentiate healthy proteins in inflamed organs has also been identified as a CAR Treg target. In rheumatoid arthritis, the accumulation of citrullinated vimentin in the joint has led to the development of CAR Tregs that aim to migrate and initiate immunosuppression directly at the inflammatory site without engaging with additional cellular components in the environment[79]. One advantage of this process is that the cellular machinery can be engineered to engage a diverse range of targets through the CAR or TCR selected to be expressed, alongside adjusting the payload that is delivered by these cells to be optimal for the immune environment[80]. However, the barriers for this process relate to selection of a highly-specific molecule that does not promote off-target effects, due to recognition of inappropriate organs or engagement in conditions of low cellular affinity[81]. This will require detailed

understanding of the mechanisms by which irAEs are induced to define dysregulated protein expression that may be targeted by cellular therapies.

Remodeling the Interior – Assessing the Impact of Multiple Immune Stimuli towards irAEs.

A distinct advantage of preclinical models, compared to utilizing clinical trial or real-world data sources, is the opportunity for mechanistic insight relating to the effects of additional immunomodulating stimuli. By using a layered approach, we can start to assess whether a variety of different immune alterations either preceding, coinciding with the initiation of, or following ICI exposure impact therapeutic response and irAE development. These can involve things that an individual cannot modify, such as genetics, sex, and age, as well as environmental exposures relating to location and lifestyle, which together could heighten or subdue the immune response leading to irAEs. Understanding these interactions through complex multi-dimensional modeling systems will help to influence clinical decision-making (Figure 4).

Host Genetics

As patient cohorts grow, and their therapeutic outcomes relating to both response and toxicity mature, the relationship between genetic susceptibility and irAEs continues to be further explored. In some cases, genetic determinants that have previously been related to risk for spontaneous autoimmunity have been shown to also be consistently observed in irAEs. For instance, an enrichment in HLA-DR4 was observed in ICI-induced autoimmune diabetes, which is also associated with conventional type 1 diabetes (T1D)[13]. Alternatively, in ICI-induced hypophysitis HLA-DQ0602 appears to be enriched, whereas HLA-DQ8 and DR53 are seen to be the dominant HLA type in the sporadic form of the disease, illustrating potentially divergent genetic susceptibility and mechanisms of antigen presentation for pathologies directed to the same organ[12]. Determining additional genetic variants associated with irAEs by genome-wide association study (GWAS) may assist in uncovering other genomic alterations that impact immune function. In ICI-treated patients with a diverse range of cancer types, development of severe irAEs independent of tissue-type was related to modifications to the *IL7* gene, leading to alterations in lymphocyte stability and activity[82,83]. When possible, mimicking these genetic changes in preclinical models, which in mouse strains is becoming increasingly possible through CRISPR/Cas9 gene-editing, could assist in further defining the effect of genetic alterations towards irAEs.

Variability in susceptibility of different mouse strains to irAEs has also provided a unique opportunity to understand the potential for genetic variation to modify the immune response to ICI treatment. Enhancing the utility of the NOD mouse strain is the intricate understanding of genes that underpin predisposition to spontaneous autoimmunity that may also contribute to irAEs. Genetic mapping of loci that exacerbate T1D, referred to as insulin-dependent diabetes (*IDD*) loci, has elucidated multiple genes that confer resistance to the development of T1D, many of which relate to immune function[32]. In response to anti-PD-L1, it was shown that the *IDD9* loci, which contains a series of genes related to TNF signaling, was protective against the development of ICI-induced autoimmune

diabetes[84]. For a small proportion of cancer patients, disrupting the PD-1/PD-L1 axis was able to induce autoimmune diabetes even in individuals that had HLA types that are strongly protective against spontaneous T1D[85]. Mouse models that interrogate changes to the selection and expansion of antigen-specific T cells due to polymorphisms in the major histocompatibility (MHC) II gene, I-A^{g7}, in NOD mice are also protected from T1D[86]. Examining whether disrupting immune checkpoints in NOD and other mouse strains that are resistant to autoimmunity due to genetic modifications, will help to expose their role in contributing to a threshold for immune tolerance that may be unveiled by ICI treatment.

Collaborative Cross (CC) mouse strains also represent a unique tool that could be leveraged to investigate the influence of genetic diversity towards modulating therapeutic outcomes relating towards the development of ICI-induced toxicity. These mice form a reproducible panel of recombinant inbred mice derived from 8 founder strains that are largely autoimmune-resistant (C57BL/6, 129S1, A/J), autoimmune-sensitive [NOD, New Zealand Obese (NZO)], and inbred wild (CAST/EiJ, PWK/PhJ, and WSB/EiJ) mouse strains. The use of the subsequent offspring that arise from mixing these diverse genetic backgrounds allows for identification of genetic associations with disease outcomes. This provides an alternate opportunity to examine the impact of genetic heterogeneity towards complex traits in a more scalable approach than the numbers that would be needed for GWAS studies for tissue-specific irAEs. CC mice have also shown promise in answering increasingly complex, multidisciplinary research questions, such as identifying that behavioral outcomes in mice are dependent on both the interaction with their genetics and microbiota[87] or identifying genetic variants that provide immune protection to vaccines[88]. This highlights both the complexity of and need for diverse preclinical modeling strategies that incorporate the influence of multiple immunomodulatory stimuli, underpinned by genetic changes, to recapitulate the complexity of the immune response that initiates irAEs in cancer patients receiving ICI treatment.

Sex

Emerging evidence suggests that females may have worse outcomes to ICI treatment due to poor visibility of tumors in part due to insufficient antigen presentation that fails to promote anti-tumor immunity in response to ICI treatment[89–91]. Furthermore, female mice were shown to have increased B16 melanoma burden of experimental lung metastasis compared to male mice, however, this protection that was associated to sex-bias was lost in the setting of castration and appeared to be testosterone-dependent[92]. In contrast, the presence of androgen receptor signaling in male mice with prostate cancer was shown to disable T cell function and ICI efficacy, which was reversed by combining anti-PD-L1 with androgen deprivation therapy and enzalutamide (an androgen receptor inhibitor)[93]. These differences in ICI responsiveness towards tumor also indicate that there may be variability in the immune response that drives irAEs due to sex-dependent differences. While many conventional forms of autoimmunity display a sex-bias directed towards higher prevalence of disease being observed in females, to date a sex association with irAEs has not been robustly reported[94]. This may be in part confounded by the fact that males may display better therapeutic responses, which is associated with both extended treatment periods and irAE development. Therefore, teasing out the interplay between sex hormones, as well as

other sex-specific events such as pregnancy, through both pharmacological and physical deprivation of sex hormone sensing should be performed to understand the impact towards irAE-mediated immune reactivity to ICI treatment.

Age

While ICI treatment appears to be well-tolerated clinically spanning a broad range of age groups, from pediatrics to older adults, it is unclear but possible that different irAE profiles could emerge with age. Aged tumor-bearing C57BL/6 and BALB/c mice treated with anti-PD-1/PD-L1 showed greater infiltration of lymphocyte aggregates in tissues compared to those of young mice[95]. This appeared to be due to $CD4^+$-mediated production of IL-21, which heightened B cell infiltration and immunoglobulin deposition, and could be overcome by both IL-21 receptor-deficiency or B cell depletion[95]. Similarly, in response to a combination of immunomodulatory agents, including IL-2 alongside either an agonistic anti-CD40 or immunostimulatory CpG, aged mice displayed greater morbidity and mortality compared to young mice due to an enhanced cytokine-mediated inflammatory response[96]. Accumulation of visceral adiposity in aged mice contributed to the development of immunotoxicity, as these deleterious immune responses were suppressed in mice who underwent caloric restriction[96]. Unlike autoimmune-prone mouse strains that display alterations in immune homeostasis from an early age, chemical induction has the advantage that it can be initiated at different stages of mouse development and will be a useful tool to examine the effect of age in initiating irAEs. Understanding modifications to the immune response that are acquired with age, particularly relating to changes in the diversity of the T cell repertoire and accumulation of antigen-specific T cells, will be of interest to define their impact towards irAE both phenotypically and mechanistically.

Microbiome

Microbial composition has been increasingly identified as a key modulator of the immune response to ICI treatment. Consistent with this, disrupting the commensal microbiota through antibiotic administration preceding initiation of ICI treatment in cancer patients has been shown to reduce anti-tumor immunity and survival benefit[97,98]. Given that the presence of irAEs is also associated with response, it is likely that changing the microbiome can also influence the development of irAEs in response to ICI treatment. However, in a study which discerned the bacterial associations with treatment response and severe immunotoxicity, there was minimal overlap between these therapeutic outcomes. For ICI-treated melanoma patients that developed severe toxicity, a higher abundance of *Bacteroides intestinalis* was identified[99]. Colonization of *B. intestinalis* in melanoma tumor-bearing C57BL/6 mice following antibiotic treatment also heightened gut toxicity through enhanced IL-1 signaling, which could be suppressed by antibody blockade of the IL-1 receptor[99]. Additionally, an intact gut microbiome, was shown to amplify liver toxicity in response to either anti-CD40 or anti-CD137, with antibiotics or the use of germ-free mice able to minimize the effect[66]. Unlike clinical reports for ICI treatment, ablating irAEs through microbial depletion did not appear to impact anti-tumor immunity initiated by anti-CD40 or anti-CD137 either with or without anti-PD-1 treatment, indicative that antibiotics could be given alongside this combination in difficult to treat tumor settings to minimize the risk for irAEs.

While most studies have assessed the influence of changes to gut microbiota towards the development of irAEs, understanding the impact of microbial colonization in alternate organs or the tumor also requires further consideration. Bacteria are ubiquitously found in regions of the TME, and their presence has been shown to coincide with heightened immunosuppression, which is consistent with increased expression of immune checkpoints, such as PD-1 and CTLA-4[100]. It is possible that ICI treatment will unleash an immune response towards the cancer as well as bacteria residing intratumorally, and this could create both a source for neoantigens and cross-reactivity at other sites dependent on the distribution in other organs. By assessing the microbial profile across multiple tissues in a clinical setting, we can start to isolate strains of interest to be tested in preclinical mouse models, such as germ-free mice or in the presence of antibiotic depletion, the latter of which is more comparable with clinical conditions, to determine the effects of microbial dysbiosis. Alternatively, mouse models that are exposed to a diverse range of microbes from the environment, including pathogenic microbes that are excluded from specific-pathogen-free animal facilities, such as the use of cohoused pet shop mice with strains of interest can also be helpful for understanding differences in microbial composition and the effect towards ICI-initiated irAEs.

Diet

Consistent with the ability of the microbiome to modulate both anti-tumor immunity and irAEs in response to ICI treatment, it is likely that diet will also contribute to critically altering these therapeutic outcomes through both direct and indirect mechanisms. Examining the influence of different dietary components towards irAEs is best performed in mouse models where the diet is easily monitored and manipulable. To date, investigations of preclinical mouse models have shown evidence that salt content and dietary obesity is able to modulate the risk of irAEs, predominantly by bolstering cytokine production that then causes deleterious immune activation towards tissues[47,101]. Diet-induced obesity in mice has been shown to cause increased tumor growth without treatment, with mixed evidence relating to its ability to promote improved responsiveness, which is consistent with clinical data that has indicated a mixed relationship between BMI and survival outcome[102,103]. In contrast, ketogenic diets have been suggested to amplify ICI-induced tumor control through T cell-dependent activation of ketone bodies in mouse models that are resistant to ICI treatment, without identification of the impact towards irAEs[104]. This suggests that ICI-treated cancer patients may be at high risk for exacerbating irAEs with reduced benefit towards their tumor, dependent on diet.

The use of a wide range of supplements in cancer patients is not uncommon, however, in addition to their purported benefits may be changes to immune function that could alter treatment response to ICIs. The use of supplements is somewhat easier to define than the composition of an individual’s diet, which may allow for integration of epidemiological data from real-world cohorts alongside preclinical studies to improve our mechanistic understanding for whether supplements modulate irAE frequency and severity. Self-reporting of Vitamin D supplementation in a retrospective cohort was shown to be protective to the development of colitis, which has similarly been observed in spontaneous gastrointestinal autoimmunity such as Crohn’s disease and ulcerative colitis[105]. Whether this

impacts additional irAEs, or causes suppression specifically in the gut will be of interest to explore preclinically. This may also indicate that it could be necessary to screen for vitamin D concentrations in the peripheral blood to use it as a prophylactic agent to protect against gastrointestinal toxicity, particularly in patients receiving CTLA-4 inhibitors. Additionally, a growing range of supplements that provide improved maintenance of muscle tone is being investigated for their ability to improve Eastern Cooperative Oncology Group (ECOG) performance status score, which is an assessment of a patient's independent function, and has been associated with improved survival outcomes to ICI treatment[106]. Creatine supplementation has entered clinical trials to potentially improve and prolong patient fitness during exposure to multiple lines of cancer treatment. Importantly, this supplement has also been shown to augment anti-tumor immunity by enhancing $CD8^+$ T cells in mouse tumor models and combining effectively with ICI treatment[107]. Whether this also modifies outcomes to irAEs, or whether the diverse range of supplements available or additional modulators of dietary outcomes such as exercise and lifestyle mediators can promote differences in the immune response is necessary.

Comorbidities

Cancer is a common occurrence in patients with multiple comorbidities including pre-existing autoimmunity, infection, as well as chronic or metabolic disorders, all of which can heighten inflammation and require long-term medication[108–110]. Many of these inflammatory conditions were initially considered contraindications for the use of ICI treatment due to the risk for promoting or exacerbating irAEs. However, the cancer-curing potential of ICIs outweighs the risk for morbidity and mortality in these patient populations and has led to cautious inclusion of these drugs in patients with pre-existing immune-mediated diseases[111]. While it may be possible to dissect the interaction between the presence of different comorbidities and their associated treatments with the risk for developing irAEs through analysis of real-world patient cohorts, preclinical models will be needed to further define the mechanism by which comorbidities alter therapeutic outcomes and lead to exacerbation of irAEs in response to ICI treatment as well as to develop additional strategies to alleviate these safety concerns.

Already in preclinical mouse models, some comorbidities have been assessed for their influence towards anti-tumor immunity. Expanding on these studies to define their impact towards irAEs will be a necessary next step. Development of non-alcoholic steatohepatitis (NASH) in both humans and mice is a risk factor for hepatocellular carcinoma (HCC). The prevalence of NASH is increased in individuals with obesity, high cholesterol, and type 2 diabetes, indicative of a group of comorbidities which are unfortunately common in cancer patients. While it was shown that NASH-induced HCC worsens the response to anti-PD-1 treatment in patients, and similarly in mice PD-1 limits $CD8^+$ T cells from driving progression to HCC, the interaction between NASH and irAE outcomes was not defined[112]. NASH causes tissue damage and scarring in the liver, therefore it is likely that anti-PD-1 may exacerbate cells drawn to the tissue site and amplify a deleterious response, as well as accelerate tumor growth.

Treatment options continue to rapidly improve for a broad range of diseases, however the ability to define whether unrelated, but well-tolerated drug combinations interact to alter therapeutic efficacy and safety is often difficult to assess clinically and should be prioritized for modeling potential negative outcomes. Aspirin is used in a broad range of indications, such as pain management, inflammation, and a blood thinner to reduce heart attack and stroke, but can also heighten the risk of gastrointestinal bleeding, highlighting the need for careful consideration of its use in a combinatorial setting. Excitement about targeting the cyclooxygenase pathway to drive anti-tumor immunity has led to clinical trials being performed in combination with ICI treatment (NCT03396952, NCT03728179, NCT03638297)[113,114]. In NCT03396952, which was a study of advanced melanoma treated with high-dose aspirin, pembrolizumab (anti-PD-1) and ipilimumab, perhaps predictably aspirin heightened the risk of gastrointestinal irAEs, such as diarrhea and colitis, but also led to a surprising increase in hypophysitis (https://clinicaltrials.gov/ct2/show/results/NCT03396952 - Study Results for NCT03396952). Assessing this combination in not only autoimmune-resistant but autoimmune-prone mouse strains may have aided risk identification for irAEs. It may also be necessary to collect a broad array of tissues and profiles of blood chemistry in preclinical mouse models to define potential tissues that may be most problematic.

Tumor involvement

In cancer patients, certain tumor-related features have been shown to impact the therapeutic response to ICI treatment. This includes the tumor mutational burden (TMB), with cancer types with higher TMB afforded greater survival benefit[115]. This has led to the approval of ICI use in a cancer-agnostic setting where patients have tumors with either high TMB, and/or in which the tumor is deficient in DNA mismatch repair genes, leading to microsatellite instability, which correlates with greater therapeutic response. Consistent with the relationship between both irAEs or TMB and survival benefit from ICI treatment, a correlation between these good prognostic indicators has also been identified[116]. It is unclear whether connectivity is due to the potential increase of neoantigens within the TME causing heightened immune activation or due to cross-reactivity with self-tissue, such as in the case of anti-melanoma immune reactivity and skin depigmentation in the form of vitiligo or the expansion of intratumoral muscle-specific T cells and the connectivity with myocarditis[15,22]. Using preclinical models, it would be of interest to modify syngeneic tumors in mice either by enriching with specific tissue-associated antigens to determine whether the ICI response in the tumor generates a tissue-directed irAE response in an autoimmune-resistant setting, or whether increasing the TMB through exposure to methods of mutagenesis may heighten irAE risk.

In addition to the neoantigen load of the tumor, the site in which metastases are present can also impact ICI treatment efficacy. For melanoma and non-small cell lung cancer (NSCLC) patients, liver metastases reduced the survival benefit provided by ICI treatment, which correlated with less $CD8^+$ T cells within the tumor[117]. Importantly, this clinical finding was also able to be modeled using a syngeneic tumor system in C57BL/6 mice, in which paired tumors located in the liver and subcutaneous setting caused reduced responsiveness to ICI treatment[118]. This was found to be due to an antigen-dependent mechanism, in which Tregs

required targeting in order to overcome liver-mediated immunosuppression against tumors treated with anti-PD-1[118]. Whether similar effects from differences in tumor-bearing site, including the location of metastasis, can change the interaction with the systemic immune response and therefore the development of irAEs remains unknown. However, indications that the primary tumor site can play a role in amplifying the risk of certain irAEs is apparent, for instance pneumonitis is more common in lung cancer patients, but it remains unclear whether this is due to additional immune destruction within that organ due to an overzealous immune response or due to antigen recognition through immune cross-reactivity.

Under Construction – Enhancing Preclinical Modeling to Mimic Clinical Responses

Developing model systems for irAEs has lagged in comparison to efforts to understand clinically beneficial therapeutic combinations that promote anti-tumor immunity. This is in part due to ICI treatment being initially deployed in cancers with extremely poor survival rates, leading to the risk for toxicity being considered acceptable given the potential therapeutic benefit. However, as both the landscape of the cancer patient population being treated with these therapies evolves alongside the availability of multiple immunomodulatory treatments, a re-evaluation of risk versus reward requires further exploration. For instance, using ICIs in indications that are already being treated with curative intent, highlights the need for more selectivity in who is exposed to these treatments to prevent the development of severe or non-resolving autoimmunity without providing additional efficacy relating to the tumor response. Here, we have defined the available model systems and possible scenarios that may be employed to be most informative for replicating clinical conditions. Understanding therapeutic strategies that promote anti-tumor immunity without initiating irAEs or that inhibit irAEs without disrupting anti-tumor immunity by modulating both the host and tumor immune environments will be essential to improve patient outcomes. These models will need to continually evolve as we learn more about the heterogeneity of patient populations, even for those with clinically similar irAEs, as well as underlying immune signatures that dictate irAE susceptibility. It will also be necessary to ensure that the preclinical models mimic the breadth of diverse immunomodulatory conditions observed in cancer patients to improve both phenotypic and mechanistic understanding of irAEs and their associated treatment.

As the motivation to combine ICI treatment with other conventional and targeted therapies grows, mindfulness about the timing and order of treatment regimens will be critical and the use of preclinical models will be invaluable to evaluate potential clinically relevant changes to the frequency and severity of immunotoxicities. Similarly, within the clinic, deciding when to discontinue ICIs in patients that are responding to treatment remains a complex decision performed on a case-by-case basis. It is often unclear whether longer treatment duration is better, especially in cases where patients have had a rapid, complete response, or whether continual treatment may heighten the risk for toxicity. Using preclinical models to examine whether the persistent or transient pressure of modulating immunoregulatory pathways through ICI treatment alongside other changes to immunomodulatory stimuli that may impact the long-term risk of irAE development will be a critical endeavor. It is

also evident that the blanket approach to dampening immune activation in irAEs, through treatments such as corticosteroids or cytokine inhibitors, may lead to tumor escape. This emphasizes the need for localized drug activity, which may be accomplished by multi-valent therapies that are drawn to a specific site either due to recognition of a tissue-specific protein or an inflammatory gradient will be necessary to heighten specificity and sensitivity of therapeutic effects.

Finally, considering these clinical challenges, but also the growing rate of knowledge relating to the characterization of the response related to immune-mediated diseases, there will likely be opportunities for personalized immune-based treatments for both cancer and autoimmunity. Preclinical models will play an essential role in supporting these findings and transitioning a broad range of therapeutic modalities to clinical utility due to superior safety and efficacy.

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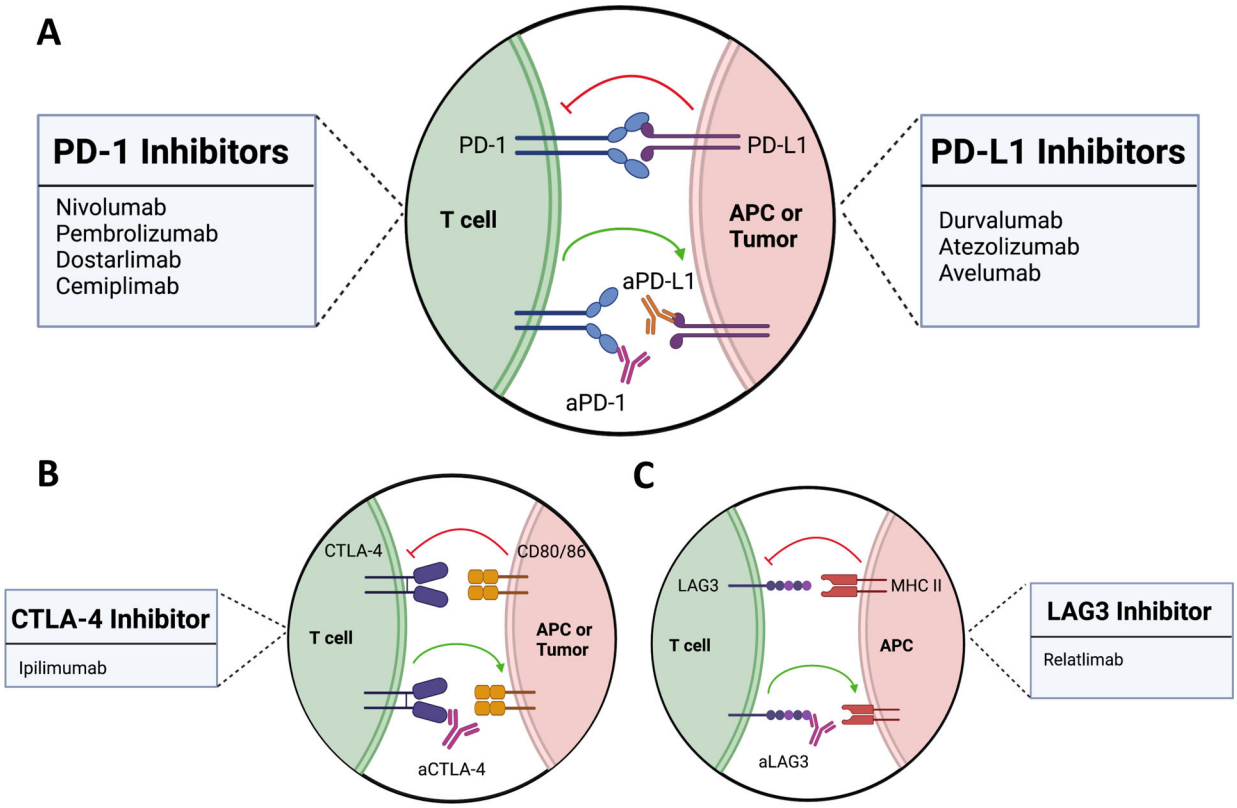

Figure 1. Immune checkpoint inhibitors enhance immune activation.

Clinically approved immune checkpoint inhibitors targeting either **(A)** PD-1, PD-L1, **(B)** CTLA-4, and **(C)** LAG-3 are highlighted. When these molecules are engaged in the tumor microenvironment or in the periphery to their respective ligands they display reduced activation, which is detrimental to tumor control but is able to protect against tissue damage. By giving immune checkpoint inhibitors (ICIs), there is a reinvigoration of the immune response, leading to in some cases improved anti-tumor immunity, but also the risk for the development of immune-related adverse events.

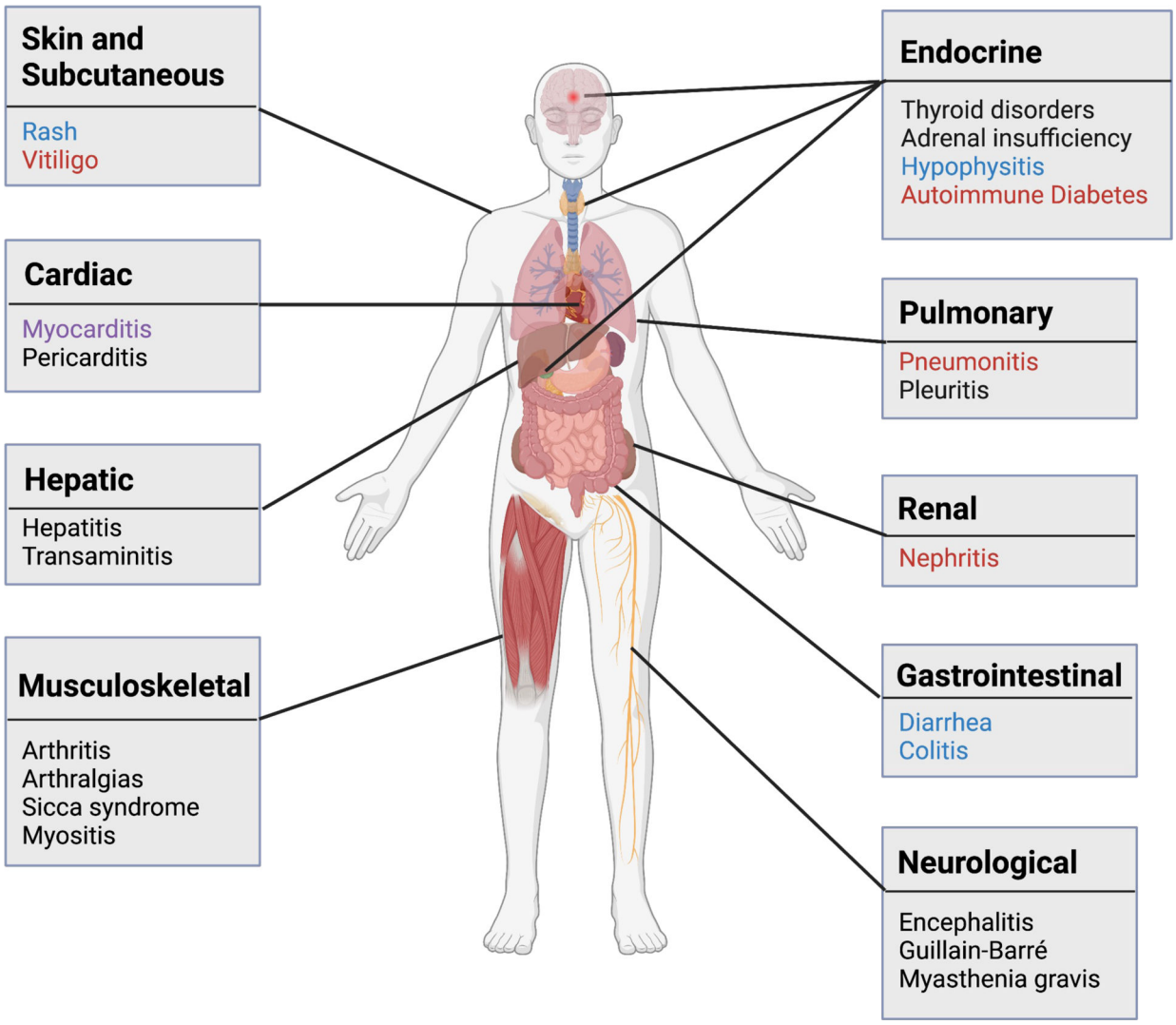

Figure 2. Immune checkpoint inhibitors initiate a broad spectrum of immune-related adverse events.

Immune checkpoint inhibitors (ICIs) can cause a diverse range of immunotoxicities that affect a broad range of organ systems throughout the body. However, some treatments have a higher propensity to induce immunotoxicities in certain tissues over others. From patient cohorts and preclinical models, it is evident that targeting the PD-1/PD-L1 pathway causes higher incidence of the immunotoxicities shown in red, whereas therapies that inhibit CTLA-4 lead to the initiation of those immunotoxicities shown in blue, those listed in black are observed at similar frequencies with either ICI treatment. Myocarditis is shown in purple as it appears to solely be observed following combination treatment. Combination treatment causes greater frequency and severity of immune-related adverse events, as well as accelerates the onset for when they occur following treatment initiation.

Model	Advantages	Disadvantages
in silico	• Data acquired • Increased patients numbers • Create consortia with shared interests • Cost-saving	• Ethical concerns for patient sharing • Complexity of sample annotation • Reproducibility of assay
Organoid	• Display cell-cell interactions • Small amount of starting material required • Recapitulate the genetics and therapeutic sensitivity of patients • Cost-saving	• Difficult to maintain • Heterogenity can create difficulties in reproducibility • Difficulty in establishing immune migration an infiltration • Only for certain tissue types
Mouse	• Multiple methods for amplifying toxicity • Amenable to genetic and environmental modifications • Able to test in large numbers • Easy to maintain • Humanized models available	• Resistance to toxicity • Not all strains have tumor models available • Immune system differs from humans • Drug surrogates often needed
Non-Human Primates	• Strong similarities in biological and behavioral aspects to humans • Able to use clinically utilized drugs • Useful for toxicology and pharmacokinetix studies	• Greater ethical considerations for experimentation • Expensive and difficult to manipulate • Unable to easily compare tumor response • Limited species selection
Canine	• Cancer patients • Similar environmental exposure as owners • Genetically diverse breeds • Tumors resistant to ICI treatment • Potential for biopsy or autopsy of tumor and organs	• Different drugs to humans • Limitations in tools • Often heavily pre-treated with conventional therapies • Toxicity observed at a lower rate than humans • Ethical and experimental limitations

Figure 3. The utility of diverse preclinical modeling systems to identify the mechanism for immune-related adverse events.

A broad range of preclinical models are needed to define mechanistic interactions that relate to immune checkpoint inhibitor initiated immune-related adverse events (irAEs). However, these models have both benefits and limitations relating to their use, which include both ethical and practical restrictions for their use.

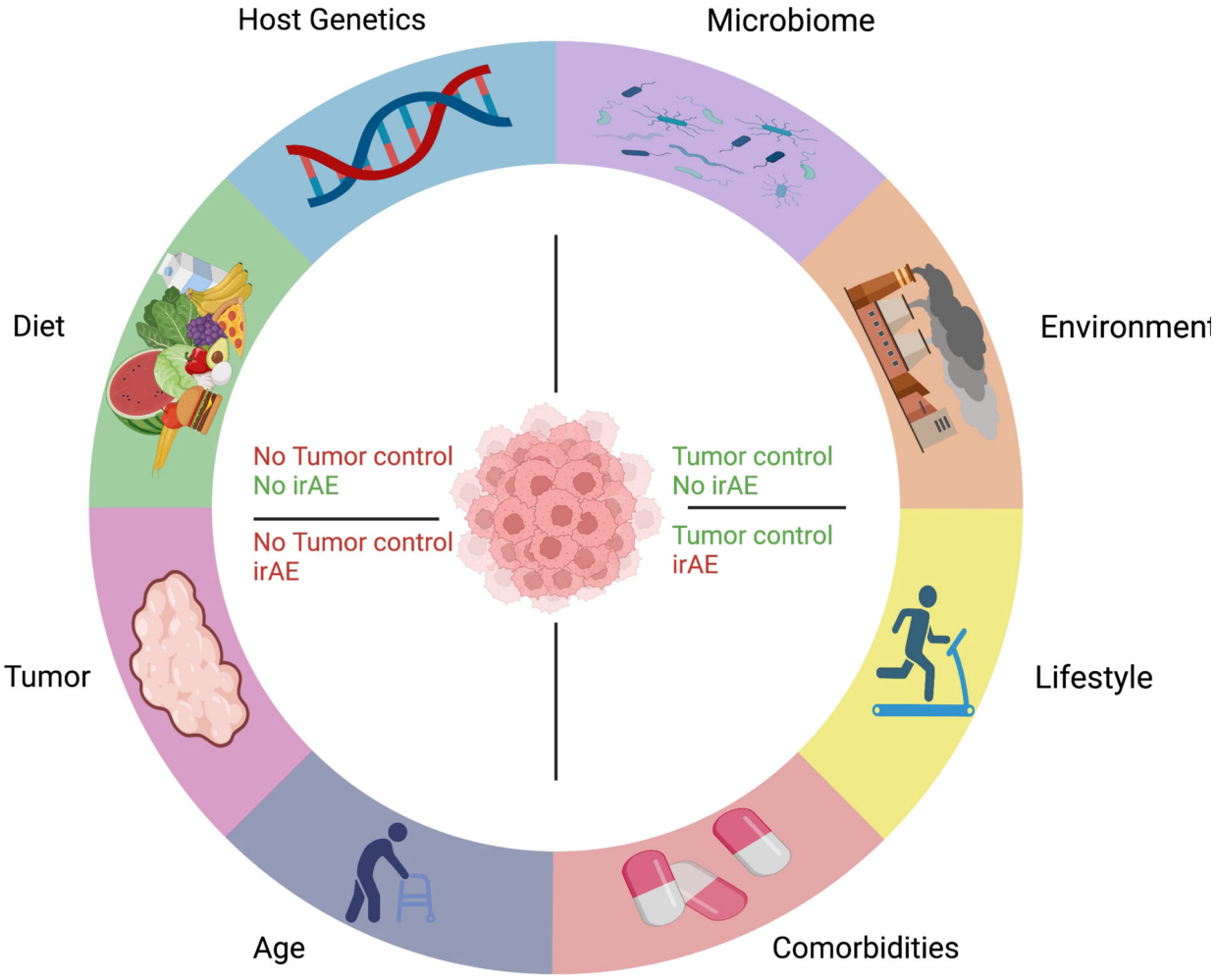

Figure 4. Immunomodulatory stimuli that lead to alterations in anti-tumor immunity and immune-related adverse events in response to immune checkpoint inhibitors.

Multiple immunomodulatory factors contribute to shaping systemic immunity, and this can lead to different outcomes in response to immune checkpoint inhibitors (ICIs) in terms of their impact towards tumor growth and immunotoxicity. Some immune-altering conditions are difficult to change, such as genetic variability, sex, and age, whereas others evolve across an individual's lifespan but can also be rapidly transformed, such as microbiome, diet, comorbidities, exercise. Intricate modeling systems will help to identify the interplay between pre-existing conditions that impact the immune response and their influence towards clinical outcomes relating to the presence or absence of irAEs and responsiveness of a tumor to ICI treatment.

Table 1.

Preclinical mouse models of irAEs and associated treatments.

Model	Toxicity susceptibility	Immunotherapy	irAE	irAE mechanism	irAE treatment	Ref.
Genetic susceptibility						
NOD mice	Autoimmune-prone	Anti PD-1 or anti-PD-L1	Autoimmune diabetes	Increased frequency of islet antigen-specific T cells producing IFNγ.		30
NOD mice	Autoimmune-prone	Anti-PD-L1	Autoimmune diabetes	Increased IFN-γ-producing CD8+ T cells in islets. β cell changes relating to IFN-γ and TNFα exposure.	Combined anti-TNFα and anti-IFNγ reduces ICI-induced diabetes.	119
NOD mice	Autoimmune-prone	Anti-PD-L1	Autoimmune diabetes	Increased immune cell infiltrate, including antigen-specific T cells in the islets.	JAK1/2 inhibitor prevents and reverses ICI-induced diabetes	74
NOD mice	Autoimmune-prone	Anti-PD-1	Autoimmune diabetes	Recruitment of monocyte-derived macrophages by T cells elicits β cell death by nitric oxide production.	Anti-CD4, anti-CD8, clodrolip depletion of macrophages, or an iNOS inhibitor were shown to protect against ICI-induced diabetes.	120
NOD mice	Autoimmune-prone	Anti-PD-1	Autoimmune diabetes	Treg expansion due to F5111 overcomes the increased pro-inflammatory T cell infiltrate.	Prophylactic F5111 (an IL-2 antibody fusion protein) protects against ICI-induced diabetes.	72
NOD insulin-dependent diabetes (*Idd)* congenic mouse strains	Autoimmune-prone	Anti-PD-L1	Autoimmune diabetes	Heightened cytokine production and proliferation of antigen-specific T cells in response to anti-PD-L1 could be significantly reduced in NOD mice congenic for *IDD* loci.	Combinations of *IDD* loci that protect against spontaneous autoimmune diabetes were able to reduce ICI-induced diabetes.	84
NOD mice	Autoimmune-prone	Anti-PD-L1	Sjögren's syndrome	Increased T and B cells in the salivary gland, particularly IFNγ+Tbet+CD4+ Th1 cells.	Anti-IFNγ improved salivary secretion.	121
NOD mice	Autoimmune-prone	Combination anti–PD-1 and anti-CTLA-4	Multiple irAEs, focus on thyroid disease	Increased of intrathyroidal IL-17A-expressing immune cells.	Anti-IL-17A neutralizing therapy reduced irAEs.	34
NOD mice	Autoimmune-prone	Combination anti–PD-1 and anti-CTLA-4	Thyroid disease	Increased of intrathyroidal IL-21-expressing CD4+ T cells.	IL-21 receptor-deficient mice have reduced thyroid disease.	63
CBA/J mice	Autoimmune-prone and immunization with thyroglobulin	Anti-PD-1	Thyroiditis	Increased effector and central memory CD4+ T cells in both lymph node and thyroid.	Anti-CD4, but not anti-CD20-mediated B cell depletion, prevented thyroid disease. Anti-CD8 provided	122

					partial protection.	
SJL/J and C57BL/6 mice	Autoimmune-prone	Anti-CTLA-4	Hypophysitis	Complement activation from antibody binding to ectopic CTLA-4 expressed by cells secretinig prolactin and thyrotropin.		123
Multiple strains, focus on C57BL/6^{lpr} and MRL^{lpr} mice	Autoimmune-prone	Combination anti–PD-1 and anti-CTLA-4	Multiple irAEs, including hepatitis, pancreatitis, colitis, pneumonitis	Differential immune infiltrate dependent on irAE. CD4+ and CD8+ T cells were abundant in liver, lung and pancreas. B cells and macrophages in liver and colon.	Use of the corticosteroid, prednisolone, decreased irAE severity.	36
C57BL/6 miR-146a$^{-/-}$ mice	Genetic susceptibility and lipopolysaccharide (LPS)-induced inflammation	Anti–PD-1	Multiple irAEs, including inflammation in the lungs, liver, colon, and skin	Increased neutrophils and T cell activation producing IFNγ and perforin	Therapeutic miR-146a mimic reduced severity of irAEs.	124
C57BL/6 CD11c^{Cre}*Stat3*$^{f/f}$ mice	Autoimmune-prone	Anti-CTLA-4	Colitis	Increased neutrophil score and production of proinflammatory cytokines, such as IL-1, IFNγ, IL-17, IL-6 in the colon.	Antibiotic treatment prior to anti-IL-6 and ICI treatment reduced immune infiltrate into the colon.	125
C57BL/6 LDLR$^{-/-}$ or APOE$^{-/-}$ mice	Autoimmune-prone	Combination anti–PD-1 and anti-CTLA-4	Multiple irAEs, particular focus on atherosclerosis and cardiac dysfunction	Increased aortic CD8+ T cells, leading to increased T cell to macrophage ratio, and higher endothelium expression of adhesion molecules.		126
C57BL/6 PD-1$^{-/-}$ mice	Autoimmune-prone	Combination epacadostat (IDO1 inhibitor) and anti-CTLA-4	Hepatitis	Increased CD4+ and CD8+ T cell infiltrate and proliferation.		127
C57BL/6 PD-1$^{-/-}$ mice	Autoimmune-prone	Combination epacadostat and anti-CTLA-4 or anti-CD137	Hepatitis	Both treatments enhanced T cell function and macrophage production of proinflammatory markers. However, treatment-specifc modulation of immune profiles were observed.	Anti-CD8 depletion attenuated liver toxicity from epacadostat and anti-CTLA-4.	128
C57BL/6 CTLA-4$^{+/-}$PD-1$^{-/-}$ mice	Autoimmune-prone	Genetic deficiency of PD-1 and haploinsufficiency of CTLA-4 mimicking combination ICI treatment	Myocarditis	Increased immune infiltrate, particularly CD8+ T cells and macrophages.	Abatacept (CTLA-4-Ig) treatment decreased cardiac dysfunction.	42
C57BL/6 CTLA-4$^{+/-}$PD-1$^{-/-}$ mice	Autoimmune-prone	Genetic deficiency of PD-1 and haploinsufficiency of CTLA-4 mimicking combination ICI treatment	Myocarditis	Cardiac-specific T cells recognizing α-myosin.	Anti-CD8, but not anti-CD4, depletion protects against cardiac irAEs.	22
Modification to cellular composition						

C57BL/6 and BALB/c Foxp3-DTR mice	Treg depletion	Anti-CD137 following transient Treg depletion	Multiple irAEs, including colitis and hepatitis.	Increased in CD4+ and CD8+ T cell abundance and proliferation in multiple tissues.	Anti-TNF reduced irAE severity in anti-CD137 and Treg delpeted mice.	43
C57BL/6 and BALB/c Foxp3-DTR mice	Treg depletion	Anti-CD40 following transient Treg depletion	Multiple irAEs, including poor clinical score, weight loss, hepatitis and colitis	Increase in circulating TNFα and IL-6 concentrations.	Anti-TNF, but not anti-IL-6R, reduced irAEs.	60
C57BL/6 Foxp3-DTR mice	Treg depletion	Systemic or local microneedle delivery of anti-CTLA-4	Multiple irAEs, including splenomegaly, blepharitis, lung, liver, and colon inflammation		Local microneedle delivery of anti-CTLA-4 to a tumor displayed reduced irAEs compared to systemic treatment.	129
C57BL/6 RAG1−/− mice	Naive CD4+ T cell transfer	Anti-CTLA-4	Muliple irAEs, including inflammation in colon, liver, lung and pancreas	increased TNFα production in CD4+ T cells.	Modified anti-CTLA-4 with a dual variable domain shielded by anti-prostate specific cancer antigen (PSCA) binding, leading to increased specificity with reduced toxicity.	130
CB17-SCID (severe combined immunodeficiency) mice	Naive CD4+ T cell transfer	Anti-CTLA-4 with intact Fc effector function	Enterocolitis, weight loss	Loss of Treg-mediated immunosuppression in the colon.	Anti-CTLA-4 that does not engage Fcγr activity reduced toxicity.	131
C57BL/6 mice	Immunization with myelin-oligodendrocyte glycoprotein peptide in complete Freund's adjuvant and pertussis toxin	anti-CTLA-4 with and without GVAX (GM-CSF vaccine)	Experimental autoimmune encephalomyelitis	High gene expression of *IL6* was identified in ICI-induced colitis and tumors that were non-responsive to ICI treatment.	Anti-IL-6 reduced the severity of EAE, which is enhanced by exposure to ICI treatment.	70
BALB/c mice	Immuniziation with α-myosin heavy chain fragment prior to ICI treatment	Anti-PD-1	Experimental autoimmune myocarditis	Increase in CD4+ T cells and macrophages, alongside expression of *IL1β, IL-6* and *COLA I* in the heart.		132
BALB/c g L7^{Cre} haemagglutinin (HA)$^{lox\text{-}stop}$ mice	Expression of HA in Purkinje cells and HA-expressing tumor cells, delivery of HA-specific T cells	Anti-CTLA-4	Paraneoplatic neurological disorder, reduced locomotion, weight loss	IFNγ and TNFα producing CD8+ T cell migration to the cerebellum		133
Chemical induced organ toxicity						
C57Bl/6 mice	Dextran sulfate sodium (DSS)	Combination anti–PD-1 and anti-CTLA-4	Colitis, weight loss	Increased *TNF* gene expression in patients with both colon irAEs and conventional ulcerative colitis.	Anti-TNF and Etanercept (TNFR2-IgG) reduced irAEs without impacting anti-tumor immunity.	46
C57BL/6 mice	DSS	Anti-CTLA-4 and dendritic cell-derived	Colitis, weight loss, increased enzymes relating	Increased ratio of IL-17-producing	Conjugated αCTLA-4 with DCNV-TA is	134

		nanovesicles presenting tumor antigens (DCNV-TA)	to liver and kidney function	CD4+ T cells to Tregs and CD4+ Th1 cells.	delivered specifically to tumor-specific T cells without initiating irAEs.	
C57BL/6 mice	DSS	PI-3065 (phosphoinositide 3-kinase δ inhibitor)	Colitis, weight loss	Continuous treatment with PI-3065 enhanced colonic Th17 cells, reduced Tregs, and altered their function by limiting *IL1R1* and *IL10* gene expression.	Intermittent dosing of PI-3065 reduced irAEs and amplified anti-tumor immunity compared to a continuous dosing regimen.	135
C57BL/6 mice	DSS	Anti-CTLA-4	Colitis, weight loss	Anti-CTLA-4 limits the suppressive function of Tregs by reducing IL-22 and IL-10 production.	*Bifidobacterium breve* and *Lactobacillus rhamnosum* reduce weight loss and toxicity	136
C57BL/6 mice	Amodiaquine	Anti-CTLA-4	Liver toxicity	Increased macrophage, B cell and T cell infiltrate. Upregulated granzyme B and perforin in CD8+ T cells, indicative of greater killing capacity.		137
Microbiome						
C57BL/6 mice	Gut microbiome	Agonistic anti-CD40 and anti-CD137	Hepatotoxicity, colitis, cytokine release syndrome (CRS)	Upregulation of myeloid-mediated inflammatory responses from anti-CD40 and CD8-mediated organ damage by anti-CD137.	Antibiotic or germ-free microbial depletion or deficiency in MYD88 reduced toxicity from both therapeutic agents.	66
C57BL/6 mice	Gut microbiome, specifically Bacteroides intestinalis	Combination anti–PD-1 and anti-CTLA-4	Colitis	Expression of *IL1b* was upregulated in inflammed colon and was increased in response to *B. intestinalis*.	Anti-IL-1R1 antagonist reduced inflammation.	99
C57BL/6 mice	*Staphylococcus epidermidis*	Anti–CTLA-4	Skin inflammation and thickness	Increased CD4+ and CD8+ T cells producing IL-17 and IFNγ in skin.	Deficiency of *RORC* in T cells, but not global loss of IFNγ signaling, prevented skin irAEs.	138
Rag2-deficient 129/SvEv	*Heliobacter hepaticus*	Anti-CTLA-4	Colitis	Increased IFNγ and IL-2 expression alongside loss of Treg-mediated immunosuppression.		139
Diet						
C57BL/6 mice	High salt content	Anti-CTLA-4	Pneumonitits, increased inflammatory markers in blood	Increased expression of proinflammatory cytokines (*IFNG, IL1B*) and inflammasome-related genes in lung-infiltrating CD4+ T cells.	Low salt diet enhanced anti-tumor immunity without irAEs.	101

C57BL/6 mice	Western diet and Imiquimod	Anti-PD-1	Skin inflammation and thickness	Increased IL-17 and PD-1 expression in gamma delta T cells with exposure to western diet, which may be targeted by anti-PD-1.	Control diet to limit dietary obesity reduced inflammatory response.	47
NOD-H2^{h4} mice	Iodinated water and autoimmune-prone mice	Anti–PD-1 or anti-CTLA-4 alone or in combination	Thyroiditis	IL-12 and IL-6 concentrations correlated with thyroid disease severity alongside increased CD103-expressing CD4+ T cells in the thyroid.		140
Age						
C57BL/6 mice	Age (18–24 months)	Anti–PD-1	Multiple irAEs, including lung, liver, and kidney toxicity	Increased infiltrate of B cells, CD4+ and CD8+ T cells, with the T cell subets expressing IL-21, which could also promote CXCL13.	Inhibiting IL-21 or CXCL13 signaling either therapeutically or genetically prevents immune infiltration of tissues.	95
C57BL/6 mice	Aged (22 months)	Anti CD40 and IL-2	Multiple irAEs, including lung, liver, and GI toxicities	Increased circulating IL-6, IFNγ, and TNFa, produced at least in part due to the presence of macrophages.	Macrophage depletion or targeting TNF signaling reduced toxicity more than T and NK cell depletion or inhibiting IFN receptor signaling.	141
Humanized mouse systems						
Immunodeficient C57BL/6 Rag2$^{-/-}$IL2r$\gamma^{-/-}$ mice	Human peripheral blood mononuclear cells (PBMCs)	Combination ipilimumab and nivolumab	Liver and colon toxicity	Increased *TNF* gene expression in patients with both colon irAEs and conventional ulcerative colitis.	Etanercept reduced irAEs without abrogating tumor control.	46
Immunodeficient NOD-SCID IL2r$\gamma^{-/-}$ (NSG) mice	Humanized with CD34+ cells	Ipilimumab	Multiple irAEs, including inflammation in liver, lung and salivary gland		CD24Fc reduced immune infiltrate in tissues and promoted survival.	75
NOD-scid IL2rγ^{mut} (NOG) mice	Humanized with CD34+ hematopoeitic stem cells, with bone marrow, liver, thymus (BLT)	Anti-PD-1 (nivolumab)	Multiple irAEs, dose-dependent survival curve observed.	Increased CD45RO+ memory T cells in the peripheral blood.		49
Young C57BL/6 with a humanized CTLA4 (Ctla4$^{h/h}$) mice	Humanized CTLA-4	Combination ipilimumab and anti-mouse PD-1	Multiple irAEs, including inflammation in liver, lung, kidney, salivary gland, heart, colon and anemia	Increased memory and autoreactive T cells alongside decreased Tregs.	Modified anti-CTLA-4 (L3D10) maintained anti-tumor immunity without initiating irAEs.	50
Young C57BL/6 CTLA-4$^{h/h}$ and mice	Humanized CTLA-4	Combination ipilimumab and anti-mouse PD-1	Multiple irAEs, including inflammation in liver, lung, salivary gland, and heart		CD24Fc reduced irAEs without compromising anti-tumor immunity.	75

Balb/c PD-1$^{h/h}$CTLA-4$^{h/h}$ mice	Humanized CTLA-4 and PD-1	Combination ipilimumab and nivolumab with collagen specific antibodies	Athritis and pneumonitis	Increased TNF-α-expressing CD4+ and CD8+ T cells.	Anti-TNFα decreased severity of irAEs.	142
Unknown Predisposition						
C57BL/6 mice	Unknown	Anti-PD-1	Enterocolitis	Increase in vascularization and CD8+ T cell infiltrate in the colon alongside upregulation of pAKT and pS6, both connected to the mTOR pathway.	Sirolimus (mTOR inhibitor) decreased severity of anti-PD-1-induced colitis.	143
C57BL/6 mice	Unknown	Anti-PD-1	Cardiotoxicity	Increased PD-L1 expression in cardiac endothelium leads to a breakdown in immune tolerance and increased T cell infiltration following ICI treatment.	Anti-TNFa protected against cardiotoxicity without reducing antitumor immunity.	144
C57BL/6 mice	Unknown	Anti-PD-L1 or anti-CTLA-4	Ovarian follicles destruction	Increased CD8+ T cell infiltrate and TNFa expression in the ovary. Follicles also display higher frequency of cleaved caspase-3 in ICI-treated mice.	BID-deficient mice prevent apoptosis of ovarian follicles.	145
C57Bl/6 mice	Unknown	anti-CD40	Weight loss, colon and liver toxicity	Increased IFNγ from T cells drives IL-12-producing Kupffer cells to promote neutrophil toxicity.	Anti-IL-12 or -IFNγ neutralization reduced liver and colon toxicity but inhibited anti-tumor immunity.	146
C57BL/6 mice	Unknown	Anti-CD40 prior to gemcitabine (chemotherapy)	Liver toxicity	Increased macrophages and myeloid-derived suppressor cells in liver.	Anti-colony stimulating factor (CSF)-1R reduced myeloid cell infiltrate to attenuate liver damage.	147
Balb/c mice	Unknown	Anti-CTLA-4 with intact Fc effector function and anti-PD-1	Colitis	Increased immune cell infiltrate and upregulation of genes-associated with cytokines (IL-21, IFNγ), chemokines (CXCL5, CXCL10, CXCL11) and cytotoxicity (granzyme B and K).	Anti-CD4 depletion reduced colon inflammation, as did anti-CD8 and anti-CSF-1R to a lesser extent.	131
A/J mice	Unknown	Anti-PD-1	Multiple irAEs, focus on myocarditis	Increased myeloid and lymphocytic immune infiltrate in heart. CD8+ T cells display increased expression of genes associated with cytotoxicity and activation.		148